Title
Intracellular membrane traffic of class I major histocompatibility complex molecules and transferrin receptor

Author
Bennett, Elizabeth McBain

Publication Date
2000

Peer reviewed|Thesis/dissertation

Intracellular Membrane Traffic of Class I Major Histocompatibility Complex Molecules and Transferrin Receptor: A Study of Viral and Dominant-Negative Inhibitors

by

Elizabeth McBain Bennett

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

[redacted]

Date University Librarian

Degree Conferred: .....

Dedication

For Mumsie and DDO

with all my gratitude for your unconditional love and support.

I made it!

Acknowledgements

I would like to thank the following people for their support and encouragement during my graduate education:

Advisors and Collaborators

Frances M. Brodsky, my advisor, for providing a supportive environment in which to pursue my research. Thank you for your help and advice and your interest in my scientific and personal development.

Lenore Pereira and Peter Walter, members of my thesis committee, for your encouragement and input during the past six years.

Fred Maxfield, for welcoming me into your lab and allowing me to monopolize your postdoc's time for an entire month.

Sharron Lin, for being a true superstar and allowing me to monopolize your time while in the Maxfield lab. Thank you for your scientific guidance and your kind words.

Jon Yewdell and Jack Bennink, for your generosity with reagents and advice.

Lab Members

Andy Wilde, for getting me started with a successful rotation.

Pat Bresnahan, for teaching me techniques of cell biology and immunology.

Shu-Hui Liu, whose scientific word I take as gospel, for sharing your knowledge and your time.

Larry Lem, my lab big brother, for always showing me the way.

Dave Riethof, for discussing the important things in life, like romance and fashion.

Diane Wakeham, for never telling me I'm a computer dunce whenever I'm being a computer dunce.

Alejandra Solache, for being a good friend.

Jean Chen, for being the smile I see when I look up.

I would also like to thank all members of the lab, past and present, who provided friendship and entertainment: Steve, Debbie, Bill, Ernest, Lin, Barrie, Diana, Joel, Tina, Victoria, Angela, Mhairi, Christine, Noriko, and Peter (our token Y chromosome).

GW Hooper Foundation

Rong Wang, for being a wonderful friend. Thank you for your input into my scientific and personal life, and thank you for sharing Christopher.

Dan Kalman, for sharing reagents, tweezers, and scope time.

Nancy Quintrell and **Jean Jackson**, for making our area of the floor a place I enjoyed.

Lois Koren, for your strength and inspiration.

Thank you to everyone in the Hooper Foundation for your friendship.

UCSF Office of Technology Management

Joel Kirschbaum, Julia Owens, Jane Fedor, and **Jennifer Cygan**, for an enjoyable and educational internship.

Friends and Family

Francie Chu, the world's greatest biology teacher, for letting me be a part of your classroom.

Paul DiGregorio, my career advisor, for helping me find a place to go from here.

Heather and Alison Anderson, for letting me be the middle sister.

Thank you to the following people for making me feel loved each day: **Julie, Meg, Carrie, Jana, Billy, Amy, and Ollie**.

And finally **my parents**, to whom this work is dedicated. Without your love and support none of my accomplishments would be possible. Thanks for seeing me through the best and worste of times (tee hee).

This work was supported by National Institutes of Health Grants GM38093, GM57657, and AI45865 (to Frances M. Brodsky) and National Institutes of Health Training Grant GM07175-23 and a scholarship from Achievement Rewards for College Scientists (ARCS) Foundation (to Elizabeth M. Bennett).

Scientific Contributors

The work presented in this dissertation resulted in two publications which are reproduced here (Chapter 2 and Appendix) and a third manuscript currently in preparation (Chapter 3). Some of this research involved the work of collaborators. The publications are listed below with the contributions of the various co-authors.

Bennett, E.M., J.W. Yewdell, J.R. Bennink, and F.M. Brodsky. 1999. Cutting edge: Adenovirus has two mechanisms for affecting class I MHC expression. *J. Immunol.*:5049-5052.

This publication is reproduced in Chapter 2. All of the data presented are the work of Elizabeth Bennett. Drs. Jonathan Yewdell and Jack Bennink provided reagents and experimental guidance. Dr. Frances Brodsky provided intellectual and editorial input.

Brodsky, F.M., L. Lem, A. Solache, and **E.M. Bennett**. 1999. Human pathogen subversion of antigen presentation. *Immunol. Rev. 1*68:199-215.

This publication is reproduced in the Appendix. Elizabeth Bennett contributed Figures 1 and 2 and editorial input for the sections on class I MHC molecules. Dr. Lawrence Lem contributed Figures 3-6 and editorial input for the sections on class II MHC molecules. Dr. Alejandra Solache contributed editorial input. The text of this manuscript is the work of Dr. Frances Brodsky.

Bennett, E.M., Lin, S.X., Maxfield, F.R., and F.M. Brodsky. 2000. Clathrin Hub expression affects endosome distribution with minimal impact on receptor recycling. *Manuscript in preparation.*

This manuscript is based on the work presented in Chapter 3. Elizabeth Bennett performed most of the experiments presented. Dr. Sharron Lin helped perform experiments involving fluorescently labeled transferrin and low density lipoprotein and did confocal imaging of labeled cells. Drs. Frances Brodsky and Frederick Maxfield provided intellectual and editorial input.

Abstract

Intracelluar Membrane Traffick of Class I Major Histocompatibility Complex Molecules and Transferrin Receptor: A Study of Viral and Dominant-Negative Inhibitors

Elizabeth McBain Bennett

Class I major histocompatibility complex molecules play a fundamental role in our immune response against viral infection. As viruses and hosts have coevolved, viruses have developed strategies to prevent class I MHC antigen presentation so as to escape immune detection. We set out to more fully define mechanisms by which viruses inhibit class I MHC expression. The project had two aims: to better define the effect of adenovirus E19 on class I molecule assembly and to develop reagents and techniques to study constitutive and HIV Nef-induced class I endocytosis. Adenovirus E19 had previously been shown to bind class I heavy chains and retain them in the endoplasmic reticulum. However, even class I alleles which do not bind well to E19 are delayed in their maturation by E19. While investigating this phenomenon we identified a novel function of E19 in inhibiting the interaction of class I molecules with TAP. E19 is the first viral protein shown to affect class I assembly at this step. Inhibition of class I molecule/TAP association may disrupt peptide loading of class I molecules and account for

delayed maturation of class I alleles that do not bind tightly to E19. Tools to study class I endocytosis were generated while investigating the role of endosomal clathrin-coated buds. The role for these clathrin-coated buds is unclear, though participation in receptor sorting and recycling has been proposed. Using the dominant-negative clathrin inhibitor Hub, we explored the effect of clathrin inhibition on the trafficking of transferrin receptor and on the organization of early endosomes. While we did not confirm a role for clathrin in receptor sorting and recycling, we did identify two novel effects of clathrin inhibition: redistribution of early endosomes into a perinuclear aggregate and disruption of the spatial relationship between AP2-coated vesicles and the actin cytoskeleton. These results support a model in which the actin cytoskeleton maintains the peripheral distribution of endosomes, and in the absence of association between endosomes and actin the early endosomal compartment collapses into the perinuclear area. Together these projects set the stage to investigate viral inhibition of membrane trafficking of class I MHC molecules.

Table of Contents

List of Tables

Chapter 1

Chapter 5

List of Figures and Illustrations

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Abbreveiations

ABC	ATP-binding cassette
AP	Adaptor protein
APLP	Amyloid precursor-like protein
β_2m	Beta-2-microglobulin
BDM	2,3-Butanedione monoxime
BSA	Bovine serum albumin
C_6-NBD-SM	6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-hexanoyl)sphingosyl phosphocholine
CCV	Clathrin-coated vesicle
CTL	Cytolytic T lymphocyte
DiI	3,3'-Dioctadecylindocarbocyanine
DMEM	Dulbecco's modified Eagle medium
DTT	Dithiothreitol
EBNA	Epstein-Barr virus nuclear antigen
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EndoH	Endoglycosidase H
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
G418	Geneticin
GSH	Glutathione
HCMV	Human cytomegalovirus
HIP1	Huntingtin interacting protein 1
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IE	Immediate early
KSHV	Kaposi's sarcoma-associate herpes virus
hsp	Heat shock protein

kD	Kilodalton
LCL	Lymphoblastoid cell line
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LMP	Low molecular weight protein
M6PR	Mannose-6-phosphate receptor
mAb	Monoclonal antibody
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
NK	Natural killer
NP	Nucleoprotein
NP40	Nonidet P40
PACS	Phosphofurin acidic cluster protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pfu	Plaque forming unit
RME	Receptor-mediated endocytosis
RRX	Rhodamine Red X
SDS	Sodium dodecyl sulfate
TAP	Transporter associated with antigen processing
Tf	Transferrin
TfR	Transferrin receptor
TGN	Trans Golgi network
TMD	Transmembrane domain
Vac	Vaccinia virus

Chapter 1

Introduction

The text of Chapter 1 is the work of Elizabeth Bennett. Some of the figures were created by others; some figures have been published and are reproduced here with permission. Figures 1.1 and 1.2 are reproduced with permission from *Immunological Reviews*, 1999, Volume 168:5-11, copyright Munksgaard International Publishers Ltd. Figures 1.5 and 1.6 are modified with permission from *Immunological Reviews*, 1999, Volume 168:199-215, copyright Munksgaard International Publishers Ltd. These figures were all created by Elizabeth Bennett. Figure 1.4 was created by Elizabeth Bennett with the help of Diane Wakeham, a graduate student in the lab. Figure 1.7 C was created by Dr. John Heuser at the Washington University School of Medicine and is reproduced with permission from *The Journal of Cell Biology*, 1987, Volume105: 1999-2009, copyright Rockefeller University Press. Figure 1.9 was created by Dr. Frances Brodsky. Figure 1.10 was created by Elizabeth Bennett based on the work of Dr. Frederick

Introduction

The interplay between a host's immune system and the immune evasion tactics of infectious pathogens highlights the opposing evolutionary forces faced by pathogen and host. Persistent threat of infection drives the immune system to develop elaborate pathways for ridding the body of infectious agents. Pathogens, in response to an evolving host immune system, must develop unique strategies to escape immune surveillance so as to establish infection and avoid clearance. Consideration of this ongoing struggle offers a unique and fascinating insight into the forces that drive evolution.

Bacteria and viruses have developed many mechanisms for immune evasion (Andersson et al., 1985; Burgert and Kvist, 1985). These tactics range from accessing immune-privileged sights to actively disrupting key components of the immune system. Additionally, pathogens might utilize components of the immune system for their own advantage. Strategies for immune evasion have been broadly grouped into three categories--stealth, sabotage, and exploitation--as presented by Dr. Frances Brodsky (Brodsky, 1999) (Fig. 1.1 and 1.2). The focus of this dissertation are sabotage tactics of viruses which inhibit antigen presentation by class I major histocompatibility complex (MHC) molecules.

While the primary focus of this work is viral inhibition of class I MHC molecule expression, the results of an investigation into the role of clathrin and the cytoskeleton in

Figure 1.1

Mechanisms of pathogen immune evasion

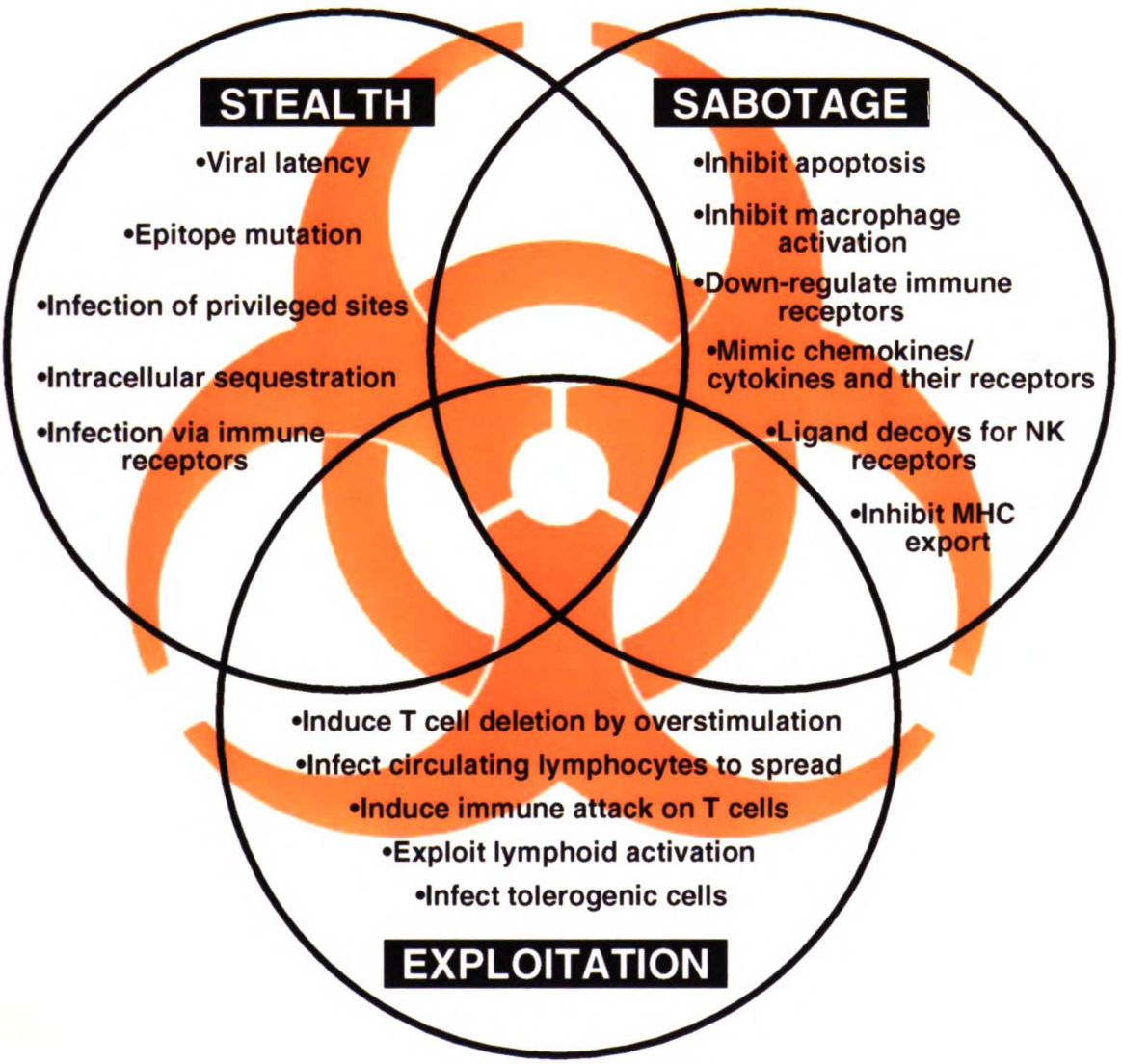


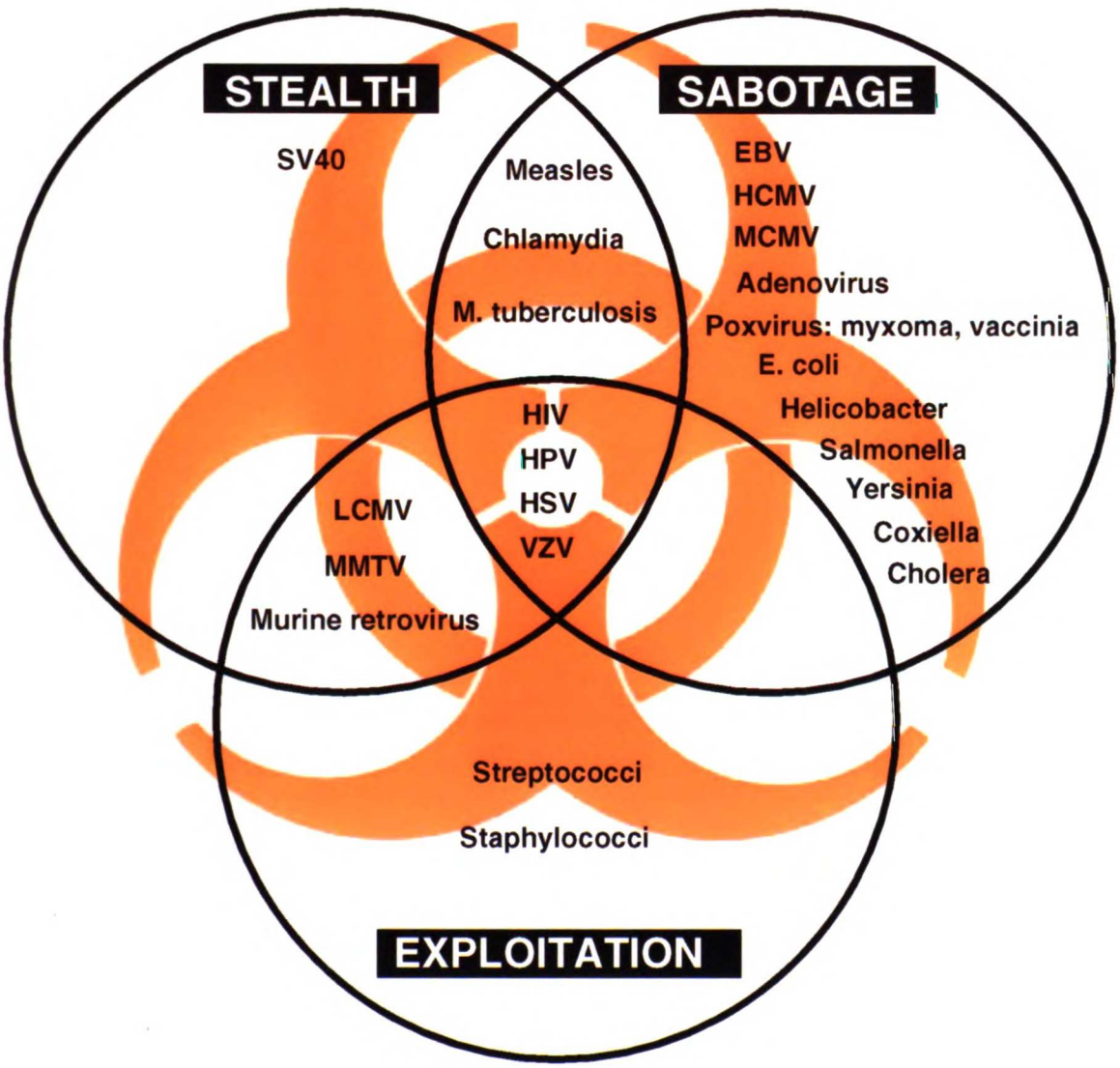
Figure 1.1. **Mechanisms of pathogen immune evasion.**

Immune evasion strategies can be grouped into three categories: stealth, sabotage, and exploitation. Stealth includes strategies to avoid immune detection. Sabotage includes mechanisms to disrupt specific functions of the immune system. Exploitation includes the ability of pathogens to use immunological processes to their own advantage.

Reproduced with permission from *Immunological Reviews*, 1999, Volume 168:5-11.

Figure 1.2

Pathogen deployment of immune evasion strategies



EBV - Epstein-Barr virus

HCMV - Human cytomegalovirus

HIV - Human immunodeficiency virus

HPV - Human papilloma virus

HSV - Herpes simplex virus

LCMV - Lymphocytic choriomeningitis virus

MCMV - Murine cytomegalovirus

MMTV - Mouse mammary tumor virus

SV40 - Simian virus 40

VZV - Varicella zoster virus

Figure 1.2. **Pathogen deployment of immune evasion strategies.**

Pathogens which possess immune evasion strategies of stealth, sabotage, and exploitation are shown. Pathogens which use more than one strategy are shown in regions of overlap. Reproduced with permission from *Immunological Reviews*, 1999, Volume 168:5-11.

transferrin receptor trafficking will also be presented. This second project stemmed from the development of tools to study the role of clathrin in class I MHC molecule endocytosis. The two projects share a common theme of intracellular protein trafficking pathways and the use of inhibitors to study these pathways.

This chapter is divided into three sections: Class I MHC molecules, Viral inhibition of class I MHC expression, and the Role of clathrin and the cytoskeleton in transferrin receptor trafficking. The first section will introduce class I MHC molecules, their function and structure, and their assembly and intracellular trafficking. The second section will introduce viral mechanisms for downregulation of class I molecules and highlight evidence for the coevolution of virus and host. The final section will introduce the intracellular trafficking pathway of transferrin receptor and the role of clathrin and the cytoskeleton in receptor endocytosis and recycling.

Class I major histocompatibility complex molecules

MHC molecules play a crucial role in signaling to T cells the presence of infectious pathogens. The MHC locus is located on the short arm of human chromosome six. It encodes both class I and class II MHC molecules, as well as accessory proteins necessary for the proper assembly and intracellular trafficking of MHC proteins (Parham, 1999) (Fig. 1.3). Class I MHC molecules mainly present peptides derived from intracellular pathogens (e.g. viruses), while class II MHC molecules present

Figure 1.3. Genetic organization of the human major histocompatibility complex (chromosome 6)

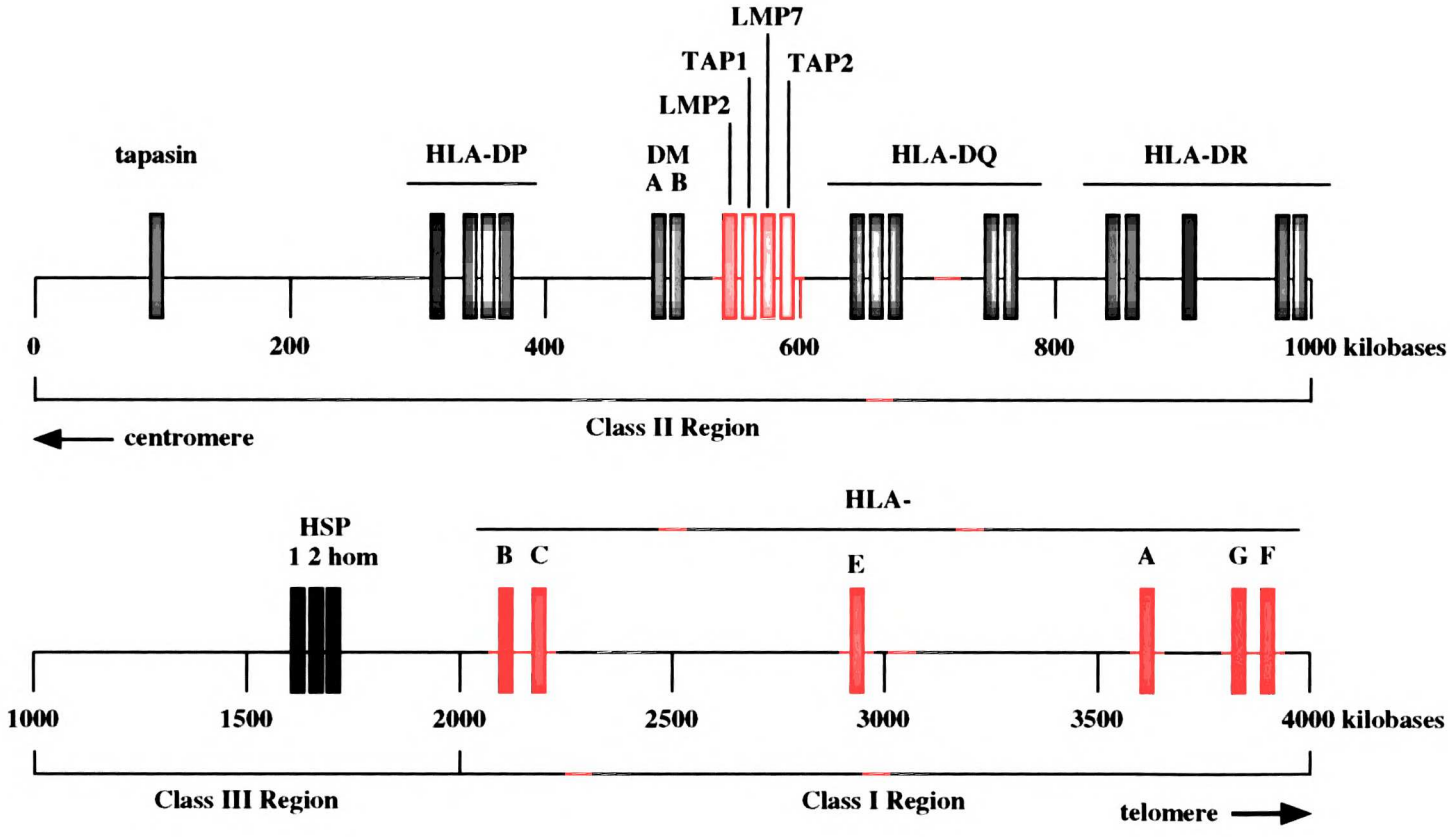


Figure 1.3. **Genetic organization of the human major histocompatibility complex.** The human MHC is found on the short arm of chromosome 6. The region is approximately 4000 base pairs and is divided into three subregions: the class I MHC, the class II MHC, and the class III MHC. Though class I and class II MHC molecules are encoded in their respective regions, some accessory proteins involved in class I MHC molecule assembly are encoded in the class II locus, including tapasin, the TAP1 and TAP2 subunits, and low molecular weight proteins (LMP) 2 and 7 which are subunits of the proteasome. Heat shock protein (hsp) 70-1, -2, and an hsp70 homolog are encoded in the class III region and may be involved in antigen processing (Pierce et al., 1991). Additional genes are encoded in the MHC region but are not shown in this figure.

extracellularly derived peptides (e.g. bacterial peptides). The presentation of non-self peptides by MHC molecules activates T cells, triggering an immune response against the foreign pathogen.

Class I MHC molecules: Function and structure

Function

Class I MHC molecules are heterotrimeric membrane proteins consisting of a polymorphic, membrane-bound heavy chain, a soluble, noncovalently associated light chain called β2-microglobulin (β2m), and a peptide of approximately 8-10 amino acids (Fig. 1.4). Class I heavy chains are encoded in the MHC in loci designated human leukocyte antigen (HLA)-A, -B, and -C (Fig. 1.3). Nonclassical class I heavy chains are encoded in loci designated HAL-E, -F, and -G and exhibit more restricted expression profiles and specialized functions compared to classical class I molecules (Le Bouteiller and Blaschitz, 1999; Leibson, 1998; Wainwright et al., 2000). β_2m is nonpolymorphic and is encoded outside the MHC on human chromosome 15.

Class I molecules present bound peptides at the plasma membrane to $CD8^+$ cytolytic T cells (CTL) (Doherty and Zinkernagel, 1975; Zinkernagel and Doherty, 1975). The antigenic peptides displayed by class I molecules are derived primarily from intracellular proteins and serve as a representative sampling of the protein population within a cell. The presentation of exogenous antigens by class I MHC molecules has

Figure 1.4

Crystal structure of HLA-A2 with decameric peptide

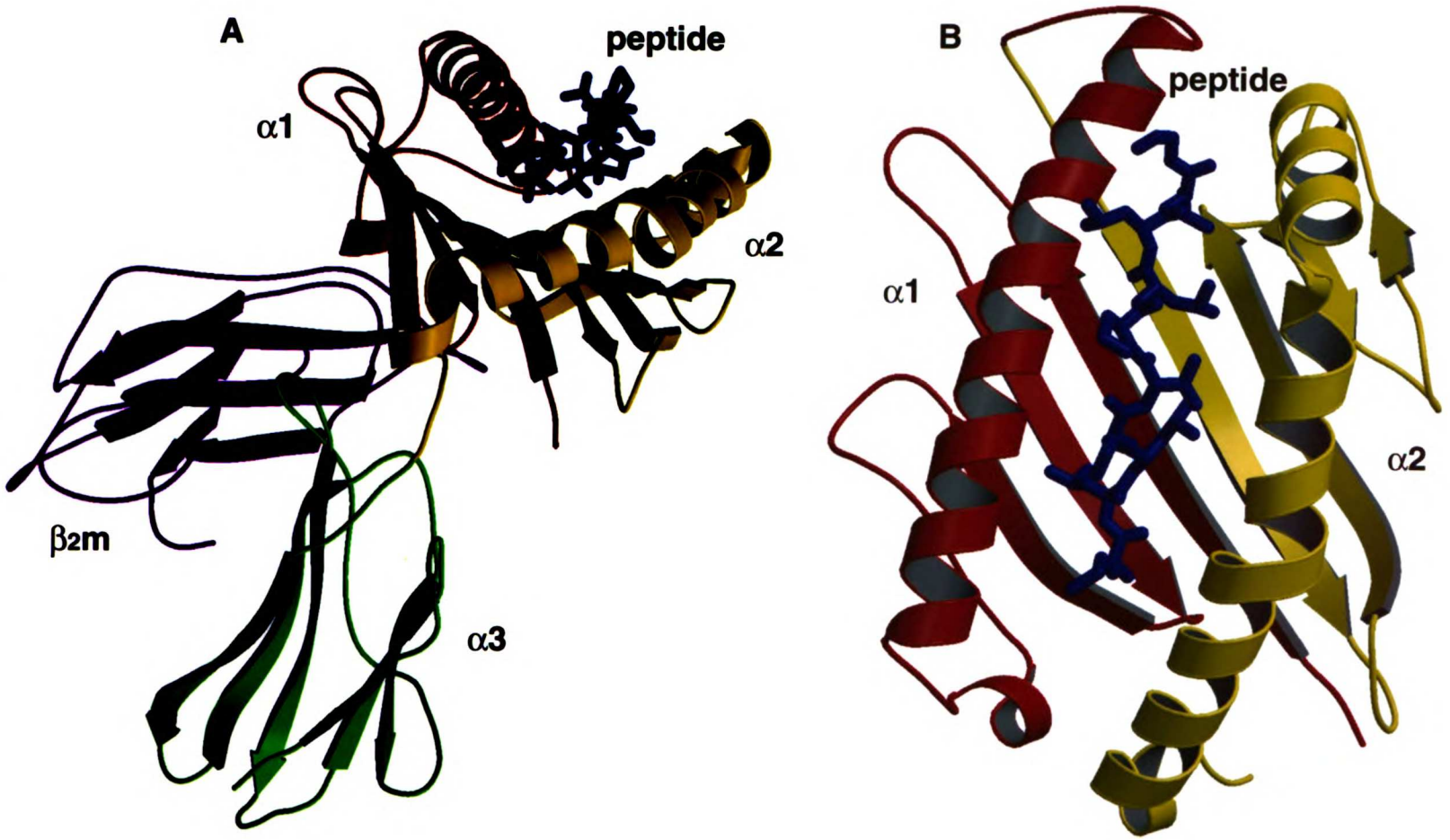


Figure 1.4. **Crystal structure of HLA-A2 with decameric peptide.**

(A) The class I MHC heavy chain contains three domains designated α_1 (red), α_2 (yellow), and α_3 (green). The α_1 and α_2 domains form a peptide binding groove--the α helices of these domains form the edges of the groove, and the β sheet portions form the floor. In this figure the peptide binding groove is occupied by a decameric peptide from calreticulin (shown in blue). The membrane-proximal α_3 domain and β_2m (shown in purple) both adopt immunoglobulin-type folds.

(B) Top-down view of the class I MHC molecule peptide binding groove.

Figure 1.4 was generated from coordinates 2CLR deposited in the Protein Data Bank (Collins et al., 1994) using Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994) at the Molecular Structure Group facilities at UCSF.

been reported (Rock, 1996), though the mechanisms by which exogenous antigens are processed for presentation by class I molecules have not been fully determined. Presentation of non-self peptides signals to CTLs the presence of a pathogen (most commonly an intracellular virus). Non-self peptides activate CTLs to initiate a cytotoxic attack against the antigen presenting cell (Harty et al., 2000). This system of peptide recognition allows the immune system to selectively destroy cells harboring viruses and other intracellular pathogens.

Structure

The crystal structure of a class I MHC molecule (Collins et al., 1994) (Fig. 1.4) reveals three heavy chain domains designated $\alpha 1$, $\alpha 2$, and $\alpha 3$. Both $\alpha 1$ and $\alpha 2$ are composed of a prominent alpha helix and an antiparallel β-pleated sheet. These two domains form a peptide binding groove which runs between the two alpha helices with the β sheets serving as a floor. The membrane-proximal $\alpha 3$ domain adopts an immunoglobulin type fold, as does β_2m. β_2m associates with the class I heavy chain via the $\alpha 3$ domain and the underside of the β sheets of the $\alpha 1$ and $\alpha 2$ domains.

Class I MHC molecules: Assembly and trafficking

The assembly of class I MHC molecules takes place within the lumen of the

endoplasmic reticulum (ER) where a variety of molecular chaperones and accessory proteins aid in the folding and assembly of the class I molecule (Pamer and Cresswell, 1998; Solheim, 1999). Once assembled and loaded with peptide, class I molecules exit the ER and traffic to the surface of the cell where displayed peptides can be monitored by CTLs. The assembly pathway for class I molecules is shown in Fig. 1.5. The molecules involved in class I assembly and the steps in the assembly process are presented below.

Components of the class I MHC assembly pathway

BiP

BiP is an hsp70 homologue which has been identified as both the immunoglobulin heavy chain binding protein (Haas and Wabl, 1983) and the glucose regulated protein grp78 (Pouyssegur et al., 1977). As part of the quality control machinery within the ER, BiP interacts with nascent polypeptides during their translocation into the ER lumen. Through ATP-dependent cycles of binding and release (Flynn et al., 1989), BiP prevents protein aggregation and maintains polypeptides in a folding-competent state until they achieve their native conformation (Gething and Sambrook, 1992). Upon achieving its native conformation, the newly folded protein no longer displays accessible hydrophobic patches to which BiP can bind, and the chaperone is released. BiP also associates in a prolonged fashion with misfolded proteins (Hurtley and Helenius, 1989) and is thought to play a role in targeting these proteins to the cytosol for degradation (Plemper et al.,

Figure 1.5. Assembly and trafficking pathways of class I MHC molecules

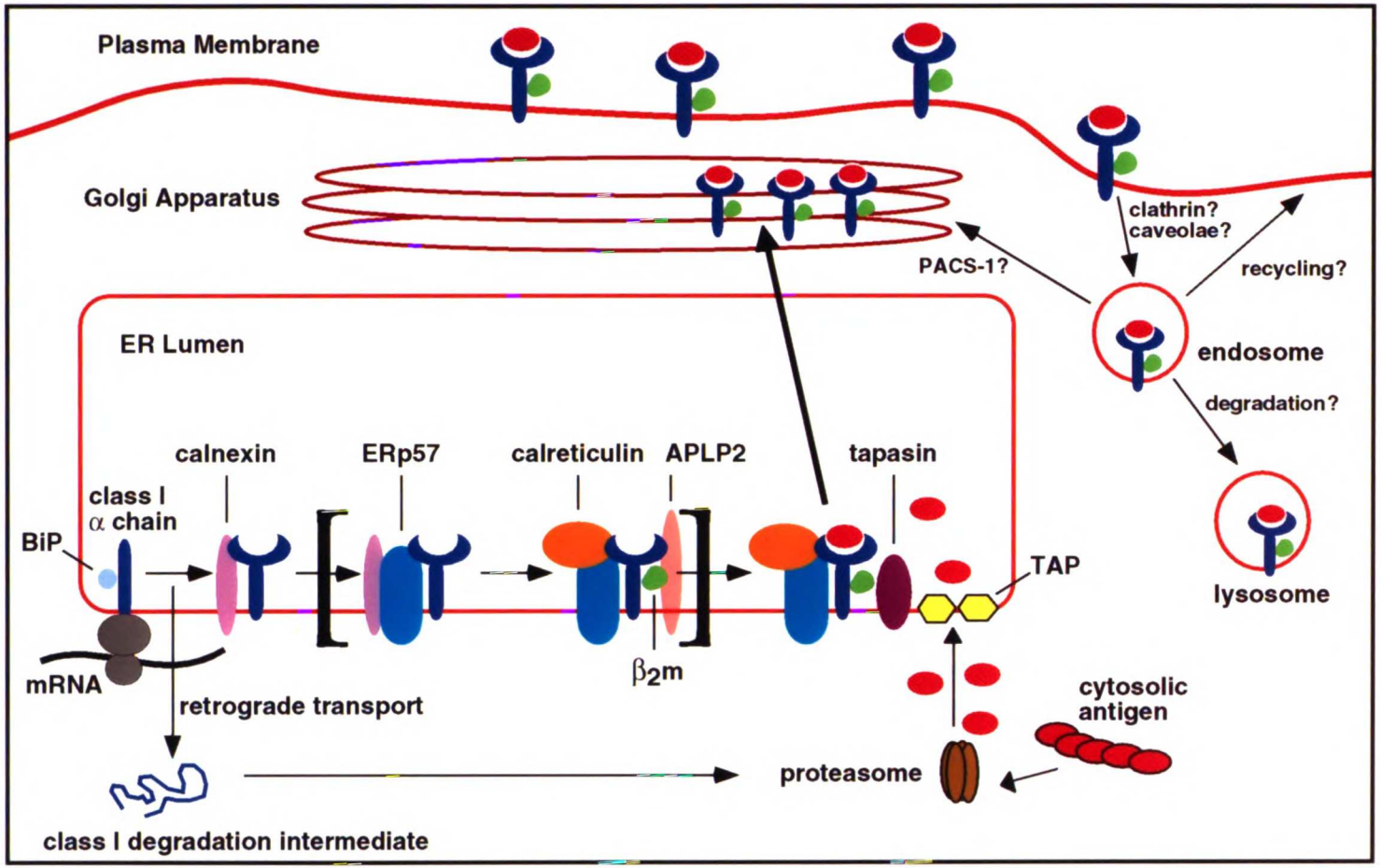


Figure 1.5. **Assembly and trafficking pathways of class I MHC molecules.** Class I MHC molecule assembly and trafficking are described in the test. Brackets indicate areas of uncertainty with regard to the role of particular ER chaperons or the order in which they interact with class I molecules. Question marks indicate processes that are uncertain or for which mechanisms remain unclear. Modified with permission from *Immunological Reviews*, 1999, Volume 168: 199-215.

1997). BiP is found in association with free class I heavy chains (Kahn-Perles et al., 1994; Nössner and Parham, 1995) and not with heavy chain-β_2m dimers, indicating that BiP plays a role in the very early stages of class I heavy chain folding. Not surprisingly, the association of BiP with nascent class I heavy chains was first observed the β_2m-negative Daudi cell line (Sege et al., 1981). The discovery of class I heavy chain retrograde transport (Wiertz et al., 1996a) and the association in yeast of BiP with the Sec61p translocation machinery (Plemper et al., 1997) suggest that in addition to stabilizing nascent class I heavy chains, BiP might aid in the transport of misfolded class I molecules from the ER to the cytosol for degradation (Nössner and Parham, 1995).

In addition to BiP, other heat shock proteins have been implicated in the class I assembly pathway. In particular, the heat shock protein grp96 has been hypothesized to participate in peptide loading of class I molecules by acting as a peptide receptor in the ER and transferring the peptides to empty class I molecules (Srivastava et al., 1994).

Calnexin

Calnexin is an ER-resident, membrane-bound chaperone that interacts with nascent polypeptides and acts to both stabilize the proteins and retain unfolded proteins in the ER (Bergeron et al., 1994). Like BiP, calnexin associates with free class I heavy chains (Galvin et al., 1992; Nössner and Parham, 1995), though heavy chain-β_2m dimers

have been observed in association with calnexin (Carreno et al., 1995). Calnexin stabilizes free heavy chains and aids in their folding (Vassilakos et al., 1996). In mutant cells that synthesize incompletely assembled class I molecules, class I heavy chains accumulate in the ER and show a prolonged association with calnexin (Degen et al., 1992) By retaining unfolded proteins in the ER (Rajagopalan and Brenner, 1994), calnexin helps ensure that misassembled class I molecules do not traffic to the cell surface. Association with calnexin is thought to account for the different ER-to-Golgi transit times between different class I alleles, presumably because different alleles achieve their native conformation and dissociate from calnexin with different kinetics (Degen and Williams, 1991; Williams et al., 1985) Interestingly, an interaction with calnexin is not obligatory for proper class I assembly, as class I peptide loading and surface expression occur normally in calnexin-negative cells (Sadasivan et al., 1995; Scott and Dawson, 1995). It is possible that BiP can compensate for the lack of calnexin, suggesting a redundant role for the two chaperones.

Calnexin is a lectin and shows a marked preference for glycoproteins which carry N-linked oligosaccharides (Ou et al., 1993; Williams, 1995) that have been processed to the monoglucosylated form (Hammond et al., 1994). However, calnexin associates with proteins which lack N-linked oligosaccharides (Rajagopalan et al., 1994), and the association of calnexin with class I MHC molecules, which carry N-linked oligosaccharide moieties, is mediated by a portion of the class I molecule that does not

carry carbohydrate modifications (Margolese et al., 1993) A model has been proposed whereby the initial interaction of calnexin with nascent polypeptides is mediated by calnexin's recognition of monoglucosylated N-linked oligosaccharides, but subsequent interactions depend more heavily on protein-protein interactions (Zhang et al., 1995). Calnexin and BiP are found in association with different pools of free class I heavy chains (Nössner and Parham, 1995) In light of BiP's proposed role in retrograde transport of misfolded proteins, it has been proposed that calnexin interacts with class I molecules on their way to forming functional complexes whereas BiP might interact with misfolded class I molecules that are ultimately degraded.

Calreticulin

Calreticulin is a soluble ER-resident protein which, like calnexin, is a lectin and associates with class I molecules in a carbohydrate-dependent manner (Krause and Michalak, 1997), although calreticulin and calnexin associate with different glycoforms of the class I heavy chain (Harris et al., 1998; Zhang and Salter, 1998). The association of class I molecules with calreticulin temporally follows association with calnexin. Whereas calnexin associates only with free class I heavy chains, calreticulin interacts with heavy chain-β_2m dimers and remains associated with class I dimers even as they associate with the transporter associated with antigen processing (TAP) (Sadasivan et al., 1996; Solheim et al., 1997). An association of calreticulin with β_2m in the absence of

class I heavy chains has been observed, though the extent to which this result is due to the presence of nonclassical class I molecules is unclear (Solheim et al., 1997). Though the exact role for calreticulin in the assembly of class I MHC molecules is not well defined, the chaperone is an integral part of the class I peptide loading complex (Cresswell et al., 1999b) and presumably stabilizes empty class I dimers until they acquire peptide.

ERp57

ERp57 is a ER-resident protein which associates transiently with newly synthesized polypeptides (Oliver et al., 1997) and is found in association with class I MHC molecules (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). Like calnexin and calreticulin, ERp57 requires a properly trimmed, monoglucosylated glycoprotein as a substrate (Van der Wal et al., 1998), though ERp57 itself lacks lectin-like properties. The requirement for a glycosylated substrate may reflect that ERp57 associates with substrate indirectly via calnexin or calreticulin (High et al., 2000; Zapun et al., 1998). In fact, a model has been proposed whereby ERp57 acts in concert with calnexin and calreticulin to aid in protein folding (Elliott et al., 1997; Oliver et al., 1999).

ERP57 has been shown to possess both thiol-dependent reductase (Hirano et al., 1995; Srivastava et al., 1991) and cysteine protease (Urade and Kito, 1992; Urade et al., 1997) activities. ERp57 is a member of the thiol oxidoreductase family of proteins

(Ferrari and Söling, 1999) and is thought to facilitate proper disulfide bond formation in its substrates. Class I MHC molecules contain two intracellular disulfide bonds, and association of class I molecules with ERp57 coincides with the formation of these bonds (Farmery et al., 2000). One disulfide bond in particular, found in the α2 portion of the peptide binding groove, is required for proper peptide acquisition by class I molecules (Warburton et al., 1994). It has been hypothesized that formation of this disulfide bond is needed for proper interaction of class I dimers with the peptide loading complex, and a role for ERp57 in formation of this bond is under investigation (Cresswell et al., 1999a). Additionally, the proteolytic activity of ERp57 may contribute to peptide trimming or protein degradation in the ER (Otsu et al., 1995; Snyder et al., 1994).

Transporter associated with antigen processing (TAP)

TAP transports peptides from the cytosol to the lumen of the ER where they are loaded onto newly synthesized class I MHC molecules (Elliott, 1997; Lankat-Buttgereit and Tampé, 1999). TAP is a member of the ATP-binding cassette (ABC) family of transporters (Higgins, 1992), which also includes the yeast sterile 6 protein (Kuchler et al., 1989), the cystic fibrosis transmembrane regulator (Riordan et al., 1989), and P-glycoprotein (Horio et al., 1988). TAP is composed of two subunits termed TAP1 and TAP2 (Androlewicz et al., 1994; Spies et al., 1992). The two subunits are 64% homologous at the protein level (Lankat-Buttgereit and Tampé, 1999), and each contains

six putative transmembrane domains (TMD) and a cytosolic ATP-biding domain. The subunits come together to form a 12 TMD channel through which peptides pass in an ATP-dependent fashion (Androlewicz et al., 1993; Neefjes et al., 1993). The transport of peptides by TAP occurs in two steps (van Endert et al., 1994). First, peptides associate with the cytosolic domain of TAP in an ATP-independent manner (Uebel et al., 1995) followed by a conformational change in TAP and ATP hydrolysis which drives peptide transport (Neumann and Tampé, 1999)

TAP exhibits selectivity in the peptides it transports (Uebel et al., 1997), which affects the peptide repertoire bound by class I MHC molecules (Daniel et al., 1998) Most of the peptides transported by TAP are generated in the cytosol by the proteolytic activity of the proteasome (Rock et al., 1994), a multisubunit, ATP-dependent protease (Baumeister et al., 1998). Two of the catalytic subunits of the proteasome (low molecular weight proteins (LMP) 2 and 7) are encoded in the MHC (Glynne et al., 1991; Kelly et al., 1991; Ortiz-Navarrete et al., 1991), and LMP2 and LMP7 knockout mice display reduced class I MHC surface expression (Fehling et al., 1994; Van Kaer et al., 1994). Other cytosolic proteases may contribute to peptide generation (Beninga et al., 1998; Vinitsky et al., 1997), and additional trimming of peptides can occur in the ER lumen (Elliott et al., 1995; Roelse et al., 1994; Snyder et al., 1994).

Within the ER lumen, class I heavy chain-β_2m dimers associate with TAP to acquire antigenic peptide (Ortmann et al., 1994; Suh et al., 1994). Association of class I

molecules with TAP has been shown to be via the TAP1 subunit (Androlewicz et al., 1994). Though there is a report of class I molecules interacting with TAP2 (Powis, 1997), the particular TAP2 allele studied was from rat and may not reflect the situation in human cells. The affinity of different class I alleles for TAP varies (Neisig et al., 1996), though differences in TAP affinity may result from different affinities for tapasin or other members of the class I loading complex. There are reports of β_2m itself associating with TAP (Fig. 2.4 and Solheim et al., 1997). The interaction may be mediated by nonclassical class I molecules, though Solheim et al. addressed this possibility and concluded that nonclassical molecules were not responsible.

Under physiological conditions TAP is phosphorylated, which seems to promote formation of the class I peptide loading complex but inhibit the transport of peptides by TAP (Li et al., 2000). Phosphorylation may regulate the activity of TAP as has been observed for other members of the ABC family of transporters (Higgins, 1992).

Tapasin

Tapasin was first observed as a coprecipitating protein with TAP (Ortmann et al., 1994) and was later identified as an ER-resident glycoprotein which binds independently to both class I MHC molecules and TAP, serving as a bridge between the two molecules (Li et al., 1997; Sadasivan et al., 1996). It has since been discovered that tapasin cements the formation of a class I peptide loading complex, consisting of class I heavy chain-β_2m

dimer, calreticulin, Erp57, tapasin, and TAP (Cresswell et al., 1999b) in a ratio of four class I and tapasin molecules per TAP molecule (Ortmann et al., 1997). In addition to bridging elements of the class I peptide loading complex to TAP, some data support a role for tapasin in mediating the association of class I molecules with calreticulin and ERp57 (Bangia et al., 1999; Hughes and Cresswell, 1998), though not all studies have supported such a model (Lindquist et al., 1998; Solheim et al., 1997).

The absence of a functional tapasin molecule in the 721.220 cell line (Copeman et al., 1998) results in a lack of class I-TAP interaction (Grandea et al., 1995) and diminished class I expression on the cell surface (Grandea et al., 1995; Ortmann et al., 1997), highlighting the importance of tapasin in the assembly of class I MHC molecules. However, while tapasin is required for the assembly of most class I molecules, some alleles are expressed on the cell surface in the absence of tapasin (Lewis et al., 1998; Peh et al., 1998). The N-terminus of tapasin is responsible for association with class I MHC molecules, calreticulin, and ERp57, while the C-terminus mediates interaction with TAP (Bangia et al., 1999). Tapasin has been shown to affect the binding of peptides to the cytosolic side of TAP (Li et al., 2000), consistent with an interaction between TAP and the cytosolic, C-terminal domain of tapasin. Soluble tapasin, lacking its cytosolic domain, associates with class I dimers but does not associate with TAP (Lehner et al., 1998). Interestingly, soluble tapasin restores class I surface expression in 721.220 cells, despite its inability to bind TAP.

In addition to bridging elements of the class I peptide loading complex, tapasin has also been credited with peptide editing (ensuring that class I molecules are loaded with optimally biding peptides) (Barnden et al., 2000; Sijts and Pamer, 1997) and with stabilizing empty class I dimers (Grandea et al., 1997) and retaining them in the ER until they acquire peptide (Schoenhals et al., 1999). Though further study is needed to clarify the myriad roles of tapasin, the protein clearly plays a prominent role in assembly of class I MHC molecules (Androlewicz, 1999).

APLP2

Amyloid precursor-like protein 2 (APLP2) binds class I MHC heavy chain-β_2m dimers and is the most recent ER protein implicated in the assembly of class I molecules (Sester et al., 2000). The protein is a member of the mammalian amyloid precursor-like protein family and shares homology with the amyloid precursor protein associated with plaques seen in the brains of Alzheimer's patients. APLP2 was recently shown to be identical to p100/110 which was found in association with class I MHC molecules in the presence of the adenovirus E19 protein (Sester et al., 2000) (discussed in detail below). In cells expressing adenovirus E19, murine class I MHC molecules show enhanced association with a protein termed "p100/110" (Feuerbach and Burgert, 1993), and this protein can be displaced by the binding of antigenic peptide to class I molecules. This finding led to the idea that p100/110 might facilitate class I MHC peptide loading. With

the identification of p100/110 as APLP2, it is now hypothesized that APLP2 plays a role in peptide acquisition by class I molecules, though the function of APLP2 remains to be fully elucidated.

Assembly of class I MHC molecules

The assembly pathway of class I MHC molecules is shown in Fig. 1.5. As class I heavy chains are synthesized, they are targeted to the rough ER and enter the ER lumen where they are modified by the addition of one N-linked oligosaccharide at position 86 of the α1 domain (Maloy, 1987). Class I heavy chains interact with BiP and calnexin, and as they adopt a stable conformation they bind β_2m. Class I heavy chain-β_2m dimers then interact with ERp57, calreticulin, APLP2, tapasin, and TAP.

Uncertainty remains about the exact order in which different chaperones interact with class I molecules and whether various interactions are direct or indirect. For example, the order in which class I molecules interact with ERp57 and calnexin has not been resolved. Kinetic studies show that calnexin association with class I heavy chains precedes association of ERp57 (Morrice and Powis, 1998). However, ERp57 has been observed to coprecipitate with calnexin and to associate with class I heavy chains in the absence of β_2m (Lindquist et al., 1998). These results suggest that ERp57 associates with class I heavy chains very early, although these observations were made in murine cells.

The proposal that ERp57 interacts with its substrate via calnexin or calreticulin may reconcile these apparently conflicting results (Zapun et al., 1998) by allowing ERp57 to bind calnexin- or calreticulin-associated class I molecules.

It is unclear to what extent association of ERp57 and calreticulin with class I molecules is dependent upon tapasin. While some researchers have observed no association of ERp57 or calreticulin with class I molecules in the tapasin-negative cell line 721.220 (Hughes and Cresswell, 1998), others have observed coprecipitation of class I molecules with ERp57 and calreticulin in the same cells (Lindquist et al., 1998; Solheim et al., 1997).

The association of calnexin, calreticulin, ERp57, and TAP with class I molecules are all sensitive to treatment with castanospermine (Lindquist et al., 1998; Sadasivan et al., 1996; Vassilakos et al., 1996). Whether this indicates a direct, carbohydrate-dependent interaction of each of these molecules with class I MHC proteins or whether certain castanospermine-sensitive chaperones mediate association of the class I molecules with other components is unclear. There is already evidence that ERp57 interacts with class I molecules via calnexin or calreticulin (Zapun et al., 1998) and that tapasin mediates the association of class I molecules with calreticulin and ERp57 (Hughes and Cresswell, 1998). Further study is needed to elucidate the exact nature of the interactions between various components of the class I assembly pathway.

Despite the uncertainty as to the order in which these components participate in

class I assembly, in the final stage of assembly tapasin cements the formation of a multimeric peptide loading complex, and TAP-transported peptides are loaded onto class I molecules. Trimeric class I complexes then exit the ER, traffic through the Golgi apparatus, and follow a default pathway to the cell surface.

Generally only peptide-loaded class I complexes exit from the ER. Empty class I dimers that progress beyond the ER recycle back from the intermediate compartment for another opportunity to interact with TAP and acquire peptide (Bresnahan et al., 1997). Class I molecules which ultimately fail to fold properly or acquire peptide are degraded.

Degradation of misfolded or empty class I MHC molecules

Degradation of class I MHC molecules which fail to properly assemble occurs in the cytosol by the action of the proteasome. Retrograde transport of class I MHC molecules from the ER to the cytosol was first demonstrated in cells expressing the human cytomegalovirus US11 gene product (Wiertz et al., 1996a). It was later shown that retrograde transport proceeded via the same Sec61 complex responsible for importing class I molecules into the ER (Wiertz et al., 1996b). These studies showed that the class I heavy chain is first deglycosylated by the activity of an N-glycanase and then degraded in the cytosol by the proteasome. The phenomenon of retrograde transport and proteasomal degradation has also been demonstrated in cells which lack β_2m or are deficient for peptide transport, indicating that this pathway functions normally in non

virally infected cells as a way to degrade empty or misfolded class I molecules (Hughes et al., 1997).

ER chaperones are thought to mediate the degradation of class I molecules. Inhibition of class I-calnexin association accelerates degradation of class I molecules, suggesting that calnexin stabilizes nascent class I molecules that would otherwise be targeted for degradation before having time to properly assemble (Wilson et al., 2000). The observation in yeast of an interaction between BiP and the Sec61 translocation machinery (Plemper et al., 1997) suggests that BiP might help mediate the transport of misfolded class I molecules to the cytosol, as has been proposed by Nössner et al. (Nössner and Parham, 1995).

In the absence of a functional TAP class I molecules have been shown to accumulate in the intermediate compartment (Raposo et al., 1995). The association of ubiquitin and ubiquitin-conjugating enzymes with the intermediate compartment suggests that class I molecules might also be targeted for degradation directly from this post-ER/pre-Golgi compartment.

Endocytosis of class I MHC molecules

Compared to our substantial knowledge of class I MHC assembly, far less is known about mechanisms that control levels of class I expression at the plasma membrane. The fate of class I molecules following antigen presentation has not been

elucidated. Some studies have hinted at internalization of class I molecules, while others have suggested that class I molecules are clipped from the cell surface by the action of a metalloprotease (Anderson et al., 1998; Demaria et al., 1994). Recent discoveries of viral proteins which induce class I MHC internalization (Coscoy and Ganem, 2000; Ishido et al., 2000; Schwartz et al., 1996) have heightened our interest in mechanisms of class I endocytosis. One of the aims of this dissertation is to determine the role of clathrin-coated vesicles (CCV) in the endocytosis of class I MHC molecules in different cell types and to determine whether internalized class I molecules recycle to the plasma membrane or are degraded.

Prior studies of class I MHC molecule endocytosis have yielded contradictory results, most likely attributable to species- and cell-specific differences and to inconsistencies in experimental procedures. Clathrin-coated vesicles have been implicated in class I endocytosis in T lymphocytes and macrophages (Dasgupta et al., 1988; Machy et al., 1987b), while caveolae are thought to mediate uptake in fibroblasts (Huet et al., 1980; Machy et al., 1987a; Stang et al., 1997). A dynamin-1 mutant which inhibits clathrin-mediated endocytosis had no effect on the surface expression of class I molecules in HeLa cells, indicating that in these cells clathrin is probably not involved in constitutive class I endocytosis (Le Gall et al., 2000). Endocytosis of class I molecules in T lymphocytes and macrophages is spontaneous (Dasgupta et al., 1988; Hochman et al., 1991; Machy et al., 1987b; Tse and Pernis, 1984), but in fibroblasts crosslinking

antibodies are required to induce endocytosis (Huet et al., 1980; Machy et al., 1987a; Stang et al., 1997). No uptake of class I molecules is observed in murine B lymphocytes (Machy et al., 1982; Tse and Pernis, 1984), although in human B cells endocytosis of class I molecules is readily observed (Reid and Watts, 1990).

The intracellular fate of endocytosed class I MHC molecules is as unclear as the mechanisms of class I endocytosis. In macrophages, endocytosed class I molecules can be seen in the trans Golgi network (TGN) (Dasgupta et al., 1988). In B cells, class I molecules are found in an early endosomal compartment along with class II MHC molecules and transferrin (Chiu et al., 1999). In T cells and fibroblasts endocytosed class I molecules are reported to be routed to the lysosome for degradation (Huet et al., 1980; Machy et al., 1987b). Recycling of endocytosed class I MHC molecules from an endosomal compartment to the plasma membrane has been observed in a variety of cells types (Abdel Motal et al., 1993; Reid and Watts, 1990; Tse et al., 1986), and the TGN localization of endocytosed class I MHC molecules in human macrophages is suggestive of a recycling pathway (Dasgupta et al., 1988). Recycling of class I MHC molecules may allow for the presentation of exogenous antigens which intersect the trafficking pathway of class I molecules (Gromme et al., 1999). While class I molecules primarily present intracellular antigens, the presentation of exogenous antigens is known to occur (Jondal et al., 1996; Rock, 1996).

Many cell surface receptors contain specific sorting motifs in their cytoplasmic

domains which mediate endocytosis. The role of the cytoplasmic tail of class I MHC molecules (comprised of amino acids 314-341) remains unclear. Spontaneous uptake of HLA-A2 molecules in a leukemia T cell line was found to be dependent on amino acids 325-340 (Vega and Strominger, 1989). In B cells endocytosis of chimeric class I molecules consisting of the extracellular domain of HLA-C and the short cytoplasmic tail of HLA-G (comprised of only 6 amino acids) is impaired compared to that of full-length HLA-C (Davis et al., 1997). However, crosslinkage-induced endocytosis of truncated HLA-A2 molecules (which terminated at amino acid 313) proceeded at levels at or above that of full-length -A2 in the T cell line Jurkat (Gur et al., 1997). Recent studies of the HIV Nef protein have revealed a cryptic sorting motif in the cytoplasmic domain of HLA-A and -B alleles (Le Gall et al., 1998). Nef-induced internalization of class I molecules was found to be dependent upon a tyrosine at position 320 of the class I molecule. HLA-C lacks this crucial residue and is not affected by Nef. Interestingly, deletion of exon 6, which encodes position 320, had no effect on spontaneous uptake of HLA-A2 molecules in leukemic T cells (Vega and Strominger, 1989). More work is necessary to better define the role of specific amino acids in the endocytosis of class I MHC molecules.

That prior studies of class I MHC molecule endocytosis have been performed in different cell types from different species, some under normal conditions and some using crosslinkers to induce endocytosis, explains the disagreement among the data. To fully

determine mechanisms of class I endocytosis and the fate of internalized class I molecules a more unified and consistent approach will be necessary.

Understanding how levels of class I expression are regulated at the plasma membrane will give us better insight into the role class I molecules play in the immune system, perhaps in processes such as positive and negative selection in the thymus and in the presentation of exogenous antigens. Determining whether viral proteins utilize existing internalization pathways or are capable of redirecting class I molecules to normally inaccessible pathways will give us a better understanding of mechanisms by which viruses downregulate class I expression.

Viral inhibition of class I MHC expression

The discovery that adenovirus can prevent antigen presentation by retaining class I MHC molecules in the ER (Andersson et al., 1985; Burgert and Kvist, 1985) has led to an explosion of discoveries of other viral proteins which share the ability to disrupt class I MHC expression (reviewed in (Früh et al., 1999; Miller and Sedmak, 1999) (Table 1.1). Downregulation of class I MHC molecules is one of many immune evasion tactics by which viruses gain a strategic edge in establishing infection (Alcami and Koszinowski, 2000). Class I downregulation also plays a role in maintaining persistent infection and avoiding clearance by the host. For example, the ability of members of the herpesvirus family to downregulate class I MHC molecules helps explain why patients

Table 1.1. Viral inhibition of class I MHC expression.

Virus	Protein	Effect on class I MHC expression	Reference
Adenovirus	**E19**	Binds and retains class I molecules in the ER. Binds TAP and inhibits class I MHC-TAP association.	(Andersson et al., 1985; Burgert and Kvist, 1985)
HCMV	**US2, US11**	Induce retrograde transport of class I heavy chains to the cytosol for degradation.	(Wiertz et al., 1996a; Wiertz et al., 1996b)
	US3	Binds and retains class I molecules in the ER.	(Ahn et al., 1996a; Jones et al., 1996)
	US6	Binds TAP and prevents peptide transport.	(Hengel et al., 1996; Lehner et al., 1997)
	UL18, UL40	Inhibit lysis by NK cells.	(Farrell et al., 1997; Ulbrecht et al., 2000)
	pp65	Inhibits processing of HCMV IE 72 kD protein.	(Gilbert et al., 1996)
HIV	**Nef**	Induces accumulation of class I molecules in the Golgi by increased endocytosis and/or PACS-1-mediated transport.	(Piguet et al., 2000; Schwartz et al., 1996)
	Vpu	Destabilized class I heavy chains.	(Kerkau et al., 1997)
EBV	**EBNA-1**	Resistant to proteasomal degradation.	(Levitskaya et al., 1997)
HSV	**ICP47**	Binds TAP and competes for peptide binding.	(Früh et al., 1995; Hill et al., 1995)
KSHV (HHV8)	**K3, K5**	Induce endocytosis of class I molecules.	(Coscoy and Ganem, 2000; Ishido et al., 2000)

carry these viruses for life (Roizman, 1996). While class I downregulation is detrimental to the infected host, it has proven enlightening for scientists. By uncovering strategies employed by viruses to disrupt the expression of class I MHC molecules, we have gained insight into previously unknown processes involved in class I assembly and trafficking.

Adenovirus

Adenovirus types 2 and 5 (members of the group C adenoviruses) encode at least five gene products dedicated to immune evasion, four of which are encoded in the E3 region of the viral genome (Wold and Gooding, 1991) E3-19K (E19), retains class I MHC molecules in the ER, preventing their cell surface expression (Andersson et al., 1985; Burgert and Kvist, 1985). The other four proteins, E3-10.5K/14.5K, E3-14.7K, and E1B-19K affect the immune response at a later stage, preventing Fas- and tumor necrosis factor-mediated cell death (Gooding et al., 1988; Gooding et al., 1991; Shisler et al., 1997). The E3 region of the adenovirus genome is unique in that it contains an NFκB binding site, allowing for transcription of this region in lymphoid cells (Williams et al., 1990). Responsiveness to NFκB and the fact that the E3 region encodes proteins which counteract immune surveillance explains why group C adenoviruses persists in lymphoid tissue (Horwitz, 1996). The entire E3 region is dispensable for viral replication (Morin et al., 1987), yet adenoviruses continue to maintain this portion of their genome (Adrian et al., 1989a; Adrian et al., 1989b), indicating that the region confers a selective advantage

to the virus. Surprisingly, deletion of the E3 region results in more severe viral pathology in the lungs of cotton rats (Ginsberg et al., 1989). This finding is informative for two reasons: first, that adenovirus pathology is due, at least in part, to the activity of the immune system; second, that a milder pathology must be advantageous to the virus, perhaps by promoting viral spread from hosts whose infection is not obvious to those around them.

Adenovirus E19 binds class I MHC molecules and retains them in the ER

Adenovirus E19 is an ER-resident glycoprotein which binds class I MHC molecules (Kvist et al., 1978; Signäs et al., 1982) and retains them in the ER (Andersson et al., 1985; Burgert and Kvist, 1985). In $E19^+$ cells class I MHC molecules fail to progress through the Golgi apparatus and are not expressed on the cell surface. As a result, the CTL response against $E19^+$ cells is greatly diminished (Burgert et al., 1987; Cox et al., 1990).

E19 carries a dilysine-based (KKMP) ER-retention signal at its C-terminus (Jackson et al., 1990; Nilsson et al., 1989). This discovery of this motif led to the hypothesis that E19 retains bound class I molecules in the ER by virtue of its ER-retention signal and does not affect class I assembly (Cox et al., 1991). However, some researchers have suggested that E19 prevents peptide loading of class I molecules

(Feuerbach and Burgert, 1993). As discussed in the previous section, E19 enhances the association of class I molecules with APLP2, and APLP2 can be released upon class I peptide loading. While a direct effect of E19 on peptide loading has been difficult to detect (see Chapter 2), the finding that the viral protein increases the interaction of class I molecules with APLP2 suggests thatE19 may affect peptide acquisition by class I molecules. This idea is supported by the data presented in Chapter 2 of this dissertation which shows that E19 binds TAP and diminishes class I-TAP association (Bennett et al., 1999) (Fig. 2.3).

E19's ER lumenal domain mediates class I MHC binding, while its transmembrane and cytosolic domains are responsible for ER retention and dimerization of the viral protein (Pääbo et al., 1986). E19 exhibits allelic specificity towards class I MHC molecules (Beier et al., 1994; Burgert and Kvist, 1987). While E19 binds HLA-A2 and -B7 very well, it binds HLA-Aw68, -B27, and -Bw58 much less efficiently (Beier et al., 1994). Although E19 impedes the intracellular transport of these latter alleles, a fraction of HLA-Aw68, -B27, and -Bw58 molecules do eventually escape to the cell surface. The allelic specificity of E19 may mean that individuals are differentially susceptible to adenovirus infection, though this possibility has not been investigated. Studies using chimeric class I alleles and antibodies against specific portions of the class I MHC molecule have identified the $\alpha 1$ and $\alpha 2$ domains of the class I heavy chain as the E19 binding region (Beier et al., 1994; Burgert and Kvist, 1987; Feuerbach et al., 1994;

Flomenberg et al., 1994; Jeffries, 1990). That this region is also the class I peptide binding region further hints at a role for E19 in disrupting peptide loading of class I MHC molecules.

Human Cytomegalovirus (HCMV)

Of all viruses capable of inhibiting class I MHC expression, HCMV is unequivocally the most fascinating. Early studies of the effect of HCMV on the expression of class I MHC molecules revealed that in HCMV-infected cells class I molecules are inherently unstable and are degraded shortly after synthesis (Beersma et al., 1993; Warren et al., 1994; Yamashita et al., 1994). Further studies revealed that HCMV has multiple gene products capable of downregulating class I molecules (Jones et al., 1995). It is now clear that HCMV launches a formidable attack on class I expression by inhibiting multiple steps in the class I assembly pathway. HCMV has at least five gene products dedicated to preventing class I antigen presentation (Barnes and Grundy, 1992; Warren et al., 1994; Yamashita et al., 1993) and two more to preventing lysis by natural killer (NK) cells (Farrell et al., 1999; Tomasec et al., 2000). These immune evasion strategies are reflected in the ability of HCMV to establish lifelong infection in the host (Britt and Alfold, 1996). While HCMV infection is usually mild or asymptotic, reactivation of latent virus in immunocompromised hosts (particularly HIV patients and organ transplant recipients) is extremely problematic, making it of great medical interest

to better understand HCMV's immune evasion strategies. As a laboratory tool, HCMV has revealed a cytosolic degradation pathway for class I MHC molecules following their retrograde transport from the ER (Wiertz et al., 1996a; Wiertz et al., 1996b), illustrating the usefulness of pathogens for studying constitutive cellular processes.

HCMV US2 and US11 induce retrograde transport of class I MHC molecules to the cytosol for degradation

HCMV US2 and US11 are ER resident proteins capable of inducing the retrograde transport of class I MHC heavy chains from the ER to the cytosol where they are degraded by the proteasome (Wiertz et al., 1996a; Wiertz et al., 1996b). This model is based on the fact that in cells expressing US2 or US11 an intermediate class I degradation product can be recovered from the cytosol when cells are treated with proteasome inhibitors. The degradation product is a deglycosylated class I heavy chain, indicating that in $US2^+$ and $US11^+$ cells class I molecules are inserted into the ER and glycosylated but are quickly (within minutes (Wiertz et al., 1996a)) transported to cytosol where an N-glycanase removes the N-linked oligosaccharide. In $US11^+$ cells, β_2m is secreted into the cell medium, reflecting the dearth of heavy chain binding partners (Wiertz et al., 1996a). While US2 associates primarily with free class I heavy chains, heavy chain-β_2m dimers are also susceptible to retrograde transport and degradation (Jones and Sun, 1997). In the presence of US2, the class I heavy degradation product

associates with both the Sec61 complex and the proteasome (Wiertz et al., 1996b). Association with the Sec61 complex and the fact that retrograde transport is ATP-dependent suggest that class I molecules exit the ER via the same transport complex through which they enter. Association of class I molecules with the Sec61 complex can be induced by treatment with DTT, which prevents proper folding of class I molecules, indicating that even in normal cells a retrograde degradation pathway exists for misfolded class I molecules (Wiertz et al., 1996b). This finding was confirmed using TAP^- and β_2m^- cell lines in which class I molecules cannot properly assemble (Hughes et al., 1997). Treatment of these cells with proteasomal inhibitors resulted in the appearance of a class I degradation product in the cytosol.

US2 has also been shown to induce the proteasomal degradation of class II MHC molecules (Tomazin et al., 1999). Unlike class I molecules, in $US2^+$ cells treated with proteasome inhibitors class II molecules do not appear in the cytosol. Therefore class II molecules seem to be transported directly from the ER into the lumen of the proteasome. This model is supported by the observed association of the proteasome with the ER membrane (Yang et al., 1998). Whether class I molecules can also pass directly into the lumen of the proteasome remains to be established.

Though US2 and US11 exert the same effect on class I MHC molecules, some differences do exist between the two proteins. A direct interaction with class I molecules has only been observed for US2 (Wiertz et al., 1996b). Whether US11 interacts with

class I molecules directly or indirectly and whether the two viral proteins act in concert remains to be determined. Though the allelic specificity of US2 and US11 has not been studied in humans, the two viral proteins exhibit allelic preferences towards murine class I alleles (Machold et al., 1997). Additionally, US2 has also been shown to escort class I heavy chains into the cytosol, while US11 remains in the ER (Wiertz et al., 1996b).

HCMV US3 binds class I MHC molecules and retains them in the ER

The HCMV US3 protein is an ER resident glycoprotein which binds class I MHC molecules, resulting in their ER retention (Ahn et al., 1996a; Jones et al., 1996). Unlike HCMV-infected cells, $US3^+$ cells do not exhibit decreased stability of class I molecules. Rather, in the presence of US3 class I molecules do acquire peptide (Ahn et al., 1996a) but are impaired in their transport from the ER to the Golgi.

US3 is expressed during the immediate early phase of HCMV infection and precedes the expression of US2 and US11 (Tenney and Colberg-Poley, 1991). A viable model for HCMV downregulation of class I MHC molecules is that US3 retains class I molecules in the ER until US2 and US11 can transport them to the cytosol for degradation. This model is supported by the fact that US3-retained class I heavy chains are susceptible to retrograde transport and degradation in the presence of US2 and US11 (Jones and Sun, 1997).

Comparison of HCMV US3 and adenovirus E19

ER-retention of proteins containing a KKXX ER-retention motif is due to retrograde transport from the Golgi to the ER mediated by the COP1 coatomer complex (Springer et al., 1999). Via this retrieval mechanism, E19-class I complexes that exit the ER are retrieved from the Golgi and transported back to the ER. Unlike E19, the cytosolic domain of US3 does not confer ER-retention to the viral protein. Instead, US3 associates only transiently with class I molecules and then progress to the Golgi, ultimately being diverted to the lysosome for degradation (Gruhler et al., 2000). Rather than functioning via an ER-retrieval mechanism, US3 seems to impair class I trafficking by excluding class I molecules from forward transport. Because the association of US3 with class I molecules is only transient, a continuous pool of newly synthesized US3 is necessary to cause persistent ER-retention of class I complexes. A crucial observation leading to this model is that in radiolabeled cells the amount of US3 coprecipitated with class I molecules declines with time, even though the class I molecules themselves remain EndoH sensitive (Gruhler et al., 2000). This finding is similar to the data in Fig. 2.1, which shows that the levels of E19 which coprecipitate with class I molecules also diminishes with time (Bennett et al., 1999). While the ER-retaining mechanisms of US3 and E19 are clearly distinct, the tendency for the viral proteins to associate transiently with class I molecules before being replaced by newly synthesized protein seems to be a shared phenomenon.

HCMV pp65 and US6 inhibit peptide generation and transport by TAP

The HCMV 72K immediate early (IE) protein is expressed prior to HCMV inhibition of class I MHC expression. However, although T cells stimulated *in vitro* can lyse cells expressing IE, few IE-responsive CTLs are detected in HCMV-infected individuals. An HCMV matrix protein, pp65, which is released into the cytosol upon viral entry, phosphorylates IE, preventing IE from being processed by the antigen processing machinery (Gilbert et al., 1996). Whether phosphorylation prevents IE from interacting with the proteasome or diverts IE to a different degradation pathway is unclear, though the result is that IE peptides are not presented by class I molecules. The ability to prevent antigen processing is not unique to HCMV. The Epstein-Barr virus nuclear antigen (EBNA)-1 contains an internal glycine-alanine repeat motif which prevents its own processing (Levitskaya et al., 1995).

HCMV US6 is an ER-resident glycoprotein which binds TAP and inhibits the translocation of peptides into the ER (Hengel et al., 1996; Lehner et al., 1997). In the presence of US6, class I molecules fail to bind peptide and do not elicit a CTL response. US6 interacts with TAP via its lumenal domain (Ahn et al., 1997) and is found in complexes with class I dimers, tapasin, calnexin, calreticulin, and TAP, although the presence of class I heavy chain and tapasin is not required for US6-TAP association (Hengel et al., 1997). US6 does not affect the binding of peptides to TAP but inhibits the transporter from importing peptides into the ER.

HCMV infection increases TAP phosphorylation

It was recently shown that in HCMV-infected cells TAP is phosphorylated and peptide transport is reduced by 25% (Li et al., 2000). As discussed above, phosphorylation of ABC transporters regulates their activity, and phosphorylation of TAP may control levels of peptide transport. HCMV is the first virus known so far to exploit this feature of TAP.

HCMV UL18 and UL40 inhibit NK cell recognition

Natural killer cells express receptors which recognize class I MHC molecules on target cells and transmit an inhibitory signal to the NK cell (Lanier, 1998). In the absence of class I molecules, NK cells are activated and mount a cytotoxic attack against class I-negative cells. This phenomenon has been referred to as the "missing self" hypothesis (Kärre, 1995) and provides a model for how our immune system deals with pathogens which downregulate class I MHC expression. HCMV is a member of the herpesvirus family, and NK cells are known to play a crucial role in immunological control of these viruses (Biron et al., 1989). However, HCMV-infected cells do not seem to be susceptible to lysis by NK cells, indicating that the virus must have evolved mechanisms for escaping NK cell recognition. HLA-C alleles are resistant to the degratory effects of US2 and US11, and their presence on the cell surface may be sufficient to prevent an NK cell response (Schust et al., 1998). Additionally, two HCMV proteins may ward off an

NK cell attack. HCMV UL18 is a class I homologue that binds β_2m and peptide and can prevent NK cell-mediated lysis of a target cell (Beck and Barrell, 1988; Browne et al., 1990; Fahnestock et al., 1995; Farrell et al., 1997). HCMV UL40 contains a leader sequence that can be presented to NK cells by HLA-E (Ulbrecht et al., 2000). HLA-E expression is actually upregulated during HCMV infection (Tomasec et al., 2000) and might play a vital role in inhibiting an NK cell response against HCMV-infected cells.

Herpes Simplex Virus (HSV)

HSV ICP47 inhibits TAP

Though all herpes viruses establish lifelong infection in the host (Roizman, 1996), HSV is perhaps the best known virus to possess this ability. HSV-infected cells show reduced levels of class I MHC expression and are resistant to CTL lysis (Hill et al., 1994; Jennings et al., 1985; Koelle et al., 1993). The gene product responsible for loss of class I surface expression was identified as the cytosolic protein ICP47 (York et al., 1994). ICP47's cytosolic location and the instability of class I molecules in cells expressing the viral protein (York et al., 1994) hinted at a role for ICP47 in preventing the generation or translocation of antigenic peptides. This hypothesis was confirmed when it was found that TAP-mediated transport of peptides does not occur in ICP47$^+$ cells (Früh et al., 1995; Hill et al., 1995). Studies of the interaction of ICP47 with TAP revealed that ICP47 binds the peptide binding domain of TAP and competes with peptide for binding to the

transporter (Ahn et al., 1996b; Tomazin et al., 1996). In the absence of transported peptide, class I molecules do not properly assemble and are not expressed on the surface of the cell.

Human Immunodeficiency Virus (HIV)

In one sense, HIV circumvents the need for creative immune evasion strategies by infecting T cells and crippling the immune system. However, even HIV expresses at least two proteins which prevent class I MHC expression. The HIV Vpu protein degrades class I heavy chains early after their synthesis by an uncharacterized mechanism (Kerkau et al., 1997). HIV Nef diminishes surface expression of class I molecules, inhibiting the CTL response against Nef$^+$ cells (Collins et al., 1998; Schwartz et al., 1996). The effect of Nef on class I expression is discussed below.

HIV Nef induces class I MHC endocytosis and accumulation in the TGN

The HIV Nef (*ne*gative *f*actor) protein has been ascribed numerous functions, including increased viral infectivity, disruption of cellular signaling pathways, and internalization of cell surface proteins (Peter, 1998). The fact that Nef is required for efficient HIV replication and AIDS induction (Kestler et al., 1991) has led to intense efforts to better define the role of Nef in HIV pathogenesis.

Nef has been shown to decrease the surface expression of both class I MHC

molecules and CD4 , the primary receptor for HIV (Dalgleish et al., 1984; Garcia and Miller, 1991; Schwartz et al., 1996). Downregulation of class I MHC molecules is clearly advantageous to the virus form an immunological standpoint by preventing CTL lysis of HIV-infected cells (Collins et al., 1998). The purpose of CD4 downregulation was first hypothesized to be prevention of superinfection with HIV (Benson et al., 1993). However, recent evidence points to a role for Nef in increasing virion release and infectivity by preventing CD4 from interacting with the HIV envelope protein gp120 during virion budding from the cell (Lama et al., 1999; Ross et al., 1999). Nef does not affect the biosynthesis or transport of class I or CD4 molecules(Garcia and Miller, 1991; Mangasarian et al., 1997; Sanfridson et al., 1994; Schwartz et al., 1996); instead enhanced endocytosis of these proteins is the mechanism of Nef-induced downregulation (Garcia and Miller, 1991; Schwartz et al., 1996). Nef-induced endocytosis is best characterized for CD4, which is internalized in clathrin-coated vesicles in the presence of the viral protein (Mangasarian et al., 1997). The mechanism of Nef-induced class I MHC endocytosis is still being elucidated.

Early studies of the effect of Nef on class I molecule internalization focused mainly on the role of CCV. These studies followed investigations of Nef-induced CD4 internalization, which occurs via CCV. The evidence for a clathrin-mediate Nef-induced endocytic mechanism came from studies which showed that Nef colocalizes and associates with components of the clathrin adaptor proteins (AP) (Bresnahan et al., 1998;

Gall et al., 1998; Greenberg et al., 1998a; Greenberg et al., 1997; Piguet et al., 1998) (adaptor proteins are discussed in the next section) and that Nef causes an increase in the formation of clathrin-coated pits (Foti et al., 1997) and the accumulation of endosomes in T cells (Sanfridson et al., 1997). In the presence of Nef, CD4 colocalizes with AP2 (Greenberg et al., 1997), accumulates in early endosomes (Schwartz et al., 1995), and is degraded in an acidic compartment (Sanfridson et al., 1994), further supporting a clathrin-mediated, Nef-induced endocytic pathway. A model has been developed which proposes that Nef induces clathrin-mediated internalization of CD4 by connecting CD4 to components of the clathrin sorting machinery (Mangasarian et al., 1997; Oldridge and Marsh, 1998). However, mutations in Nef that inhibit internalization of CD4 do not inhibit Nef-induced class I MHC internalization (Greenberg et al., 1998a; Greenberg et al., 1997), and different Nef motifs mediate downregulation of CD4 and class I MHC molecules (Greenberg et al., 1998a; Mangasarian et al., 1999; Riggs et al., 1999). Therefore results from studies of the effect of Nef on CD4 cannot be directly extrapolated to studying the effect of Nef on class I MHC expression.

Studies of Nef-induced class I MHC internalization in lymphoid cells have suggested that like CD4, class I MHC molecules are internalized via CCV in the presence of Nef. In Nef$^+$ CEM cells class I molecules localize to endosomes and are degraded (Schwartz et al., 1996). However, a clathrin-mediated mechanism of Nef-induced class I MHC internalization has not been universally supported. In Nef$^+$ fibroblasts class I

molecules are internalized from the cell surface, accumulate in the TGN, and colocalize with AP1 (Greenberg et al., 1998b). Using the epithelial cell line HeLa, Le Gall et al. have shown that while Nef-induced CD4 internalization is dynamin-1-dependent, the internalization of class I MHC molecules induced by Nef occurs independently of dynamin (Le Gall et al., 2000). Dynamin plays a role in the formation of CCV (as discussed in the following section). Therefore these data strongly point to a non-clathrin-mediated pathway for Nef-induced class I internalization in certain cell types. A role for caveolae in Nef-induced endocytosis has not yet been investigated, but in light of the fact that caveolae are implicated in class I endocytosis in some cell types, the interaction of Nef with caveolae could be an interesting area of study.

Studies of Nef-induced class I internalization have revealed a cryptic sorting motif in the cytosolic domain of HLA-A and -B alleles, which contain a tyrosine at position 320, alanine at position 324, and aspartic acid at position 327 (Cohen et al., 1999; Le Gall et al., 1998). HLA-C lacks tyrosine 320, and HLA-E lacks the alanine and aspartic acid residues, making these alleles resistant to Nef-induced downregulation and enabling them to prevent an NK cell attack (Cohen et al., 1999). Thus HIV, like HCMV, has evolved mechanisms not only to prevent class I antigen presentation but also to respond to our immune system's backup defenses by preventing an NK cell response as well. Le Gall et al. have mutated the $Y_{320}SQA_{324}$ motif to further elucidate mechanisms of class I endocytosis (Le Gall et al., 2000). They created HLA-A2 molecules with

prototypic tyrosine-based internalization motifs: Y_{320}SQL and Y_{320}SQI. YXXΦ motifs, where X represents any amino acid, and Φ is a residue with a hydrophobic side chain, have been shown to mediate clathrin-dependent protein trafficking by interacting with the μ subunits of AP1 and AP2 (Ohno et al., 1995). When HLA-A2 molecules containing such motifs were expressed in HeLa cells they showed reduced cell surface expression. Surface expression could be decreased even further by the expression of Nef, indicating that Nef and the clathrin machinery act upon class I molecules via different class I determinants. Class I molecules with prototypic internalization motifs were internalized to the Golgi and colocalized with AP1 and rab6. This localization is identical to that for wildtype class I alleles internalized in the presence of Nef. However, while wildtype class I alleles do not show evidence of recycling from this location, alleles containing internalization motifs did recycle back to the plasma membrane, and Nef had no effect on their recycling. These data further indicate that, at least in HeLa cells, Nef acts on class I molecules via a mechanism that is distinct from clathrin-mediate processes.

Piguet et al. recently shed light on the mechanism of Nef-induced class I accumulation in the TGN (Piguet et al., 2000). Three regions of Nef are required for the viral protein to exert its effect on class I expression: a myristylation signal which tethers Nef to the plasma membrane, an SH3-binding, proline-based repeat, and a conserved cluster of acidic residues (Greenberg et al., 1998b). Nef's acidic cluster shares homology

with the PACS (phosphofurin acidic cluster sorting protein)-1 binding domains of furin and mannose-6-phosphate receptor (M6PR). PACS-1 is a coat protein which mediates trafficking of furin and M6PR from endosomes to the Golgi apparatus by linking these molecules to AP1 (Wan et al., 1998). In cells expressing antisense PACS-1 and Nef, Nef fails to relocate class I molecules to the TGN, and instead class I molecules remain on the cell surface. Nef was shown to coprecipitate with PACS-1 and seems to act as a connector between endocytosed class I molecules and the PACS-1 sorting machinery. The model presented by Piguet et al. points to a role for Nef in redirecting endocytosed class I molecules to the TGN, though whether Nef actually induces accelerated endocytosis of class I molecules is unclear. The internalization of class I molecules in the presence of Nef is not as rapid as that of CD4, and it may be that Nef exerts it primary effect on class I molecules not by accelerating their endocytosis but by redirecting endocytosed molecules to the TGN. Le Gall et al. found that class I molecules are endocytosed quite slowly in HeLa cells, with only 5% of the molecules endocytosed in 5 minutes (Le Gall et al., 2000). Class I alleles containing prototypic internalization motifs were endocytosed at a much higher rate, with 50% of the molecules internalized in 5 minutes. Although Nef increased the rate of endocytosis of class I molecules, even after 30 minutes up to 70% of the molecules remained on the cell surface. The minor effect of Nef on the rate of class I MHC endocytosis suggests that Nef's primary role is in redirecting class I molecules once they have been endocytosed.

The mechanism of Nef-induced class I MHC downregulation is a focus of this dissertation. To better understand how Nef affects trafficking of class I molecules more work is needed to first identify constitutive pathways for class I endocytosis and to determine the fate of internalized class I molecules. It will then be necessary to determine whether Nef utilizes (or alters) these pathways or accesses novel pathways. Additionally, both constitutive and Nef-induced class I endocytosis should be studied in different cell types, as mechanisms of endocytosis clearly vary with cell type. Of particular interest is the effect of Nef on class I MHC molecules in cells commonly infected by HIV, such as T cells and macrophages. Studies in these cell types will help us understand the role that class I MHC downregulation plays during actual HIV infection. Experiments to address these issues are proposed in Chapter 4.

Kaposi's sarcoma-associate herpesvirus (KSHV)

KSHV 3 and 5 induce class I MHC endocytosis

KSHV (also called human herpes virus 8) is associated with the development of Kaposi's sarcoma (Whitby and Boshoff, 1998). Two KSHV proteins, K3 and K5, have recently been shown to cause enhanced endocytosis of class I MHC molecules (Coscoy and Ganem, 2000; Ishido et al., 2000). In the presence of K3 or K5, class I molecules assemble, acquire EndoH resistance, and traffic to the surface of the cell but are then quickly internalized and degraded in a lysosomal compartment. K3 and K5 differ in their

ability to downregulate class I molecules: K3 downregulates all class I alleles, while K5 is inhibited in its ability to induce endocytosis of HLA-C and -E alleles (Ishido et al., 2000). The transmembrane and cytosolic domains of the class I heavy chain appear to mediate K3/K5-induced endocytosis (Ishido et al., 2000), though no association of the viral proteins with class I molecules has been observed (Coscoy and Ganem, 2000). It is not even clear where the viral proteins are localized in the cell, as they have been reported to reside primarily in the cytosol and at the plasma membrane (Ishido et al., 2000) as well as being associated with the ER membrane (Coscoy and Ganem, 2000). Further work is necessary to determine the mode of action of K3 and K5, although preliminary results indicate that K5-mediated class I downregulation is via endocytosis in clathrin-coated pits (Coscoy and Ganem, 2000).

Evidence for coevolution

That so many viruses have developed strategies to inhibit class I MHC expression speaks to the fact that viruses face a constant challenge from the host's immune system. Immune evasion genes are often dispensable for viral replication; viruses have acquired these genes over the course of evolution and seem to maintain them only in the face of evolutionary pressure from the host. The existence of NK cells highlights the evolutionary pressure on the host to maintain backup lines of defense against pathogens which disrupt class I MHC expression. Even here coevolution is evident, as viruses such

as HCMV, HIV, and KSHV have all evolved such that their class I downregulating processes do not affect HLA-C and -E, class I alleles known to inhibit an NK cell response (Borrego et al., 1998; Colonna et al., 1993). As our immune system continues to coevolve with viral pathogens, it will be interesting to see if in the next million years it adapts to uniquely recognize cells which express only HLA-C and -E alleles.

Figure 1.6. Viral inhibition of class I MHC moleulce assembly and trafficking and NK cell-mediated lysis

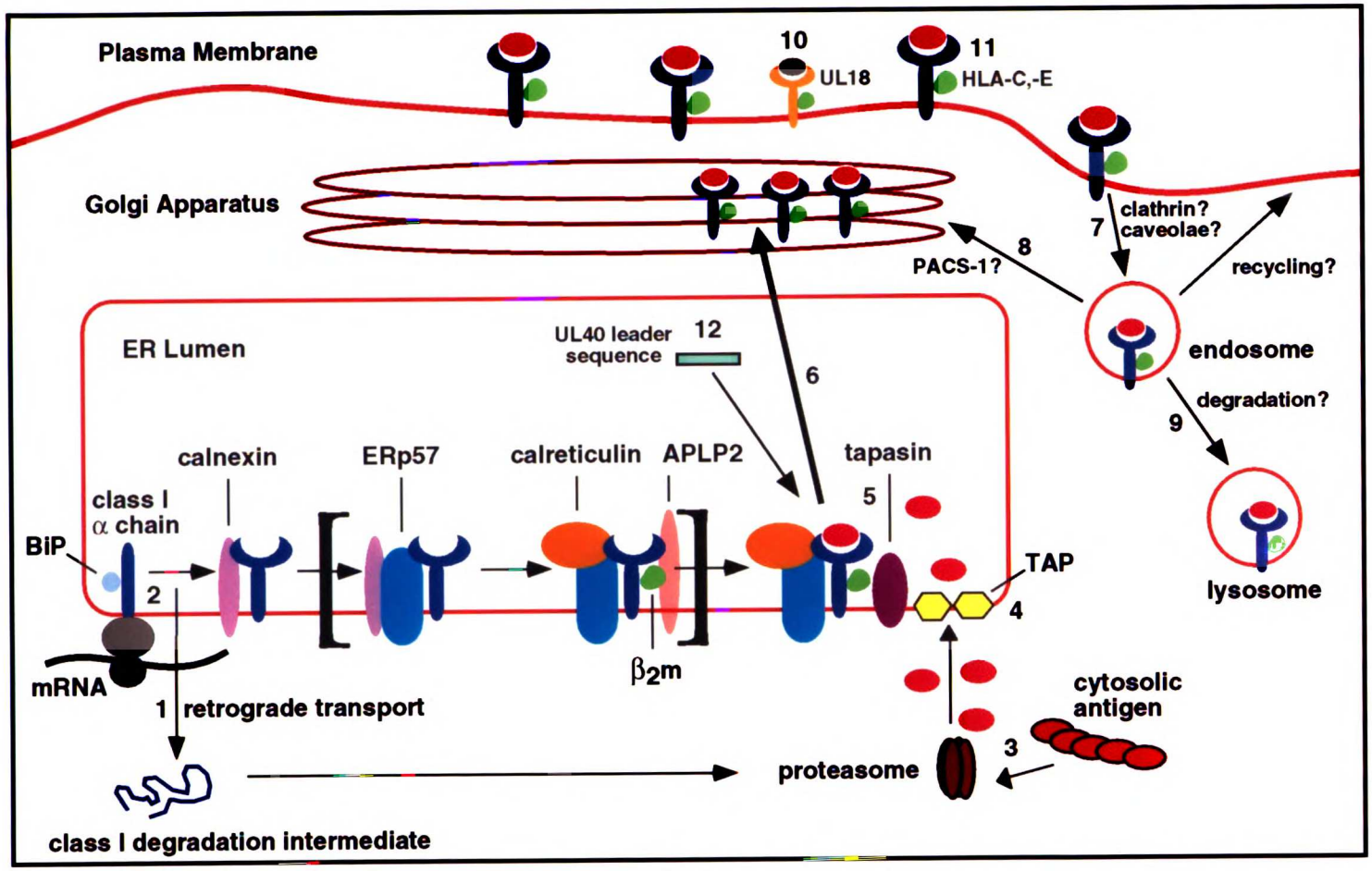


Figure 1.6. **Viral inhibition of class I MHC molecule assembly and trafficking and NK cell-mediated lysis.**

Viral gene products which inhibit class I MHC expression act at the following steps: (1) HCMV US2 and US11 induce retrograde transport of class I heavy chains into the cytosol for degradation by the proteasome. (2) HIV Vpu causes degradation of nascent class I heavy chains by an uncharacterized mechanism. (3) HCMV pp65 phosphorylates the HCMV IE 72 kD protein, preventing its antigen processing. EBV EBNA-1 contains an internal gly-ala repeat motif which prevents its own proteasomal degradation. (4) HSV ICP47 and HCMV US6 inhibit TAP. ICP47 competes with peptides for TAP binding. US6 prevents peptide transport. (5) Adenovirus E19 inhibits the association of class I molecules with TAP. (6) Adenovirus E19 and HCMV US3 retain class I molecules in the ER. (7) HIV Nef and KSHV K3 and K5 induce endocytosis of class I molecules. (8) In certain cell types HIV Nef causes accumulation of class I molecules in the TGN by inducing PACS-1-mediated transport from endosomes. (9) In certain cell types HIV Nef- and KSHV K3- and 5-induced endocytosis leads to class I degradation in the lysosome.

Viral gene products which inhibit NK cell-mediated lysis act at the following steps: (10) HCMV UL18 acts as a class I MHC mimic at the cell surface. (11) HCMV UL40 contains a leader sequence which can be presented by HLA-E. (12) HIV, HCMV, and

KSHV selectively affect HLA-A and -B class I alleles, allowing HLA-C and -E alleles to inhibit an NK cell response.

Modified with permission from Immunological Reviews, 1999, Volume 168: 199-215.

The role of clathrin and the cytoskeleton in transferrin receptor endocytosis and recycling

Eukaryotic cells contain numerous membrane-bound compartments with specialized functions. These compartments require specific protein components to maintain their function. In addition, the protein composition of the plasma membrane must be regulated to control signaling events and maintain proper uptake and secretion of various molecules. The need for temporal and spatial control of protein distribution calls for an elaborate system of protein trafficking within a cell (Rothman and Wieland, 1996). The transport of proteins between membranes occurs via membrane vesicles which acquire cargo proteins by budding from a donor membrane and deliver the proteins to a target membrane by membrane fusion. Coat proteins mediate membrane budding and trafficking events, with distinct coats responsible for protein trafficking between different membranes (Schekman and Orci, 1996). The clathrin coat mediates receptor-mediated endocytosis at the plasma membrane and lysosomal targeting of proteins from the TGN. Chapter 3 of this dissertation addresses the role of clathrin in receptor recycling following RME.

Clathrin and adaptor proteins

Clathrin-coated vesicles mediate the selective transport of integral membrane proteins between cellular membranes (reviewed in Kirchhausen, 2000). At the plasma

membrane, clathrin mediates receptor-mediated endocytosis (RME), targeting surface receptors to the endosomal compartment. At the TGN, clathrin targets lysosomal proteins to the lysosome. Clathrin molecules form triskelion subunits composed of three clathrin heavy chains and three clathrin light chains which polymerize on membranes into the distinctive polyhedral structure of a CCV. Fig. 1.7 shows the structure of the clathrin triskelion and an image of a clathrin-coated vesicle budding from the plasma membrane. Completion of the polyhedral clathrin cage causes budding vesicles to dissociate from the membrane. After an uncoating step, vesicles fuse with target organelles, delivering cargo proteins to their destination.

Adaptor proteins in the cytosol facilitate the association of clathrin triskelions with membrane proteins (Pearse and Robinson, 1990) and from a layer between the invaginating membrane and the surrounding clathrin coat (Vigers et al., 1986). AP1 acts at the TGN, while AP2 functions at the plasma membrane to mediate RME. The domain structures of AP1 and AP2 are shown in Fig. 1.8. The complexes consist of a core domain joined to two ear domains via a region called the hinge domain. The hinge region of the AP2 β subunit has been shown to associate with the terminal domain of clathrin (Greene et al., 2000; Shih et al., 1995), while the α subunit mediates interaction with lipid membranes (Chang et al., 1993). Fig. 1.9 shows the steps involved in AP2-mediated clathrin-receptor association at the plasma membrane and the subsequent vesicle budding and uncoating steps.

Figure 1.7

Clathrin structure and assembly

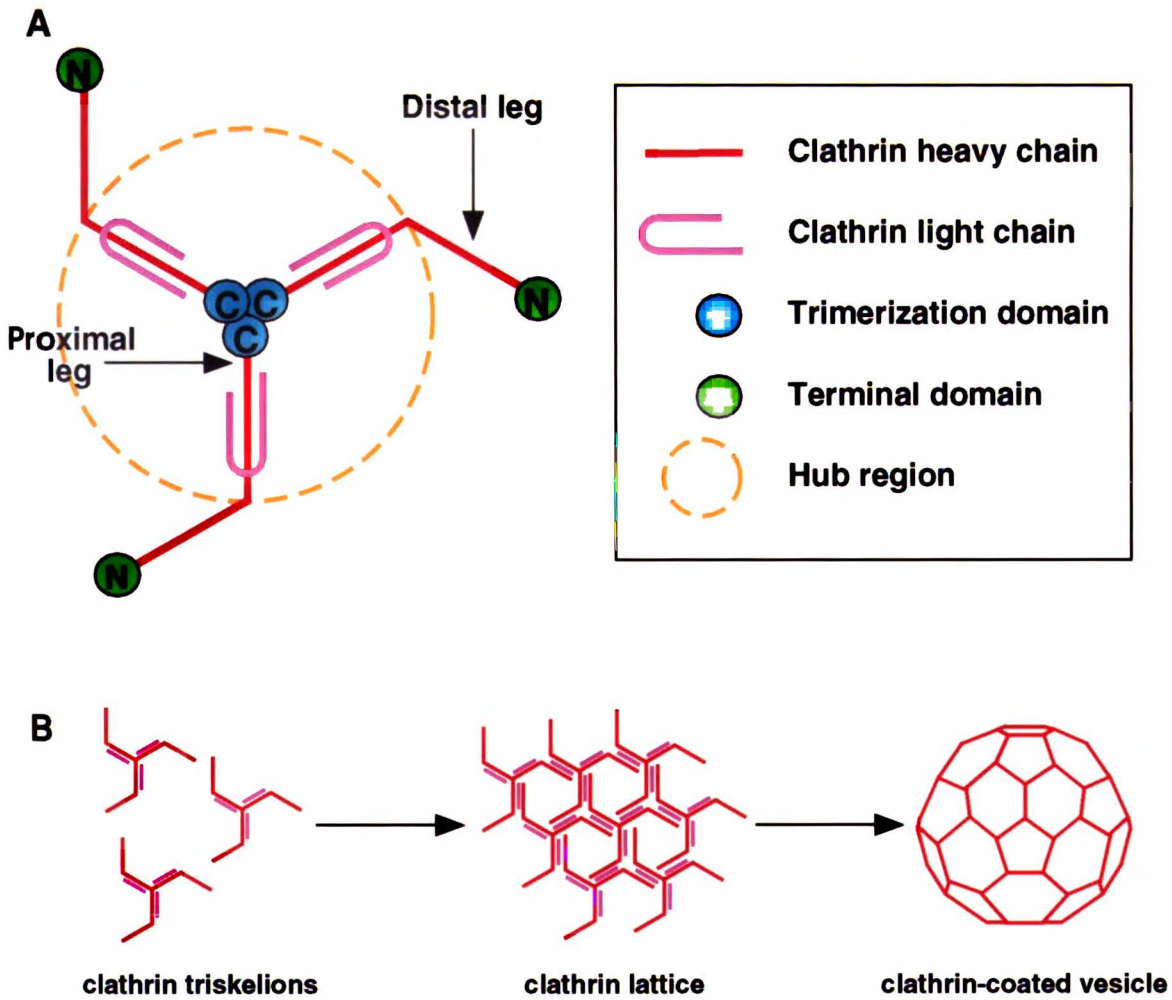


Figure 1.7. **Clathrin structure and assembly.**

(A) The clathrin triskelion is composed of three clathrin heavy chains (192 kD, shown in red) and three clathrin light chains (25-29 kD, shown in purple). The three heavy chains interact with each other via their trimerization domains (shown in blue) and with adaptor proteins and accessory molecules via their terminal domains (shown in green). Hub (outlined in orange) comprises the C-terminal third (proximal leg and trimerization domain) of the clathrin heavy chain.

(B) Clathrin triskelions self assemble into a repeating lattice. Clathrin heavy chains are nonplanar, such that polymerization on a membrane induces membrane curvature. The final polyhedral lattice of a clathrin-coated vesicle is composed of repeating pentagonal and hexagonal units. Light chains are incorporated into the final clathrin coat, though for simplicity they are not shown in this figure.

(C) Deep-etch image of the cytosolic side of a plasma membrane showing the polygonal lattices of a clathrin-coated pit and an invaginating clathrin-coated vesicle (scale bar 33 nm). Reproduced with permission from *The Journal of Cell Biology*, 1987, Volume105: 1999-2009.

Figure 1.8

Structure of clathrin adaptor protein complexes

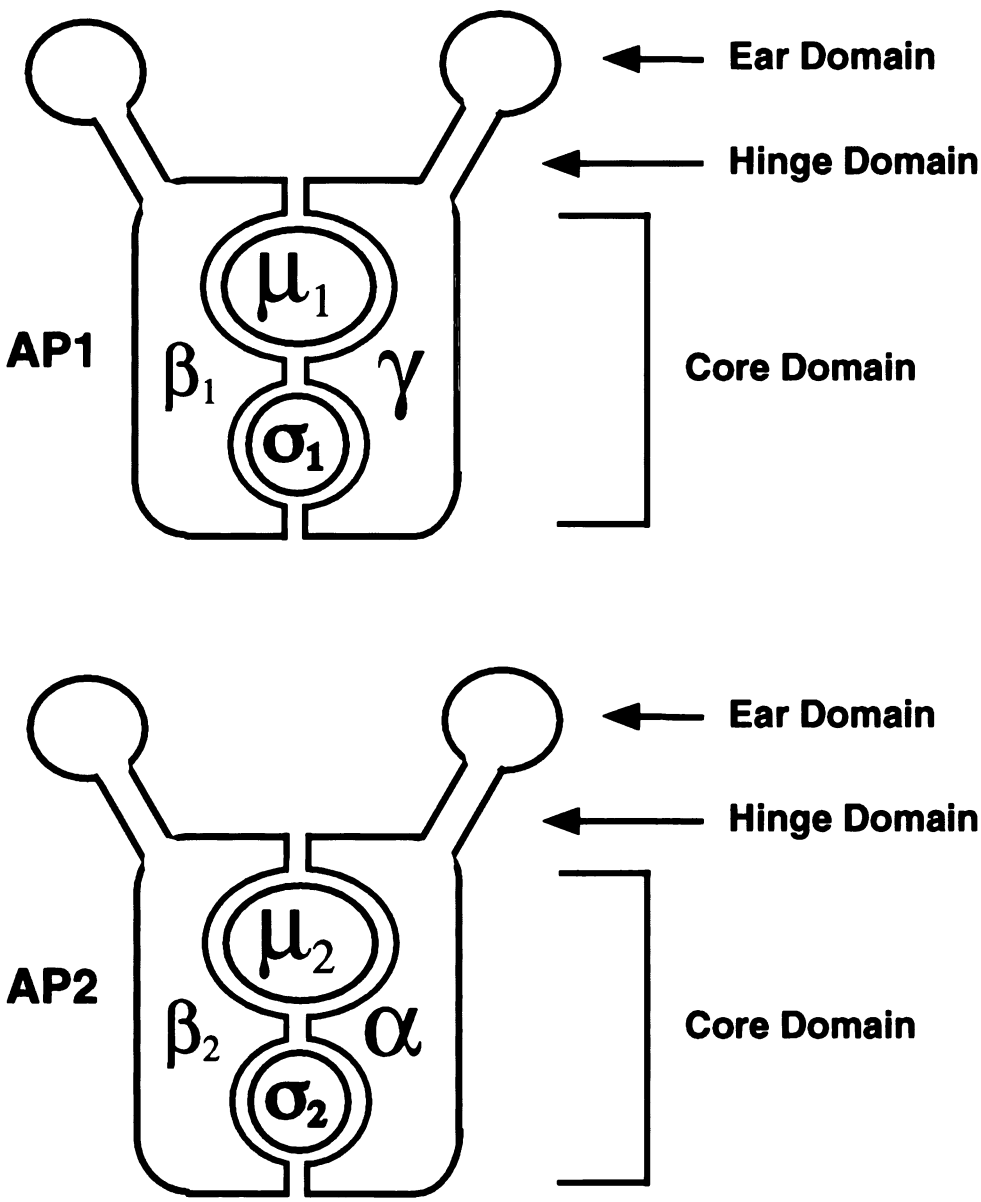


Figure 1.8. **Structure of clathrin adaptor protein complexes.**

Clathrin adaptor proteins are composed of four subunits: α, β_1, σ_1, and μ_1 for AP1 and γ, β_2, σ_2, and μ_2 for AP2. The four subunits adopt a structure consisting of three types of domains: a core domain, two ear domains, and two hinge domains which connect the ears to the core. Adaptor proteins act to connect clathrin to membrane proteins and concentrate protein cargo in developing clathrin-coated pits. AP1 functions at the TGN; AP2 functions at the plasma membrane.

Figure 1.9

Clathrin-mediated endocytosis

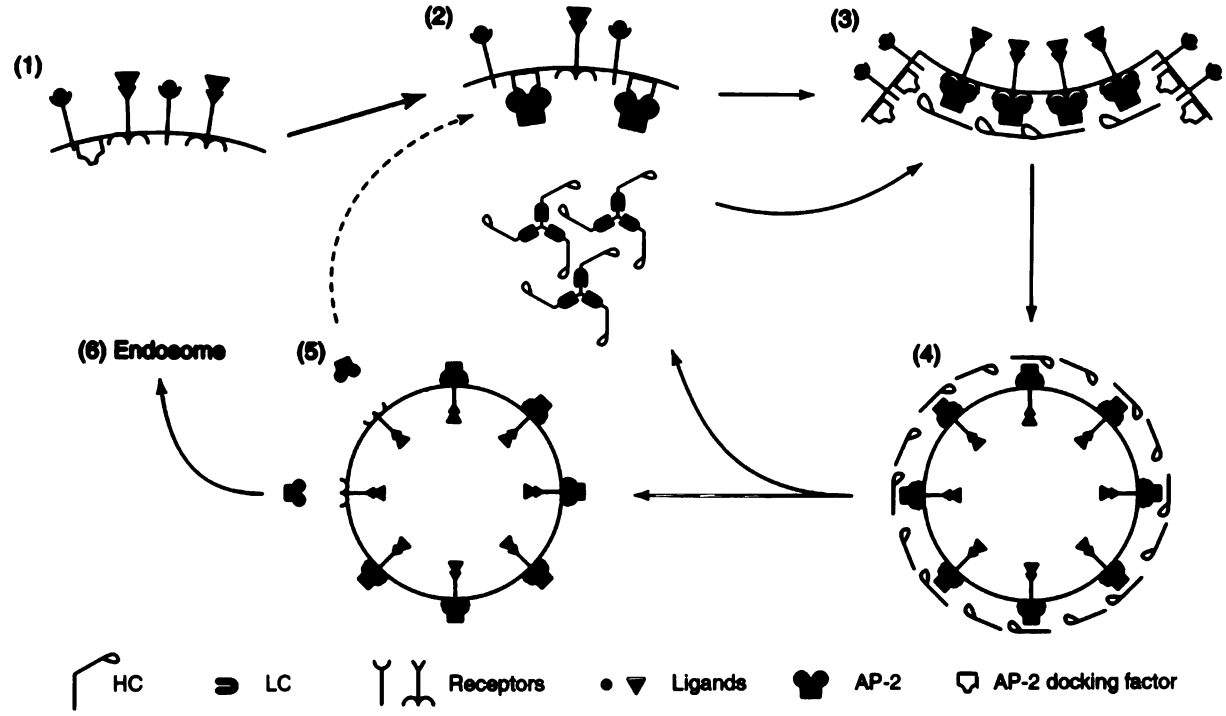


Figure 1.9. **Clathrin-mediated endocytosis.**

The sequence of events involved in clathrin-mediated endocytosis is shown in numbered steps. (1) Plasma membrane receptors (only some of which contain an AP2 recognition motif) and an unknown docking factor for AP2. (2) AP2 complexes interact with an AP2 docking factor on the plasma membrane. (3) AP2 complexes interact with motifs in the cytoplasmic domains of receptors (which contain an AP2 recognition motif) and nucleate clathrin assembly. Clathrin assembly organizes the AP-associated receptors and sorts them from non-interactive receptors. (4) The fully polymerized clathrin-coated vesicle detaches from the membrane. (5) Clathrin and AP complexes are progressively uncoated from the internalized CCV. (6) Vesicle contents are fused into an early endosome.

Early endosomal compartment: Sorting endosomes and the recycling compartment

Following RME, receptors and their ligands are delivered to an early endosomal compartment, comprised of both sorting and recycling endosomes (Ghosh et al., 1994). Sorting endosomes have a tubulovesicular morphology (Geuze et al., 1983) and contain endocytosed materials that are to be recycled to the plasma membrane as well as materials destined for the lysosome (Dunn et al., 1989). This mildly acidic compartment (Yamashiro and Maxfield, 1987) serves as the primary sorting site from which endocytosed receptors and ligands are directed to their ultimate cellular destinations. Some receptors progress to the late endocytic pathway, ultimately residing in the lysosome where they are degraded. Epidermal growth factor (EGF) receptor follow this route (Carpenter and Cohen, 1976; Fox and Das, 1979), with lysosomal degradation serving a way to regulate EGF signaling. Other receptors, such as transferrin (Tf), low density lipoprotein (LDL), and α_2-macroglobulin receptors, are sorted from the endocytic pathway and recycle to the plasma membrane for subsequent reutilization (Anderson et al., 1982; Hopkins and Trowbridge, 1983; Kaplan, 1980). These receptors are sorted from the sorting endosome to the interconnected tubules (Yamashiro et al., 1984) of the recycling compartment (Dunn et al., 1989; Mayor et al., 1993). Though still acidic, recycling endosomes maintain a slightly higher pH than sorting endosomes (Presley et al., 1993), and the two compartments are distinguishable by optical and electron microscopy (Dunn et al., 1989; Marsh et al., 1995; Marsh et al., 1986; Yamashiro et al.,

1984) and by their characteristic marker proteins (Daro et al., 1996; Sheff et al., 1999; Trischler et al., 1999). While the movement of receptors and ligands through the early endocytic compartment has been extensively studied, the mechanisms controlling their trafficking have not been fully elucidated.

Intracellular trafficking of transferrin receptor and low density lipoprotein receptor

Transferrin receptor (TfR) is the prototypical recycling receptor (Hopkins and Trowbridge, 1983.) It binds iron-loaded (holo) Tf at the cell surface, where neutral pH favors this interaction (Dautry-Varsat et al., 1983; Klausner et al., 1983a). Upon endocytosis, TfR and Tf are delivered to sorting endosomes. Under acidic conditions iron dissociates from Tf and is released to the cytosol for storage or use (Dautry-Varsat et al., 1983; Klausner et al., 1983b; Rao et al., 1983). Apotransferrin, devoid of iron, remains bound to TfR at acidic pH (Dautry-Varsat et al., 1983; Klausner et al., 1983a), and travels with the receptor through the recycling compartment back to the cell surface (Karin and Mintz, 1981; Klausner et al., 1983b), where neutral pH favors the binding of a new holotransferrin molecule. In this manner, an individual TfR molecule may cycle up to 300 times before eventually being degraded in the lysosome (Omary and Trowbridge, 1981).

Like TfR, the LDL receptor (LDLR) recycles to the plasma membrane following endocytosis (Anderson et al., 1982). An individual LDLR molecule may cycle up to 150

times in its lifetime (Brown et al., 1982). However, in the acidic environment of the endosome, LDL is released from its receptor and progresses to the lysosome (Davis et al., 1987). Because Tf and LDL are both internalized via RME to sorting endosomes and then separate from one another, with Tf traveling to the recycling compartment and LDL progressing to the lysosome, these two ligands can be used as markers to monitor endocytosis, sorting, and recycling. The trafficking pathways of Tf, TfR, LDL, and LDLR are shown in Fig. 1.10.

The existence of compensatory or complementary trafficking pathways for both the endocytosis and recycling of TfR has been hypothesized. It has been shown that TfR can enter the cell, though in a delayed fashion, despite disruption of its interaction with clathrin (Jing et al., 1990; McGraw and Maxfield, 1990). Endocytosis of TfR is greatly diminished but not abolished in the presence of clathrin inhibitors (Damke et al., 1994) and Chapter 3 of this dissertation). The detachment of CCV from the plasma membrane requires the activity of dynamin (Schmid et al., 1998). Dynamin associates with clathrin-coated pits by wrapping around the neck of a forming vesicle. Via a GTPase activity dynamin induces detachment of a CCV from the plasma membrane. Damke et al. have identified a clathrin- and dynamin-independent pathway for TfR pinocytosis from the plasma membrane (Damke et al., 1995). This pinocytic mechanism may account for the lack of complete inhibition of TfR internalization when clathrin-mediated endocytosis is impaired.

Figure 1.10

Endocytosis and recycling pathways of transferrin and low density lipoprotein receptors

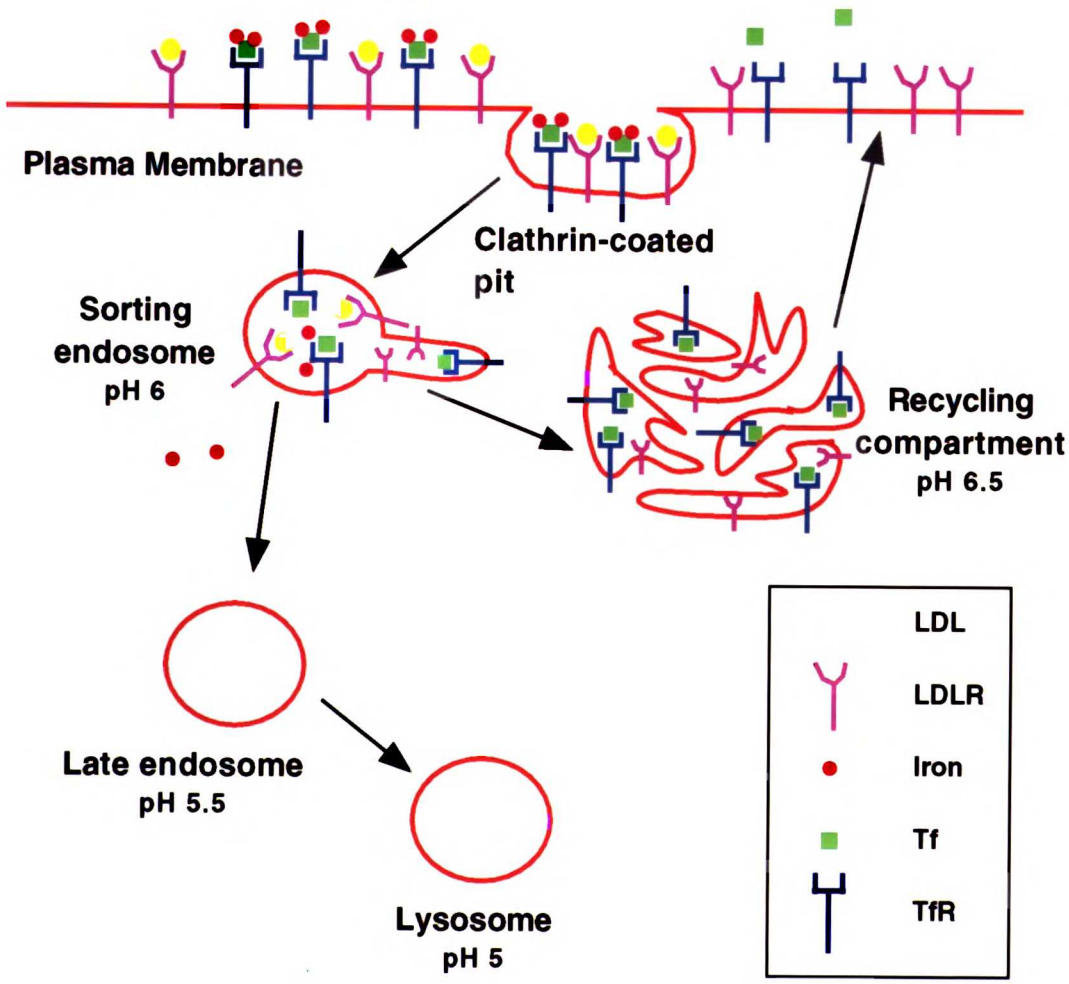


Figure 1.10. **Endocytosis and recycling pathways of transferrin and low density lipoprotein receptors.** The intracellular trafficking of TfR and LDLR is described in detail in the text. Briefly, TfR and LDLR are endocytosed from the plasma membrane in clathrin-coated vesicles. Upon entering the sorting endosome, LDL is released from LDLR, and iron is released from Tf. LDL progresses to the lysosome. LDLR and TfR/Tf traffic through the recycling compartment back to the plasma membrane. At the plasma membrane Tf is released from TfR, and both TfR and LDLR bind new ligands and cycle again. Based on the work of Dr. Frederick Maxfield, *Physiological Reviews*, 1997, Volume 77: 759-803.

Key: LDL, low density lipoprotein. LDLR, low density lipoprotein receptor. Tf, transferrin. TfR, transferrin receptor.

In addition to a secondary route of TfR internalization there are at least two pathways for TfR recycling, resulting in fast and slow pools of recycling receptors which access the recycling compartment to different degrees (Ghosh and Maxfield, 1995; Hopkins, 1983; Hopkins et al., 1994 ; Hopkins and Trowbridge, 1983; Sheff et al., 1999). Some studies have even found evidence for TfR recycling via the Golgi apparatus (Snider and Rogers, 1985; Stoorvogel et al., 1988; Woods et al., 1986), though this model has not been supported some studies (Hedman et al., 1987). The existence of multiple trafficking pathways for TfR complicates studies aimed at determining the role of clathrin in TfR transport. It must be kept in mind that experimental systems which alter protein trafficking may skew TfR distribution between its alternative trafficking routes.

The role of clathrin in receptor recycling

While it is known that recycling receptors such as TfR, LDLR, and α_2-macroglobulin receptor are endocytosed in CCV (Anderson et al., 1977; Bleil and Bretscher, 1982; Willingham et al., 1979), the degree to which clathrin mediates the sorting and subsequent recycling of the proteins has been a subject of debate. Under conditions which inhibit clathrin-mediated internalization of TfR, recycling of the receptor occurs with normal kinetics, suggesting that receptor recycling is a clathrin-independent process (Damke et al., 1994; Jing et al., 1990; McGraw and Maxfield, 1990). Physical sorting mechanisms have been proposed to mediate receptor sorting and

recycling (reviewed in Mukherjee et al., 1997). Previous data has supported the notion that recycling occurs as part of a default, bulk flow process by which lipid and associated proteins return to the plasma membrane (Mayor et al., 1993; Vergés et al., 1999). The bulk of the membrane surface area of sorting endosome lies in tubular structures, resulting in a distribution in which the majority of lipid membrane is in the tubules, while the bulk of fluid volume remains in a vesicular area (Geuze et al., 1987; Griffiths et al., 1989; Marsh et al., 1986). This geometry creates a situation in which integral membrane proteins are disproportionately concentrated in the tubules. It has been suggested that iterative budding of membrane from these sorting tubules can result in high efficiency sorting of recycling membrane and associated receptors (Dunn et al., 1989; Rome, 1985). Endosomal pH has also been shown to affect receptor recycling. Recycling of TfR, LDLR, and α_2-macroglobulin receptor were all found to be moderately inhibited by increases in endosomal pH (Basu et al., 1981; Johnson et al., 1994; Presley et al., 1993J. Cell Biol.; Van Leuven et al., 1980), though whether the inhibition is direct or indirect (for example, it could be a secondary effect of inhibited bulk membrane flow (Presley et al., 1997)) is unclear. The effect of altered endosomal pH on TfR recycling is much greater than the effect on membrane recycling (Presley et al., 1997) and is dependent upon the TfR YTRF internalization motif (Collawn et al., 1990; Johnson et al., 1993; Presley et al., 1997), indicating at least one additional level of control on TfR recycling above simple bulk membrane flow. However, whether this additional control mechanism

promotes receptor recycling or is actually a retention mechanism (for example, to retain receptors which have not released their ligand) needs to be clarified.

A compelling finding in support of a clathrin-mediated sorting/recycling pathway is the existence of clathrin-coated buds on endosomes which are enriched for Tf and TfR (Killisch et al., 1992; Stoorvogel et al., 1996; Whitney et al., 1995). Endosomal clathrin-coated buds in polarized Madin-Darby canine kidney (MDCK) cells were found to mediate the targeting of internalized TfR to the basolateral membrane (Futter et al., 1998; Odorizzi et al., 1996). The role for clathrin in this process was suggested to be to concentrate TfR in developing basolateral recycling vesicles. Interestingly, disruption of clathrin on the endosomal buds did not affect the kinetics of TfR recycling but completely abolished directional targeting to the basolateral surface, indicating that clathrin might affect the directionality of recycling.

We investigated clathrin's role in receptor sorting and recycling by studying the trafficking of Tf, TfR, and LDL in cells expressing the dominant-negative clathrin inhibitor Hub. This work is presented in Chapter 3.

Cytoskeleton involvement in receptor endocytosis and recycling

Components of the cytoskeleton are thought to play a role in receptor endocytosis and recycling, though the nature of their involvement is unclear. Actin assembly is required for endocytosis in yeast and in some mammalian cells (Kubler and Riezman,

1993; Lamaze et al., 1997; Salisbury et al., 1980; Wendland et al., 1998), though agents which disrupt actin assembly have variable effects on endocytosis in different cells types (Fujimoto et al., 2000; Sandvig and van Deurs, 1990). Multiple roles for the actin cytoskeleton in RME have been proposed (reviewed in Qualmann et al., 2000). There is evidence that actin localizes components of the endocytic machinery to sites of endocytosis (Gaidarov et al., 1999), induces membrane invagination, and aids in membrane fission and detachment of coated vesicles (Lamaze et al., 1997) Actin may also play a role in propelling endocytic vesicles through the cytosol, a hypothesis supported by the discovery of actin tails associated with endosomes (Taunton et al., 2000). While the actin framework seems to be necessary to maintain the surface organization of clathrin-coated pits, the actin cytoskeleton itself does not appear to be necessary to maintain a functional endocytic pathway (Gaidarov et al., 1999). There is some evidence of an inhibitory role for actin in RME. The cortical actin cytoskeleton has been shown to inhibit membrane traffic (Trifaró and Vitale, 1993), and the area surrounding clathrin-coated pits is devoid of actin filaments (Fujimoto et al., 2000). The discovery that the Hip1R protein (a protein closely related to the huntingtin interacting protein 1 (Hip1)) is enriched in CCV fractions and binds actin *in vitro* (Engqvist-Goldstein et al., 1999) suggests that clathrin might interact with the actin cytoskeleton with Hip1R serving as a link. Hip1R is a mammalian homolog of Sla2p, a yeast actin-binding protein involved in actin organization and endocytosis (Drubin et al., 1988; Wesp

et al., 1997). Hip1R has been shown to colocalize with markers for RME, including AP2. Engqvist-Goldstein, et al. recently reported that Hip1R binds clathrin *in vivo* and is found in association with clathrin-coated pits. Hip1R was also found to induce crosslinking of both actin and clathrin (Engqvist-Goldstein et al., 2000).

An intact microtubule network is necessary to maintain proper organization of the recycling compartment (McGraw et al., 1993), and some studies have indicated that early endosomes associate with microtubules and recycle along a polarized microtubule cytoskeleton (Hopkins et al., 1994; Marsh et al., 1995). However, the effect of microtubule disruption on receptor trafficking varies with cell type, particularly with regard to whether cells are adherent or detached (Subtil and Dautry-Varsat, 1997).

We had previously observed a linear distribution pattern of AP2-coated vesicles which suggested association of these vesicles with the actin cytoskeleton (unpublished observation, see Chapter 3). In light of the proposed role for actin in RME and our observation that AP2-coated vesicles might associate with actin, we investigated the role of the actin cytoskeleton in the trafficking of TfR. This work is presented in Chapter 3.

Aims and organization of this dissertation

The overall goal of the research presented in this dissertation was to study mechanisms of viral inhibition of antigen presentation by class I MHC molecules. To accomplish this goal I investigated the effect of adenovirus E19 on the cell biology of class I MHC molecule assembly. Additionally, I investigated the role of clathrin and the cytoskeleton in the trafficking of transferrin receptor. While undertaking this second project, I developed tools to study the effect of HIV Nef on the trafficking of class I MHC molecules. These three projects share a common theme of intracellular protein trafficking and the use of inhibitors to study trafficking pathways.

Aim 1. To determine the effect of adenovirus E19 on class I MHC assembly.

Aim 2. To determine the role of clathrin-coated buds on endosomes.

Aim 3. To determine the role of clathrin in the recycling of transferrin receptor.

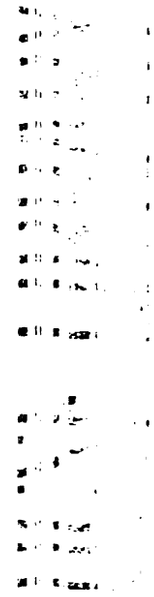
These three aims are addressed in Chapters 2 and 3.

Chapter 4 will summarize the results of this research and proposes future experiments.

Chapter 5 will give detailed descriptions of protocols and reagents developed during the course of this research.

Chapter 2

Adenovirus E19 has Two Mechanisms for Affecting Class I MHC Expression



This chapter is reproduced with permission from *The Journal of Immunology*, 1999, Volume 162:5049-5052, copyright The American Association of Immunologists.

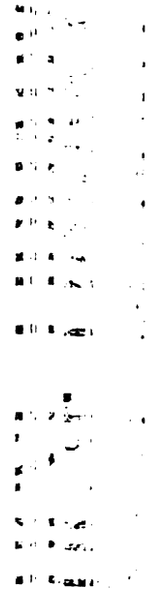
The text of Chapter 2 and the experiments presented are the work of Elizabeth Bennett. Dr. Frances Brodsky provided intellectual and editorial input. Drs. Jack Bennink and Jonathan Yewdell, both from the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health provided vaccinia virus constructs and valuable advice. Dr. Robert DeMars from the University of Wisconsin at Madison supplied B lymphoblastoid cell lines. Dr. William Wold (St. Louis University School of Medicine) provided anti-E19 rabbit serum, and Dr. Peter Cresswell (Yale University School of Medicine) provided anti-TAP1 and anti-tapasin rabbit sera.

Adenovirus E19 has Two Mechanisms for Affecting Class I MHC Expression

Elizabeth M. Bennett*‡, Jack R. Bennink§, Jonathan W. Yewdell§,

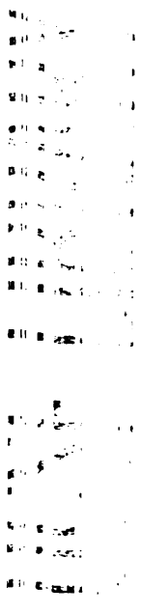
and Frances M. Brodsky*†‡

*The G. W. Hooper Foundation, Department of Microbiology and Immunology and Departments of †Biopharmaceutical Sciences and ‡Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0552. §Laboratories of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD, 20892-0440.



Abstract

Viral strategies for immune evasion include inhibition of various steps in the class I MHC assembly pathway. Here we demonstrate that adenovirus produces one gene product with a dual function in this regard. It is well established that adenovirus E19 binds class I molecules and retains them in the endoplasmic reticulum (ER). However, E19 also delays the expression of class I alleles to which it cannot tightly bind. Here we show that E19 binds TAP and acts as a tapasin inhibitor, preventing class I/TAP association. ΔE19, an E19 mutant lacking the ER-retention signal, delays maturation of class I molecules, indicating that E19's inhibition of class I/TAP interaction is sufficient to delay class I expression. These data identify tapasin inhibition as a novel mechanism of viral immune evasion and suggest that through this secondary mechanism adenovirus can affect antigen presentation by MHC alleles which it can only weakly affect by direct retention.



Introduction

Viruses with mechanisms for evading the immune system have a unique ability to establish long-term infection in the host. The adenovirus E3 19 kD protein, E19, is one of at least four E3-encoded proteins which attenuate the host immune response to adenovirus infection (Shisler et al., 1997; Wold and Gooding, 1991.). E19 has been shown to bind class I major histocompatibility (MHC) molecules and prevent their cell surface expression (Andersson et al., 1985; Burgert and Kvist, 1985), inhibiting the cytolytic T cell (CTL) response against adenovirus-infected cells (Andersson et al., 1987). It has been demonstrated that E19 contains a di-lysine endoplasmic reticulum (ER)-retention motif in its cytoplasmic domain (Jackson et al., 1990). The effect of this motif on ER retention of class I molecules has been shown by expression of the deletion mutant ΔE19, which lacks the retention motif, in murine mastocytoma cells (Cox et al., 1991). In the presence of ΔE19, a portion of H2-K^d molecules travel to the cell surface where they elicit a CTL response. No such response is detected when wild-type E19 is expressed. While this result highlights the ability of E19 to act as an ER-retention molecule, the data also suggest that E19 may be affecting class I expression by an additional mechanism. In the presence of ΔE19 the kinetics of K^d maturation are delayed almost ten-fold, and a significant portion of K^d molecules fail to reach the cell surface. Additionally, the maturation of both human and murine class I alleles which are

bound only weakly by E19 is still greatly delayed in the presence of the viral protein (Beier et al., 1994; Cox et al., 1990). These observations prompted our further investigation into the effect of E19 on the class I assembly pathway.

Class I MHC molecules are assembled in the ER as a membrane-bound heavy chain and a soluble light chain, β_2-microglobulin (β_2m). Class I heterodimers acquire peptides that are generated in the cytosol by proteasome-mediated degradation and transported into the ER lumen by the transporter associated with antigen processing (TAP). Tapasin, an ER-resident membrane protein, facilitates the interaction of class I molecules with TAP by joining the two proteins and cementing the formation of a class I assembly complex which also includes calreticulin and ERp57. (For a recent review see Lehner and Trowsdale, 1998). We hypothesized that E19 could be inhibiting an additional step in the class I assembly pathway because of the persistent effect of ΔE19 on the export of murine class I molecules. To address this question E19 was expressed in a panel of human cell lines, and the interactions of E19, class I molecules, and TAP were studied. We report here that E19 binds both class I molecules and TAP. Unlike tapasin, however, E19 binds class I molecules and TAP independently rather than simultaneously and thereby causes a decrease in class I/TAP association, which can explain the delay in class I maturation we observe in the presence of ΔE19.

Materials and Methods

Cell lines, antibodies, and virus

EBV transformed human B lymphoblastoid cell lines (LCL) were cultured in RPMI 1640 supplemented with 2mM L-glutamine and 10% FBS at 37°C in 8% CO_2. The TAP1-/TAP2-negative LCL 721.174 (Spies et al., 1992), the HLA-A-,B-,C-negative LCL 721.221 (Shimizu and DeMars, 1989), and the tapasin-negative LCL 721.220 (Greenwood et al., 1994; Sadasivan et al., 1996) transfected with HLA-B8 were from Dr. Robert DeMars. HeLa 229 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 2mM L-glutamine and 10% FBS at 37°C in 6% CO_2. The specificities of mAb W6/32 (anti-human class I MHC (Barnstable et al., 1978)) and Tw1.3 (against adenovirus E19 and ΔE19 (Cox et al., 1991)) and of polyclonal antibodies R.RING4C (Ortmann et al., 1994) and R.gp48N (Sadasivan et al., 1996) (against TAP1 and tapasin, respectively, both from Dr. Peter Cresswell), P129-143 (anti-E19, from Dr. William Wold (Hermiston et al., 1993)), anti-TAP2, and UCSF#2 (anti-HLA-A2) (Bresnahan et al., 1997) have been described. Vaccinia virus constructs encoding the E3 19 kD protein of adenovirus type 5 (E19-Vac), a deletion mutant of E19 lacking the C-terminal 6 amino acids (ΔE19-Vac), and the nucleoprotein of influenza virus (NP-Vac) are described elsewhere (Cox et al., 1991; Cox et al., 1990; Smith et al., 1987).

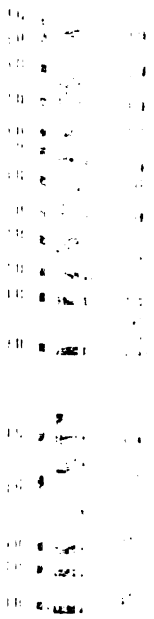
Infection and metabolic labeling

HeLa cells ($1.5x10^7$) were infected with vaccinia virus (25 plaque forming units (pfu) per cell) in 2 mL 0.2% BSA/PBS for 45 minutes at 37°C. Virus was removed, and cells were maintained in DMEM/10% FBS until 2 hours post-infection. LCL ($5x10^6$) were infected with 30 pfu/cell in 1 mL RPMI 1640/1% FBS for 2 hours at 37°C. RPMI 1640/10% FBS was added (to 10^6 cells/mL), and infection proceeded for 12 hours. For ^{35}S metabolic labeling, cells were starved in cysteine/methionine-free medium supplemented with 5% dialyzed FBS for 1 hour at 37°C. Cells were labeled by the addition of 1 mCi per 10^7 cells Tran^{35}S-Label (ICN Pharmaceuticals, Irvine, CA) for 10 minutes at 37°C, washed 3 times in ice-cold PBS, and chased in medium containing five times excess methionine at 37°C.

Immunoprecipitation and Endoglycosidase H treatment

Cells were lysed in 1% digitonin (Calbiochem, La Jolla, CA), 10 mM Tris pH 7.4, 150 mM NaCl on ice for 20 minutes. After pelleting the nuclei, lysates were precleared with protein G sepharose for 1 hour at 4°C, then incubated with 2 μg of antibody and fresh protein G sepharose for 2 hours at 4°C. Immunoprecipitates were washed and analyzed under reducing conditions by SDS-PAGE. For Endoglycosidase H (EndoH) treatment, cells were lysed in 1% Nonidet P-40 (ICN Biomedicals, Aurora, OH) and

immunoprecipitated as above. Immunoprecipitates were washed and resuspended in 200 μL 100 mM sodium citrate pH 5.6, 0.2% SDS, 150 mM 2-ME. Samples were divided in half and incubated at 37°C for 16 hours in the presence or absence of 5 mU EndoH (Boeringher Mannheim, Germany) followed by SDS-PAGE analysis. For immunoblotting proteins were transferred to nitrocellulose, incubated with antibody followed by HRP-conjugated secondary antibody (Zymed Laboratories Inc., South San Francisco, CA), and visualized by enhanced chemiluminescence (ECL) and exposure to film. Bands were scanned and quantitated using the National Institutes of Health Image 1.61 program.

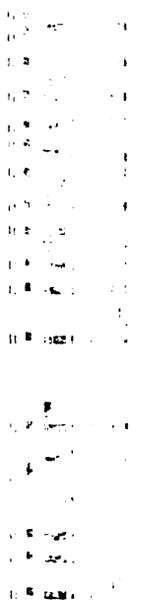


Results

Adenovirus ΔE19 delays the maturation of human class I MHC molecules

Adenovirus E19 has been shown to bind both human and murine class I MHC molecules (Signäs et al., 1982) and to prevent their cell surface expression (Andersson et al., 1985; Burgert and Kvist, 1985; Burgert et al., 1987). Though it lacks an ER-retention motif, ΔE19 caused a significant delay in the maturation of murine class I molecules (Cox et al., 1991). We wanted to determine whether the same delay is observed in human class I expression in the presence of ΔE19 and where in the secretory pathway the delay occurs. When ΔE19 was expressed in HeLa cells using a vaccinia virus vector

(ΔE19-Vac), class I molecules remained sensitive to treatment with Endoglycosidase H (EndoH) throughout a two-hour chase period (Fig. 2.1 A). Endo-H cleaves N-linked carbohydrates which have not been fully trimmed and terminally glycosylated, modifications which take place during passage through the Golgi complex. Thus EndoH sensitivity can be used as a measure of a protein's progression from the ER through the secretory pathway. Class I molecules typically begin to acquire EndoH resistance within 30 minutes (Fig. 2.1 B). Our data show that in the presence of ΔE19, human class I molecules, like murine class I molecules, are significantly delayed in export from the ER, remaining EndoH sensitive for longer than two hours. When cells were infected with a control vaccinia construct encoding the nucleoprotein of influenza virus (NP-Vac), class I molecules acquired EndoH resistance without delay, indicating that vaccinia infection itself does not affect class I maturation (Fig. 2.1 C).

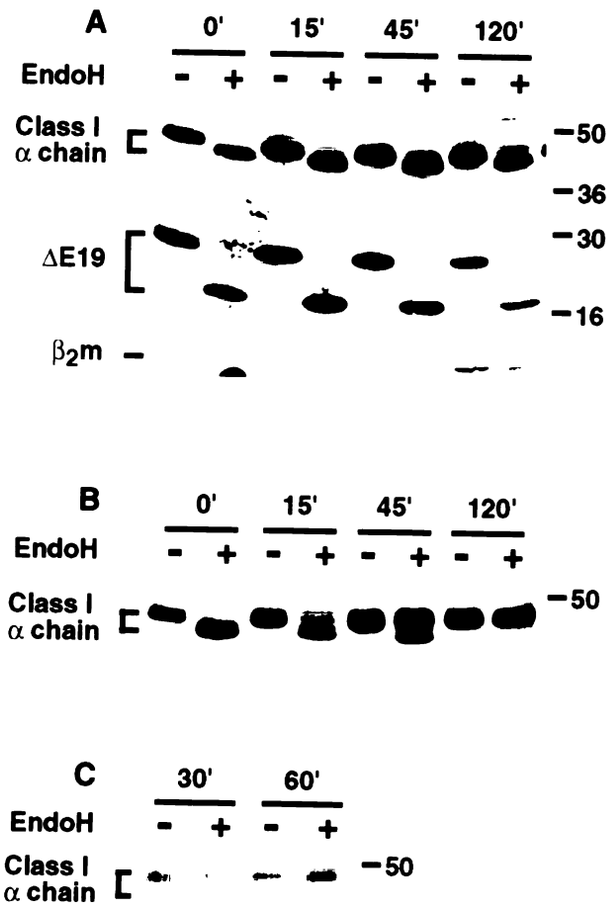


Adenovirus E19 binds the transporter associated with antigen processing (TAP)

To determine whether the inhibition of class I MHC expression observed in the presence of ΔE19 is due to a block in class I assembly, we investigated whether class I molecules bound by E19 associate with TAP. Only properly assembled class I dimers interact with TAP (Solheim et al., 1997). Thus TAP association can be used to assess whether early steps in the class I assembly pathway are intact. During the course of this

Figure 2.1

Delayed maturation of class I MHC molecules in the presence of adenovirus ΔE19



Delayed maturation of class I MHC molecules in the presence of

ΔE19.

were infected with ΔE19-Vac (A) or a control virus (NP-Vac) (C) or not

, then pulse labeled with ^{35}S-methionine (10 minutes) and chased for the

ted (minutes). Class I MHC molecules were immunoprecipitated from cell

mAb W6/32, which coprecipitates ΔE19. Immunoprecipitates were divided

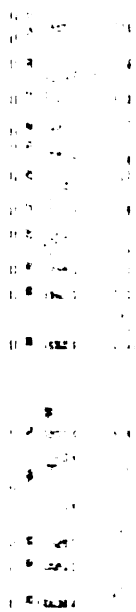
ither treated with EndoH (+) or left untreated (-), then analyzed by SDS-

visualized by autoradiography. Migration positions of uncleaved (upper

ndoH-cleaved (lower band) class I molecules and ΔE19 are indicated at the

position of β_2m. Migration positions of molecular weight marker proteins

d at the right (in kilodaltons).



investigation, we found an unexpected direct association between E19 and TAP. E19 was expressed in a panel of human B lymphoblastoid cell lines (LCL) and immunoprecipitated from cell lysates with mAb Tw1.3. Immunoblotting analysis showed that in the wildtype cell line JY, Tw1.3 coprecipitated both the class I heavy chain and TAP2 with E19 (Fig. 2.2 A). In the class I-negative cell line 721.221 the coprecipitation of TAP with E19 was maintained, indicating that the association of TAP with E19 is not dependent on class I molecules. In the reciprocal experiment, the absence of TAP (721.174 cells) did not prevent the coprecipitation of class I molecules with E19. Identical results were obtained from cells expressing ΔE19 (Fig. 2.2 B), showing that E19's ER-retention signal is not required for E19/TAP association. These data suggest that in addition to binding class I molecules, E19 binds TAP.

While the results from .221 cells indicate that E19 does not associate with TAP indirectly through classical class I MHC molecules, non-classical class I molecules, known to be expressed in .221 cells (Shimizu and DeMars, 1989), could be responsible for the coprecipitation of TAP with E19. Additionally, E19 may bind tapasin and could thereby associate with both classical and nonclassical class I molecules and TAP indirectly. To examine both of these possibilities we expressed E19 in a tapasin deficient cell line (721.220/B8). When E19 was immunoprecipitated from .220/B8 lysates, we continued to observe coprecipitation of both class I molecules and TAP (Fig. 2.2 C). In tapasin-deficient .220/B8 cells this coprecipitation cannot be attributed to tapasin. Nor is

Figure 2.2

Adenovirus E19 binds TAP

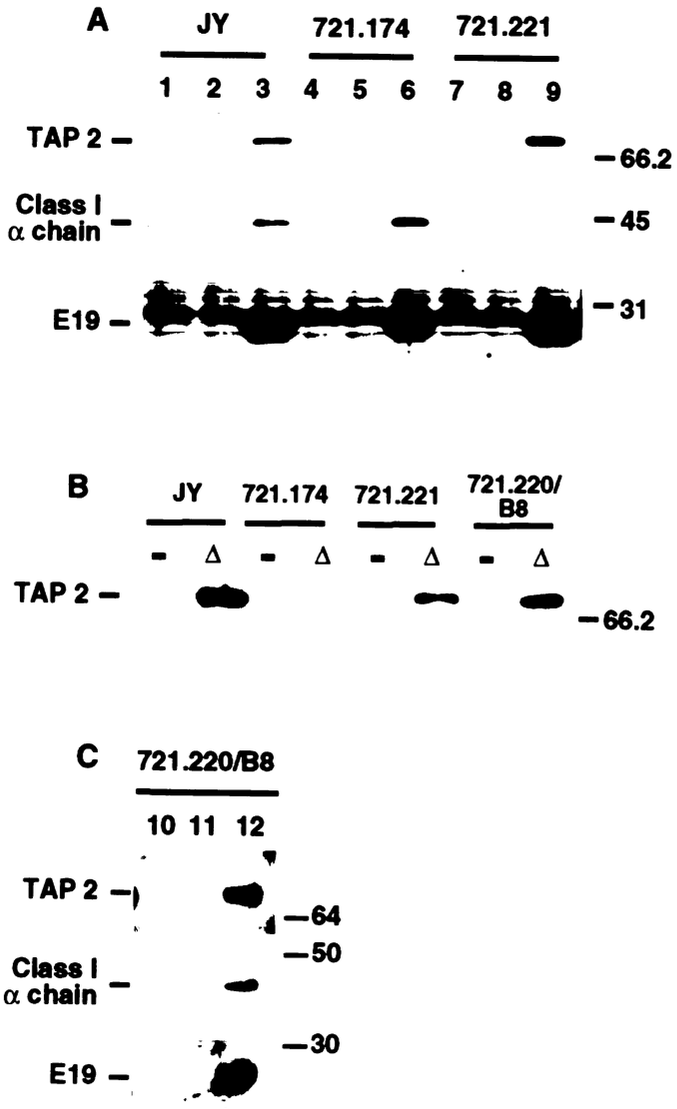


Figure 2.2 **Adenovirus E19 binds TAP.**

(A and C) Human LCL, indicated above the lanes, were uninfected or infected with E19-Vac or a control virus (NP-Vac). E19 was immunoprecipitated from cell lysates with mAb Tw1.3. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted for E19 (anti-E19 rabbit serum), class I MHC heavy chain (UCSF #2 rabbit serum), and TAP2 (anti-TAP2 rabbit serum). Migration positions of these proteins are indicated at the left. Proteins were visualized by ECL. Lanes 1, 4, 7, and 10 are uninfected; lanes 2, 5, 8, and 11 are infected with NP-Vac; lanes 3, 6, 9, and 12 are infected with E19-Vac.

(B) LCL were uninfected (-) or infected (Δ) with ΔE19-Vac. ΔE19 was immunoprecipitated with mAb Tw1.3, and TAP2 was visualized as in (A) and (C). The migration position of TAP2 is indicated at the left. JY is a wildtype LCL; 721.174 is a TAP-negative LCL; 721.221 is an HLA-A-,B-,C-negative LCL; 721.220/B8 is a tapasin-negative LCL which has been transfected with HLA-B8.

Migration positions of molecular weight markers are indicated at the right of each panel (in kilodaltons).

it likely to be attributable to E19 interaction with nonclassical class I molecules since the surface expression of HLA-E has been shown to be tapasin-dependent, which suggests that nonclassical class I molecules are not bound to TAP in these cells (Braud et al., 1997). Furthermore, the level of expression of nonclassical class I molecules in .221 cells is extremely low compared to total levels of class I molecules in other LCL (Shimizu and DeMars, 1989). However, comparable levels of TAP coprecipitated with E19 in .221 and wildtype JY cells (Fig. 2.2 A), strengthening our conclusion that nonclassical class I molecules are not solely responsible for E19-TAP association. Therefore, we conclude that E19 binds both to class I molecules and TAP.

Adenovirus E19 inhibits the interaction of class I MHC molecules with TAP

The fact that both class I MHC molecules and TAP coimmunoprecipitated with E19 in tapasin-deficient .220/B8 cells suggested that E19 might be acting as a tapasin mimic. To test this hypothesis we infected JY and .220/B8 cells with a vaccinia virus vector encoding adenovirus E19 (E19-Vac) and immunoprecipitated TAP from cell lysates with anti-TAP1 rabbit serum. In uninfected JY cells both tapasin and class I molecules co-precipitated with TAP (Fig. 2.3 A, lane 1). In the absence of tapasin, class I molecules failed to coprecipitate with TAP (Fig. 2.3 A, lane 3). The expression of E19 in .220/B8 cells did not restore the coprecipitation of class I molecules with TAP,

Figure 2.3

Inhibition of class I MHC/TAP interaction by adenovirus E19

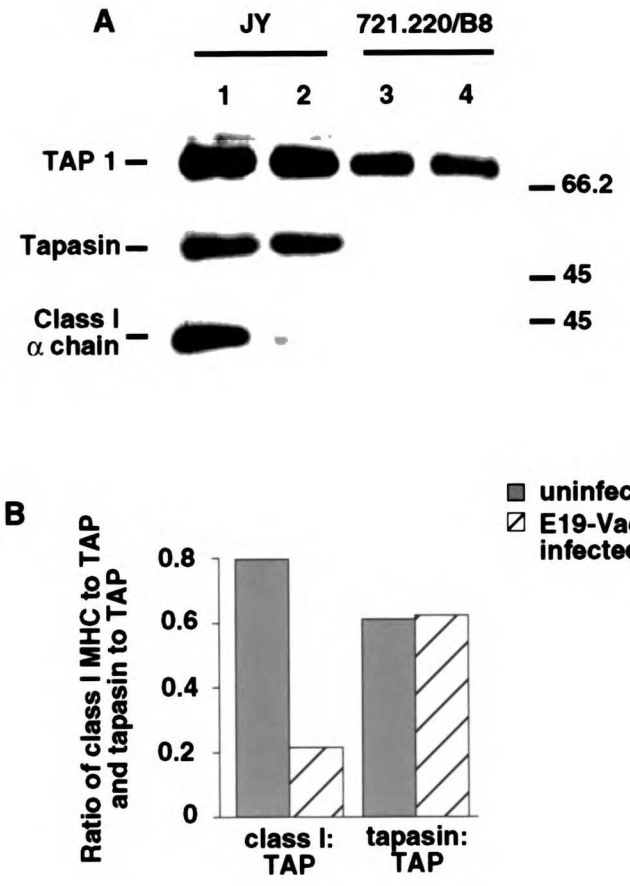


Figure 2.3 **Inhibition of class I MHC/TAP interaction by adenovirus E19.**

(A) Human LCL, indicated above the lanes, were uninfected (-) or infected (E19) with E19-Vac. Cells were lysed in 1% digitonin, and TAP was immunoprecipitated with anti-TAP1 rabbit serum R.RING4C. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted for TAP1 (R.RING4C) and class I MHC heavy chain (UCSF #2 rabbit serum), and visualized by ECL. The nitrocellulose was then stripped and reprobed for tapasin (R.gp48N rabbit serum).

(B) Protein bands from uninfected and E19-Vac infected JY cells in (A) were scanned and quantitated using the National Institutes of Health Image 1.61 program. Ratios of class I heavy chain to TAP and tapasin to TAP were calculated. This quantitation of the data in (A) was reproduced in subsequent experiments and shows a typical decrease in the ratio of class I molecules coprecipitated with TAP in the presence of E19.

indicating that E19 cannot substitute for tapasin (Fig. 2.3 A, lane 4). This is consistent with lack of sequence homology between the two proteins (Ortmann et al., 1997). Interestingly, when E19 was expressed in JY cells, the coprecipitation of class I molecules with TAP was greatly reduced (Fig. 2.3 A, lane 2). Quantitation showed that in the presence of E19 the ratio of co-precipitated class I molecules to TAP was decreased by approximately 75%, while the amount of tapasin bound to TAP was approximately the same (Fig. 2.3 B). Taken together these data indicate that E19 binds to both class I molecules and TAP independently, and in so doing prevents tapasin from bridging the two molecules. The result is a reduced steady state association between class I molecules and TAP.

Discussion

These data identify tapasin inhibition as a novel viral strategy for immune evasion and show that tapasin inhibition is a second mechanism by which adenovirus E19 interferes with maturation of class I MHC molecules. We believe E19 evolved this second strategy to inhibit expression of class I alleles to which it cannot tightly bind. Polymorphism of class I molecules poses a considerable challenge to E19 in terms of binding and retaining many different alleles. E19 does, in fact, exhibit allelic specificity towards class I molecules (Beier et al., 1994; Burgert and Kvist, 1987). By inhibiting class I/TAP association, E19 can still exert a block on the expression of class I alleles

with which it associates only weakly. Like tapasin, E19 is capable of binding both class I molecules and TAP. However, E19 is unable to bridge class I molecules to TAP, and instead appears to bind only one protein at a time. In doing so, however, E19 inhibits the ability of tapasin to join class I molecules to TAP, resulting in a reduced steady state class I/TAP association. This interference with TAP association can explain the delay in class I maturation seen in the presence of ΔE19. While tapasin inhibition allows ΔE19 to reduce the efficiency of class I assembly, it does not block it completely, as a small portion of class I molecules do become Endo-H resistant over time (Fig. 2.1 A). E19 thus provides itself with a backup mechanism enhancing ER retention and inhibition of class I expression.

Inhibition of class I MHC/TAP interaction should lead to a delay in peptide loading of class I molecules. We are unable to detect such a delay, primarily because assays which rely on the thermal instability of empty class I dimers are difficult to interpret. E19 has been shown to bind the α1/α2 domains of the class I molecule (Beier et al., 1994; Burgert and Kvist, 1987), the same region where peptide binds. E19 and ΔE19 remain bound to class I molecules following detergent lysis and heat treatment (data not shown), and therefore may be stabilizing empty dimers, rendering them undetectable. However, since we observe both inhibition of class I/TAP association in the presence of E19 and delayed kinetics of class I maturation in the presence of ΔE19,

the most viable explanation is that E19 delays peptide loading.

Viral immune evasion strategies highlight the evolutionary pressure on viruses to prevent clearance by the host and ensure their continued spread. Blocking antigen presentation is one mechanism by which viruses escape immune detection. In addition to adenovirus, human cytomegalovirus, herpes simplex virus, and human immunodeficiency virus have all been shown to prevent antigen presentation by class I MHC molecules (reviewed in Ploegh, 1998). However, adenovirus E19 is unique in that it is the only viral gene product identified so far which uses more than one mechanism to inhibit class I expression--inhibition of class I/TAP interaction and ER retention. The ability of E19 to inhibit tapasin function appears to be novel, although additional studies will determine whether other viruses use this strategy as well.

Chapter 2: Supplemental Data

The following data was produced as part of the work presented in Chapter 2 but was not included in the manuscript published in *The Journal of Immunology*, 1999, Volume 162:5049-5052.

Monoclonal antibody BBM.1, against β_2m, has been described (Brodsky et al., 1979).

In analyzing the cell lines used in the work presented in Chapter 2, experiments were performed to confirm the proposed model for how tapasin mediates the association of class I heavy chain-β_2m dimers with TAP (Sadasivan et al., 1996). This model states that class I dimers interact with TAP via tapasin and is based on the observation that class I molecules do not associate with TAP in the absence of tapasin. In testing this model and analyzing various B cell lines, we and others (Solheim et al., 1997) have observed a possible direct interaction between β_2m and TAP in the absence of class I MHC heavy chains. Fig. 2.4 shows a western blot of cell lysates and mAb BBM.1 immunoprecipitates (BBM.1 recognizes β_2m) from various B cell lines. In this experiment samples were separated by SDS-PAGE and immunoblotted for TAP1 and tapasin. In wildtype JY cells, both tapasin and TAP are readily detected in the cell lysate (lane 1) and the BBM.1 immunoprecipitate (lane 2). Interestingly, despite the absence of classical class I heavy chains in the HLA-A, -B, -C-negative cell line 721.221, both TAP and tapasin continue to precipitate with β_2m (lane 6). The observation that TAP coprecipitates with β_2m in 721.221 cells was independently made by Solheim, et al. who precleared nonclassical class I heavy chains from cell lysates before immunoprecipitating β_2m and therefore concluded that β_2m binds directly to TAP. However, the absence of a coprecipitating TAP band in the tapasin-negative cell line 721.220/B8 (lane 8) rules out a direct interaction between β_2m and TAP. If Solheim, et al. did, in fact, preclear all

Figure 2.4

β_2m associates with tapasin and TAP in the absence of classical class I MHC molecules

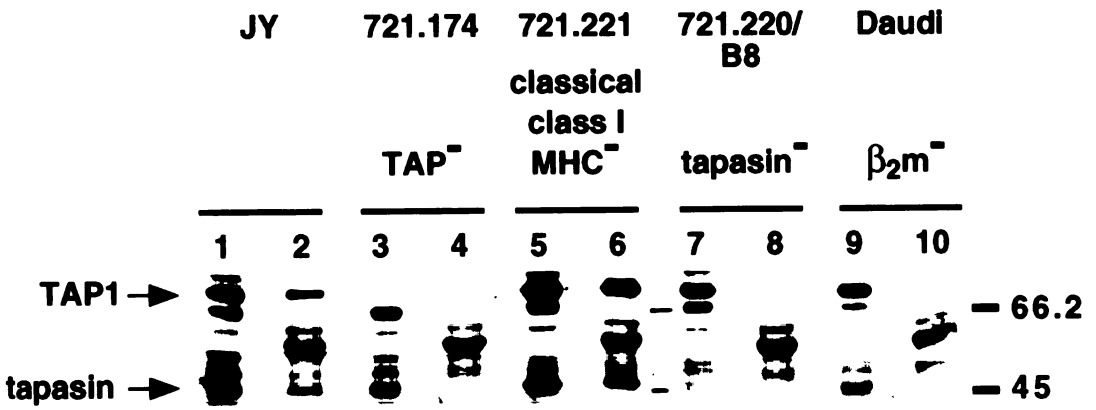


Figure 2.4. **β_2m associates with tapasin and TAP in the absence of classical class I MHC molecules.**

Various cell lines (indicated about the lanes) were lysed in 1% NP40, and mAb BBM.1 was used to immunoprecipitate β_2m. Immunoprecipitates (lanes 2, 4, 6, 8, and 10) and cell lysate (lanes 1, 3, 5, 7, and 9) were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted for TAP1 (R.RING4C) and tapasin (R.gp48N), and visualized by ECL. Migration positions of TAP1 and tapasin are indicated at the left and of molecular weight marker proteins (in kilodaltons) at the right.

nonclassical class I heavy chains prior to β_2m immunoprecipitation, then our result indicates that β_2m binds directly to tapasin, not TAP, and that tapasin mediates the coprecipitation of TAP with β_2m in 721.221 cells. Of interest is the reduced amount of tapasin coprecipitated with β_2m in the absence of TAP (lane 4). This result is conflicting if β_2m does, in fact, bind directly to tapasin.

More work is needed to reconcile how β_2m interacts with tapasin and TAP. Solheim, et al. used mAb W6/32 to preclear nonclassical class I heavy chains from 721.221 cell lysates. W6/32 has been shown to recognize nonclassical class I molecules (Shimizu et al., 1988), though Solheim, et al. do not show data confirming the absence of nonclassical heavy chains following preclearance. If Solheim, et al. did, in fact, preclear all nonclassical class I heavy chains from 721.221 lysates prior to β_2m immunoprecipitation, then β_2m must bind directly to tapasin and/or TAP via a mechanism that depends on the presence of both proteins. However, in the case that Solheim, et al. failed to fully preclear nonclassical class I heavy chains, a simple explanation is that β_2m associates with tapasin and TAP via nonclassical class I heavy chains in 721.221 cells. In the absence of tapasin, nonclassical heavy chain-β_2m dimers cannot associate with TAP (lane 8). In the absence of TAP, class I molecules do not bind peptide and are degraded (Hughes et al., 1997), leaving β_2m without its binding partner and therefore unable to associate with tapasin (lane 4). Further investigation will clarify

Chapter 3

Clathrin Hub Expression Affects Endosome Distribution with Minimal Impact on Receptor Recycling

The text of Chapter 3 and most of the experiments presented are the work of Elizabeth Bennett. Dr. Sharron Lin from the Weill Medical College of Cornell University helped perform experiments involving fluorescent labeling of cells (Figures 3.2, 3.11, and 3.13) and did confocal imaging of labeled cells (Figure 3.13). Dr. Dan Kalman and Orion Weiner from the University of California, San Francisco, helped with labeling and confocal imaging (respectively) of cells to study the spatial relationship of AP2 with the actin cytoskeleton (Figure 3.7). Drs. Frances Brodsky and Frederick Maxfield (Weill Medical College of Cornell University) provided intellectual and editorial input. Dr. Ira Tabas from Columbia University provided DiI-LDL. Dr. John Moran from the University of Michigan Medical School provided the pJM601 vector.

This chapter will be submitted for publication to *The Journal of Cell Biology*, copyright

Clathrin Hub Expression Affects Endosome Distribution

with Minimal Impact on Receptor Recycling

Elizabeth M. Bennett*‡, Sharron X. Lin§, Frederick R. Maxfield§,

and Frances M. Brodsky*†‡

*The G. W. Hooper Foundation, Department of Microbiology and Immunology and Departments of †Biopharmaceutical Sciences and ‡Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0552. §Department of Biochemistry, Weill Medical College of Cornell University, New York, NY 10021.

Abstract

Clathrin-coated buds have been observed on endosomes, though their function at this location has remained a mystery. We wanted to determine the role that these coated-buds play on endosomal membranes. We created a HeLa cell line which expresses the dominant-negative clathrin inhibitor Hub to determine the effect of clathrin inhibition on endosome function and distribution. We observed two novel effects of Hub expression: perinuclear aggregation of early endosomes and disruption of the spatial relationship between AP2-coated vesicles and the actin cytoskeleton. In non-Hub-expressing cells, AP2-coated vesicles align in linear arrays reflective of the actin cytoskeleton. Actin depolymerization, myosin inhibition, and Hub expression all disrupt these linear arrays, indicating that the actin cytoskeleton establishes a spatial arrangement which influences AP2 distribution and that clathrin is involved in mediating the relationship between AP2-coated vesicles and actin. We hypothesize that due to Hub-induced clathrin inhibition, actin cannot influence endosome distribution, leading to a collapse of early endosomes into the perinuclear region. Surprisingly, transferrin receptor sorting and recycling were unaffected by Hub expression. Our data show that clathrin is not involved in these trafficking events and that proper functioning of the early endosomal compartment is not dependent upon its cellular organization.

Introduction

Clathrin-coated vesicles (CCV) mediate the selective transport of integral membrane proteins between cellular membranes (Kirchhausen, 2000). At the plasma membrane, CCV facilitate receptor-mediated endocytosis (RME), whereby cell surface receptors and their ligands are internalized and delivered to the endocytic pathway (Schwartz, 1995). Following RME, the fates of internalized receptors and ligands vary (reviewed in (Mukherjee et al., 1997)). Some progress to the late endocytic pathway where they are degraded in lysosomes. Others, such as transferrin (Tf) and low density lipoprotein (LDL) receptors, are sorted to the recycling compartment (Dunn et al., 1989; Mayor et al., 1993), from which they return to the plasma membrane (Anderson et al., 1982; Hopkins and Trowbridge, 1983). While it is known that these receptors are endocytosed via CCV (Anderson et al., 1977; Bleil and Bretscher, 1982; Willingham et al., 1979), the degree to which clathrin mediates subsequent sorting and recycling of the proteins has been a subject of debate. The existence of clathrin-coated buds on endosomes has led us and others to investigate the role of these buds to determine if clathrin is involved in the sorting or recycling functions of early endosomes.

The early endocytic pathway is comprised of both sorting and recycling endosomes (Ghosh et al., 1994). In the sorting endosome endocytosed material that is to be recycled is sorted from material destined for the lysosome. Recycling components proceed to recycling endosomes from which they return to the plasma membrane.

Clathrin-coated buds have been observed on early endosomes (Stoorvogel et al., 1996), making it a logical hypothesis that clathrin might mediate receptor sorting and recycling. A compelling finding in support of a clathrin-dependent sorting/recycling pathway is that endosomal clathrin-coated buds are enriched for Tf and Tf receptor (TfR) (Killisch et al., 1992; Stoorvogel et al., 1996; Whitney et al., 1995). However, not all studies have supported the hypothesis that clathrin is involved in these trafficking events. Under conditions which inhibit clathrin-mediated endocytosis of TfR, recycling occurs normally (Damke et al., 1994; Jing et al., 1990; McGraw and Maxfield, 1990). Kinetic studies comparing the trafficking of TfR to the flow of bulk membrane concluded that recycling occurs as part of a bulk flow process (Mayor et al., 1993). These results left open the question as to what function endosomal clathrin-coated buds serve if they do not participate in receptor sorting or recycling. A finding that might provide a clue to the function of clathrin-coated buds on endosomes is that in polarized Madin-Darby canine kidney (MDCK) cells endosomal clathrin-coated buds were found to mediate polarized recycling of TfR to the basolateral membrane (Futter et al., 1998; Odorizzi et al., 1996). Disruption of these buds did not affect the kinetics of recycling but abolished polarized targeting to the basolateral surface, suggesting a role for clathrin in maintaining the directionality of recycling, perhaps by mediating association of early endosomes with components of the cytoskeleton.

The actin cytoskeleton is thought to play a role in RME, and is required for

endocytosis in yeast (Kubler and Riezman, 1993) and in some mammalian cells (Lamaze et al., 1997; Salisbury et al., 1980). Multiple roles for actin in RME have been proposed (Qualmann et al., 2000), including localization of components of the endocytic machinery to sites of endocytosis (Gaidarov et al., 1999), induction of membrane invagination, and membrane fission and detachment of coated vesicles (Lamaze et al., 1997). There is also some evidence for an inhibitory role of actin in RME. The cortical actin cytoskeleton has been shown to inhibit membrane traffic (Trifaró and Vitale, 1993), and the area surrounding clathrin-coated pits lacks actin filaments (Fujimoto et al., 2000). Attachment of clathrin-coated pits to the actin cytoskeleton has also been shown to act as a barrier to diffusion of coated pits at the plasma membrane (Gaidarov et al., 1999). Though evidence for a direct link between CCV and the cytoskeleton has been lacking, at least one protein, Hip1R, has been identified which binds actin *in vitro* and is enriched in CCV fractions (Engqvist-Goldstein et al., 1999).

We wanted to further investigate the role of clathrin-coated buds on endosomes by studying trafficking events in cells expressing the dominant-negative clathrin inhibitor Hub. Hub comprises the C-terminal third of the clathrin heavy chain (Liu et al., 1995) and has been shown to act as a dominant-negative clathrin inhibitor by competing for light chain binding (Liu et al., 1998). Previously Hub has been used to demonstrate clathrin's involvement in the internalization of HIV Nef-CD8 chimeras (Lu et al., 1998), in ARF-6-mediated apical internalization in MDCK cells (Altschuler et al., 1999), and in

the endocytosis of protease-activated receptor-1 (Trejo et al., 2000). We created a Hub-expressing HeLa cell line to study the effect of clathrin inhibition on endosome distribution and function. We had previously made the observation that AP2-coated vesicles organize into linear arrays reflective of the actin cytoskeleton (unpublished result). Here we show that both actin/myosin inhibition and clathrin inhibition disrupt these linear arrays, indicating that the organization of AP2-coated vesicles is influenced by the actin cytoskeleton and that this influence is mediated by clathrin. We also observed a perinuclear aggregation of sorting endosomes in the presence of Hub, suggesting that when actin's role in mediating the organization of endosomes is disrupted, endosomes collapse into the perinuclear region. Interestingly, Hub-induced redistribution of endosomes had no effect on the protein sorting or recycling functions of the early endosomal compartment.

Materials and Methods

Creation of HeLa-T7Hub cell line and cell culture reagents

Clathrin Hub (amino acids 1073-1675 (Liu et al., 1995)) with an N-terminal T7 tag was previously created by subcloning into the pET23d vector (Novagen, Madison, WI) (Liu et al., 1998). The T7Hub construct was liberated from pET23d by cleavage with *Nco*I (GibcoBRL, Rockville, MD) and treatment with Klenow fragment (Roche Diagnostics Corporation, Indianapolis, IN), followed by cleavage with *Hind*III (GibcoBRL). Vector

pJM601, created by replacing the CMV promoter of pCEP4 (Invitrogen, Carlsbad, CA) with the tetracycline operator sequence, was a gift from Dr. John Moran (University of Michigan Medical School). T7Hub was subcloned into pJM601 cleaved with *Pvu*II and *Hind*III (GibcoBRL), and the resulting construct was purified on a CsCl gradient. HeLa Tet On cells (Clontech, Palo Alto, CA) were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 20 mM HEPES, 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.2 mg/mL G418 (GibcoBRL) at 37°C in 6% CO_2. Cells were grown to 25% confluency in a 6 cm petri dish and transfected with 3 μg pJM601-T7Hub using FuGENE6 Transfection Reagent (Roche). At 36 hours, cells were transferred to medium containing 0.2 mg/mL G418 and 0.4 mg/mL hygromycin (Roche) and plated at 1000 cells per 15 cm dish. Viable colonies were selected, induced with 2 μg/mL doxycycline (Sigma, St. Louis, MO) for 48 hours, and screened for T7Hub expression by immunofluorescence as described below. As a control, HeLa Tet On cells were transfected with the pJM601 vector alone. Stable cells were carried in medium containing 10% Tet System Approved Fetal Bovine Serum (Clontech), 0.2 mg/mL G418, and 0.4 mg/mL hygromycin.

Antibodies and fluorescent reagents

Rabbit serum against clathrin light chain and monoclonal antibody (mAb) AP.6 (against

the α chain of the AP2 adaptor complex) have been described (Acton and Brodsky, 1990; Chin et al., 1989). Anti-T7 tag mAb was purchased from Novagen. mAb H68.4 (against human transferrin receptor) was purchased from Zymed (South San Francisco, CA). Anti-α-tubulin mAb was purchased from Sigma. Anti-non-muscle myosin rabbit serum was purchased from Biomedical Technologies, Inc. (Stoughton, MA). HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies were purchased from Zymed. Rhodamine red X (RRX)- and FITC-conjugated donkey anti-mouse and Cy5- and FITC-conjugated donkey anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Human transferrin (Tf) (Sigma) was iron-loaded, purified by Sephacryl S-300 (Pharmacia LKB, Uppsala, Sweden) gel-filtration chromatography, and conjugated to Alexa488 according to the manufacturer's instruction (Molecular Probes Inc., Eugene OR). For some experiments Alexa488-Tf was purchased directly from Molecular Probes. Alexa488-phalloidin, Alexa568-phalloidin, and 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-hexanoyl)sphingosyl phosphocholine (C_6-NBD-SM) were from Molecular Probes. 3,3'-dioctadecylindocarbocyanine (DiI)-labeled low density lipoprotein was a gift from Dr. Ira Tabas (Columbia University, New York).

Anti-T7Hub immunoblotting

HeLa-T7Hub cells were grown in the presence of 2 μg/mL doxycycline for 7, 24, 48, or

72 hours, or in the presence of 0, 0.2, 0.02, or 0.002 μg/mL doxycycline for 48 hours. 3 x 10^5 cells were lysed in 1% NP40, 150 mM NaCl, 10 mM Tris, pH 7.4 and separated by SDS-PAGE. Lysates were immunoblotted with anti-T7 tag mAb, followed by goat anti-mouse HRP secondary antibody. Proteins were visualized by enhanced chemiluminescence (ECL) and exposure to film.

Indirect Immunofluorescence

Cells grown on coverslips and treated with 2 μg/mL doxycycline for 48 hours were fixed for 20 minutes in PBS containing 4% formaldehyde (Ted Pella, Inc., Redding, CA). Cells were permeabilized in 0.04% saponin for 15 minutes, then blocked in PBS containing 1% cold fish gelatin, 0.1% BSA, 0.02% SDS, 0.1% NP40, and 0.02% azide for at least 1 hour. Cells were incubated with appropriate antibodies in blocking buffer for at least 1 hour, followed by incubation with fluorescent-labeled secondary antibodies for at least one hour. Cells were washed with PBS containing 0.008% saponin and 10% blocking buffer after each incubation. To label actin, following antibody incubation cells were incubated in 165 nM labeled phalloidin in PBS containing 1% BSA for 20 minutes and washed. Coverslips were mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, CA).

Fluorescent labeling of cells

Cells were grown on coverslips affixed beneath holes in the bottom of 35 mm petri dishes and treated with 2 μg/mL doxycycline for 48 hours. To study steady state Tf distribution, cells were incubated at 37°C in serum-free DMEM containing 20 mM HEPES and 5 μg/mL Alexa488-Tf for 60 minutes followed by fixation. To study Tf and LDL trafficking, cells were pulse-labeled at 37°C in serum-free DMEM/HEPES containing 5 μg/mL Alexa488-Tf and/or 5 μg/mL DiI-LDL for 3 minutes. Cells were washed with ice-cold M2 buffer (150 mM NaCl, 5 mM KCl, 1mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM HEPES, pH7.4) and incubated in chase medium (DMEM containing 0.1 mg/mL unlabelled Tf and 0.1 mM deferoxamine mesylate (Sigma)) for various lengths of time followed by fixation. Lipid labeling was accomplished by incubating cells at 37°C in M2 buffer containing 5 μM C_6-NBD-SM and 0.2% glucose for 3 minutes, followed by washing with ice-cold M2 buffer. Cell surface C_6-NBD-SM was removed with six 10-minute washes in ice-cold PBS containing 5% fatty acid-free BSA (Sigma), and cells were chased in medium at 37°C.

BDM and Cytochalasin D treatment

To study the effect of the myosin inhibitor 2,3-butadione monoxime (BDM) (Sigma) on endosomal distribution, cells were incubated in 10mM BDM at 37°C for 15 minutes prior

to fixation. To study the effect of the actin depolymerizing agent cytochalasin D (Calbiochem, San Diego, CA), cells were incubated in 5μM cytochalasin D at 37°C for 30 minutes prior to fixation.

Widefield and confocal microscopy

Indirect immunofluorescent samples mounted on glass slides were viewed with a Zeiss Axiophot fluorescence microscope (Carl Zeiss Inc., Thronwood, NY). For coverslip-bottomed dishes, widefield fluorescence microscopy was performed on a DMIRB inverted microscope (Leica Inc., Deerfield, IL). Confocal images were collected on an LSM510 laser scanning confocal unit (Carl Zeiss Inc., Thronwood, NY) attached to an Axiovert 100M inverted microscope (Zeiss). Excitation on the LSM510 laser was with a 25-mW argon laser emitting 488nm, a 1.0-nW helium/neon laser emitting at 514nm, and a 5.0-mW helium/neon laser emitting at 633 nm. Emissions were collected using a 505-530-nm band pass filter to collect Alexa488 and a 585-nm long pass filter to collect DiI emission. For confocal images, reduced excitation light was applied for control of photo-bleaching. Cross-talk of the fluorophores into the wrong detectors was negligible.

^{125}I transferrin internalization and recycling

Cells were grown in 12-well plates and treated with 2 μg/mL doxycycline for 48 hours. Cells were serum starved in serum-free DMEM containing 20 mM HEPES for 60

minutes at 37°C. To study a single round of ^{125}I Tf endocytosis and recycling, cells were incubated on ice in 500 µL serum-free DMEM/HEPES containing 0.2 µCi/mL ^{125}I Tf (NEN, Boston, MA) for 1 hour, washed with ice-cold medium, and then incubated at 37°C for various lengths of time. At each time point cells were washed twice with 500 µL ice-cold PBS. The medium and both PBS washes were combined in the "Medium" fraction. Surface ^{125}I Tf was removed by acid stripping at room temperature in 50 mM MES pH5, 0.15 M NaCl, 280 mM sucrose (Dunn et al., 1989). Cells were incubated in 500 µL stripping buffer for 1 min, followed by 500 µL fresh buffer for an additional 3 min. Cells were then washed three times with PBS. Both acid washes and all three PBS washes were combined in the "Acid" fraction. Cells were lysed in 1 mL 1% Triton X 100, 0.1 M NaOH (the "Lysate" fraction). The amount of radioactivity in each fraction was determined using a CliniGamma gamma counter (Wallac Inc., Gathersburg, MD). The percent radioactivity in each fraction was calculated as the number of counts in that fraction divided by the total number of counts recovered. To study ^{125}I Tf recycling following continuous uptake, cells were grown and serum starved as described above then incubated at 37°C in 500 µL serum-free DMEM/HEPES containing 0.2 µCi/mL ^{125}I Tf for 30 or 60 minutes. Following acid stripping, 500 µL DMEM/HEPES containing 0.5 mg/mL unlabelled Tf (Calbiochem, La Jolla, CA) were added to each well, and cells were incubated at 37°C for various lengths of time. At each time point medium and

lysate fractions were collected and the percent radioactivity in each fraction was determined as described above.

Biotinylation of transferrin receptor

Following 48 hours of 2 μg/mL doxycycline treatment, cells were detached from 10 cm petri dishes using Ca^{2+}/Mg^{2+}-free PBS containing 0.04%EDTA. Approximately $2.5x10^6$ cells per sample were incubated in 0.5 mg/mL EZ-Link NHS-SS-Biotin (Pierce, Rockford, IL) in PBS at 4°C for 30 minutes, followed by washing. To allow biotinylated receptors to internalize, cells were incubated in medium at 37°C for 20 minutes, then washed with ice-cold PBS. Biotin was stripped from the cell surface using the technique of Bretscher et al. (Bretscher and Lutter, 1988). Briefly, cells were incubated on ice in stripping buffer (50 mM glutathione (GSH) (Sigma), 75 mM NaCl, 1 mM EDTA, 75 mM NaOH, 1% BSA) for 20 minutes, followed by a second 30 minute incubation in fresh stripping buffer. Cells were then washed extensively in ice-cold PBS. To follow receptor recycling, cells were reincubated at 37°C for 15 minutes and stripped again with GSH. Cells were lysed in 1% NP40, 150 mM NaCl, 10 mM Tris, pH 7.4, and Tf receptor was immunoprecipitated using mAb H68.4. Immunoprecipitates were separated by SDS-PAGE and blotted with streptavidin HRP (Zymed). Proteins were visualized by ECL and exposure to film. Bands were scanned and quantitated using the National Institutes of Health Image 1.61 program.

Results

Characterization of HeLa-T7Hub cells

To study the role of clathrin-coated buds on endosomes, we wanted to create a cell line which would express the dominant negative clathrin inhibitor Hub in an inducible fashion. An inducible system is necessary, as constitutive Hub expression leads to decreased cell viability (unpublished observation). We created a HeLa cell line (HeLa-T7Hub) which expresses Hub with an N-terminal T7 tag under control of the tetracycline operator sequence, such that Hub expression can be induced by addition of doxycycline. Fig. 3.1 A shows levels of Hub expression following treatment of HeLa-T7Hub cells with different concentrations of doxycycline for various lengths of time. Hub expression is induced by doxycycline in both a time- and concentration-dependent manner. High levels of Hub expression were achieved by 48 hours of treatment with 2 μg/mL doxycycline, as measured by both western blotting (Fig. 3.1 A, lane 4) and immunofluorescence (Fig. 3.1 B b). This induction condition was used in all subsequent experiments.

To ensure that HeLa-T7Hub cells express a functional Hub, we looked at the effect of Hub induction on the cellular distribution of the clathrin light chain. Previous studies showed that Hub binds clathrin light chain (Liu et al., 1995) and causes a cytosolic redistribution of light chain in transiently transfected HeLa cells (Liu et al.,

Figure 3.1

Characterizaion of HeLa-T7Hub cells

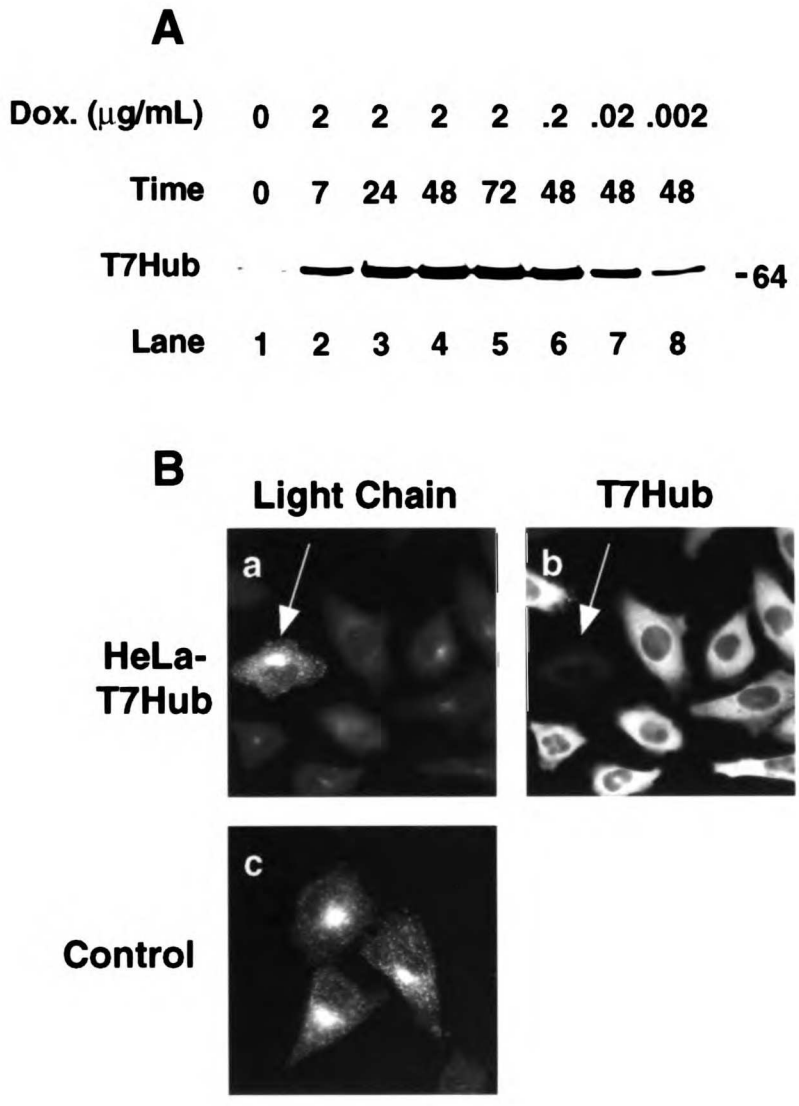


Figure 3.1. **Characterization of HeLa-T7Hub cells.**

(A) HeLa-T7Hub cells were grown in the presence of doxycycline using the concentrations and times indicated about each lane. Cell lysates were separated by SDS-PAGE and immunoblotted for T7Hub using an anti-T7 tag mAb. Proteins were visualized by ECL and exposure to film.

(B) HeLa-T7Hub cells (a and b) and control cells (c) were grown on coverslips and treated with 2 μg/mL doxycycline for 48 hours. Cells were processed for immunofluorescence and incubated with anti-clathrin light chain rabbit serum and anti-T7 tag mAb, followed by staining with FITC-conjugated donkey anti-rabbit (a and c) and RRX-conjugated donkey anti-mouse (b) secondary antibodies. Staining of control cells with the anti-T7 tag antibody was negligible (data not shown). Arrows indicate a non-Hub-expressing cell.

1998). Light chain redistribution is due to the fact that Hub does not associate with membranes. Therefore, Hub-bound light chain remains cytosolic. We looked at the distribution of clathrin light chain in HeLa-T7Hub and control cells following treatment with 2 μg/mL doxycycline for 48 hours. In control cells clathrin light chain has a punctate staining pattern indicative of association with clathrin-coated pits and vesicles (Fig. 3.1 B c) . In HeLa-T7Hub cells clathrin light chain has a cytosolic distribution as evidenced by a diffuse staining pattern and loss of staining intensity, the latter due to loss of cytosolic light chain upon cell permeabilization (Fig. 3.1 B a). Thus HeLa-T7Hub cells express a functional Hub in an inducible fashion, providing us with a convenient system for studying the effect of clathrin inhibition on various cellular trafficking processes.

Approximately 15% of HeLa-T7Hub cells did not stain brightly for Hub following doxycycline treatment (Fig. 3.1 B b, arrow). Cells which do not stain brightly for Hub do not show a redistribution of clathrin light chain (Fig. 3.1 B a, arrow) and thus may not have impaired clathrin function. Heterogeneity in Hub expression is probably due to the episomal nature of the pJM601 vector which may give rise to different copy numbers of the vector per cell or a higher tendency for cells to lose the construct altogether. Therefore, in biochemical experiments where high Hub- and low Hub-expressing cells cannot be distinguished, the measured effect of Hub on receptor trafficking may represent only 85% of the true effect.

Hub induces perinuclear aggregation of early endosomes

Because our aim was to determine the role of clathrin-coated buds on endosomes, we wanted to characterize the effect of Hub expression on the early endosomal compartment. We used Alexa488-Tf to monitor the movement of proteins through this compartment. An unexpected but striking effect of Hub expression was a distinct perinuclear aggregation of Tf-containing vesicles. While in control cells Tf-containing vesicles are dispersed throughout the cytosol (Fig. 3.2 A), in HeLa-T7Hub cells the vesicles aggregate in the perinuclear area, with hardly any vesicles in the periphery (Fig. 3.2 B). The perinuclear aggregate does not represent clustered Tf trapped on the cell surface, as an acid strip fails to eliminate it (data not shown). When cells were stained with an anti-TfR antibody, the staining pattern was identical to the pattern of Alex488-Tf localization, indicating that the receptor and its ligand are both present in the perinuclear aggregate (Fig. 3.2 D). Additionally, both DiI-LDL (Fig. 3.2 F) and C_6-NBD-sphyngomyelin (Fig. 3.2 H) also traffic through this perinuclear aggregate of endocytic vesicles, indicating that the compartment is part of the normal endocytic/recycling pathway and does not represent a Tf-specific effect. Both Tf and LDL arrive in the perinuclear aggregate rapidly after endocytosis. As the two markers sort from one another (shown in Fig. 3.12), Tf-containing vesicles remain in the perinuclear space, though they have a slightly broader distribution. Therefore we conclude that these perinuclear vesicles represent sorting and recycling early endosomes. These data are the

Figure 3.2

Hub induces perinuclear aggregation of early endosomes

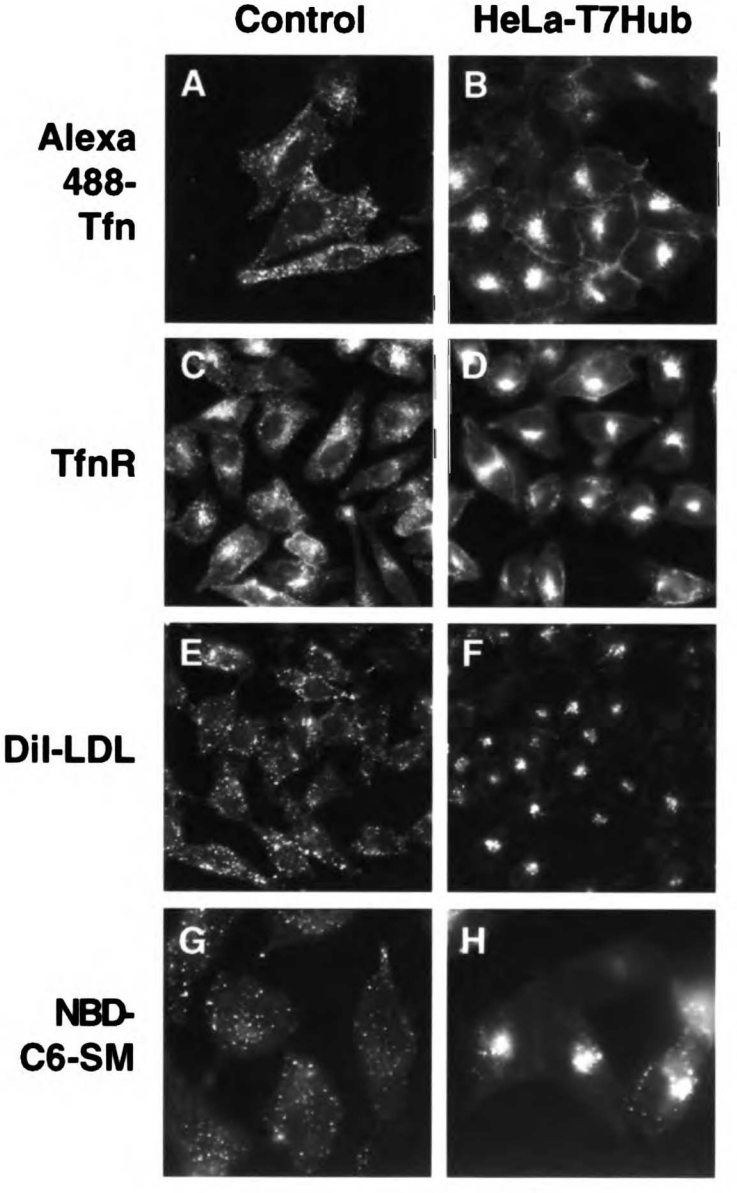


Figure 3.2. **Hub induces perinuclear aggregation of early endosomes.**

HeLa-T7Hub cells (right) and control cells (left) were grown on coverslips and treated with doxycycline.

(A and B) Cells were incubated at 37°C for 60 minutes in medium containing Alexa488-Tf and immediately photographed.

(C and D) Cells were processed for immunofluorescence and stained with mAb H68.4 which recognizes TfR, followed by RRX-conjugated goat anti-mouse secondary antibody.

(E and F) Cells were incubated at 37°C for 3 minutes in medium containing DiI-LDL, followed by a 5-minutes chase prior to photographing the cells.

(G and H) Cells were incubated at 37°C for 3 minutes in M2 buffer containing C_6-NBD-SM, and surface C_6-NBD-SM was back exchanged prior to photographing the cells.

first to show that clathrin-inhibition can lead to a redistribution of the early endosome compartment, and they point to a role for endosomal clathrin-coated buds in mediating endosome distribution.

AP2-coated vesicles organize in linear arrays in control cells but not in HeLa-T7Hub cells

Elements of the cytoskeleton have been shown to play a role in the organization of the early endocytic compartment (McGraw et al., 1993). Hub-induced redistribution of early endosomes led us to wonder whether Hub was acting by inhibiting association of early endosomes with cytoskeletal components. We had previously observed a linear staining pattern of AP2-coated vesicles that resembles the staining pattern of the actin cytoskeleton (unpublished observation and Fig. 3.3). We wanted to further investigate this apparent interaction of AP2-coated vesicles with actin. When cells were stained with mAb AP.6, which recognizes the α subunit of AP2, a dramatic difference was observed between the cellular distribution of AP2-coated vesicles in control cells and the distribution in HeLa-T7Hub cells. In control cells, AP2-coated vesicles can be seen in linear arrays (Fig. 3.3 C), while in HeLa-T7Hub cells AP2 vesicles are dispersed throughout the cytosol (Fig. 3.3 A). This result suggests that AP2-coated vesicles interact in some manner with the actin cytoskeleton.

Figure 3.3

AP2-coated vesicles organize in linear arrays in control cells but not in HeLa-T7Hub cells

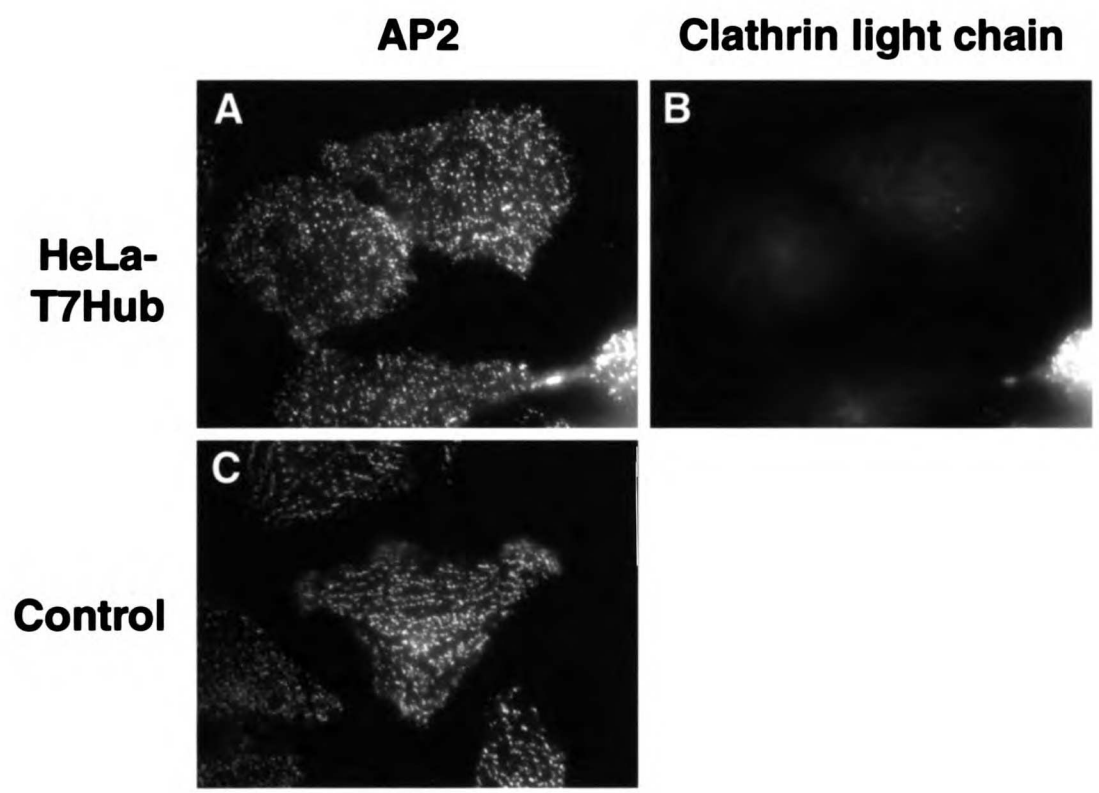


Figure 3.3. **AP2-coated vesicles organize in linear arrays in control cells but not in HeLa-T7Hub cells.**

HeLa-T7Hub (A and B) and control cells (C) were grown on coverslips, treated with doxycycline, and processed for immunofluorescence. Cells were incubated with mAb AP.6, followed by incubation with RRX-conjugated goat anti-mouse secondary antibody (A and C). To confirm that the cells in panel A express high levels of Hub, cells were also stained with anti-clathrin light chain rabbit serum followed by FITC-conjugated goat anti-mouse secondary antibody (C). The diffuse light chain staining pattern confirms the high Hub expression in these cells. Light chain staining for control cells was punctate as expected (data not shown.)

Actin establishes a spatial organization which influences AP2-coated vesicle distribution

In light of the observed linear patterns of AP2 staining, we wanted to determine whether AP2-coated vesicles directly associate with the actin cytoskeleton. Cells were costained with mAb AP.6 and fluorescently labeled phalloidin. The staining pattern of AP2 is collinear with that of actin (Fig. 3.4) suggesting that the two proteins might interact. AP2 distribution also correlates with the staining pattern of myosin (Fig. 3.5), a motor protein that associates with actin filaments. Fig. 3.6 shows cells triply stained for actin, myosin, and AP2. The merging of these images clearly shows a strong correlation between the three staining patterns. However, despite this correlation, AP2 does not fully colocalize with actin (Fig. 3.4, confocal image in Fig. 3.7). Rather, it seems that actin filaments organize a spatial distribution that AP2-coated vesicles follow.

To test whether the linear distribution of AP2-coated vesicles is actin-directed, we treated cells with cytochalasin D to induce actin depolymerization. Cytochalasin D treatment completely abolished the linear arrangement of AP2-coated vesicles (Fig. 3.8 A and B). Further, treatment of cells with the myosin inhibitor 2,3-butadione monoxime (BDM) also resulted in disruption of AP2 distribution (Fig. 3.8 C and D). These data support our hypothesis that the actin cytoskeleton establishes a spatial organization that mediates the cellular distribution of early endosomes. Further, the effect of Hub expression is identical to the effect of actin depolymerization and myosin inhibition, indicating that actin-directed AP2 distribution is clathrin-mediated. The lack of perfect

Figure 3.4

AP2 staining correlates with actin staining

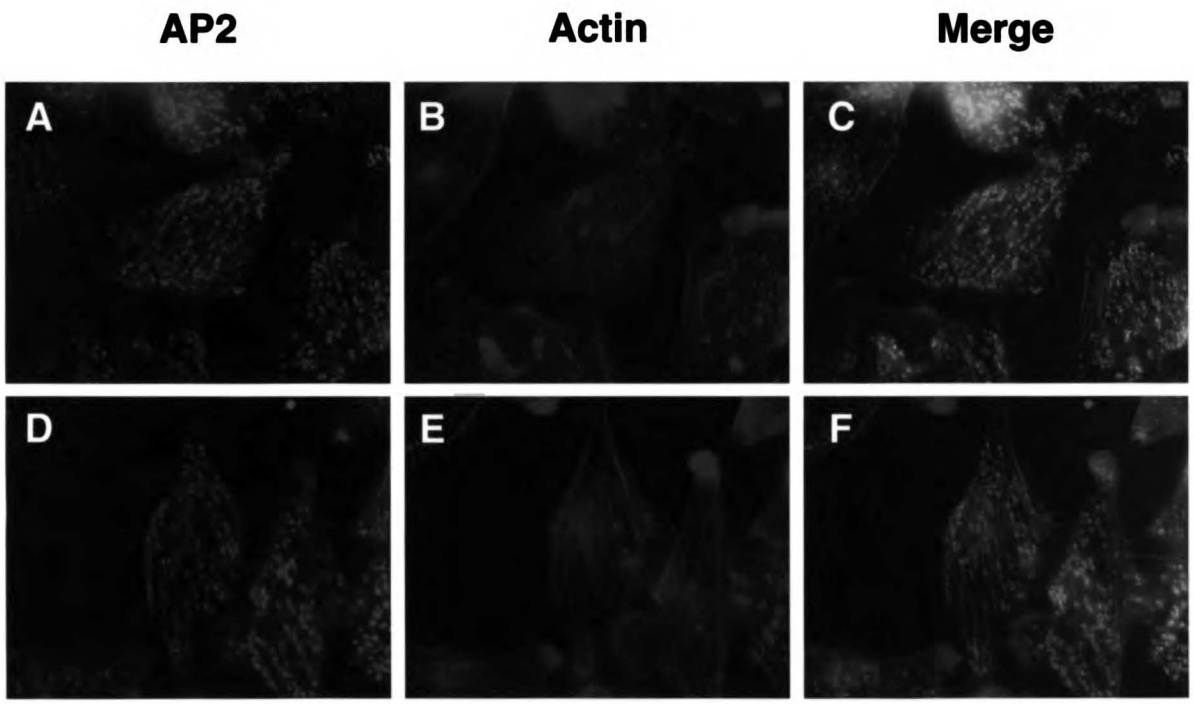


Figure 3.4. **AP2 staining correlates with actin staining.**

Cells were grown on coverslips and processed for immunofluorescence. Cells were incubated with mAb AP.6 followed by RRX-conjugate goat anti-mouse secondary antibody (A and D). Cells were then incubated with Alexa488 Phalloidin to label the actin cytoskeleton (B and E). C and F show merged images of AP2 and actin staining. Panels A-C and D-F represent two different fields of cells.

Figure 3.5

AP2 staining correlates with myosin staining

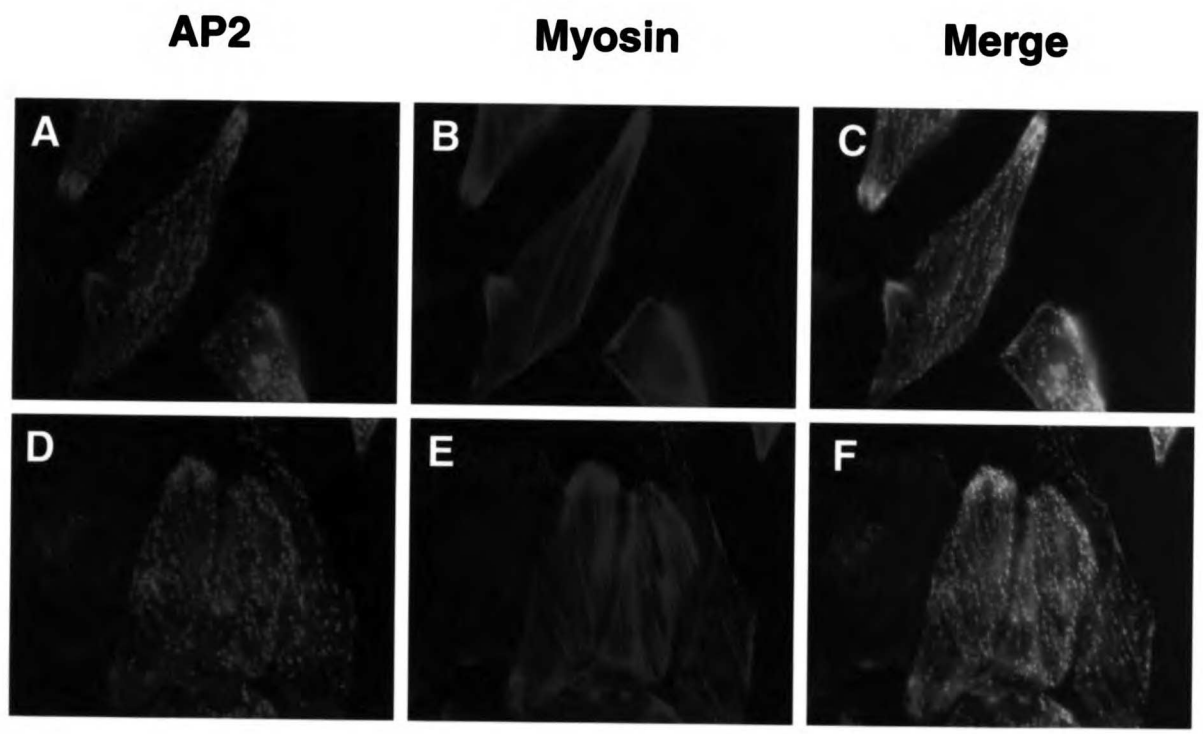


Figure 3.5. **AP2 staining correlates with myosin staining.**

Cells were incubated with mAb AP.6 and anti-myosin rabbit serum followed by RRX-conjugated goat anti-mouse (A and D) and FITC-conjugated donkey anti-rabbit (B and E) secondary antibodies. C and F show merged images of AP2 and myosin staining. Panels A-C and D-F represent two different fields of cells.

Figure 3.6

Triple staining of AP2, actin, and myosin

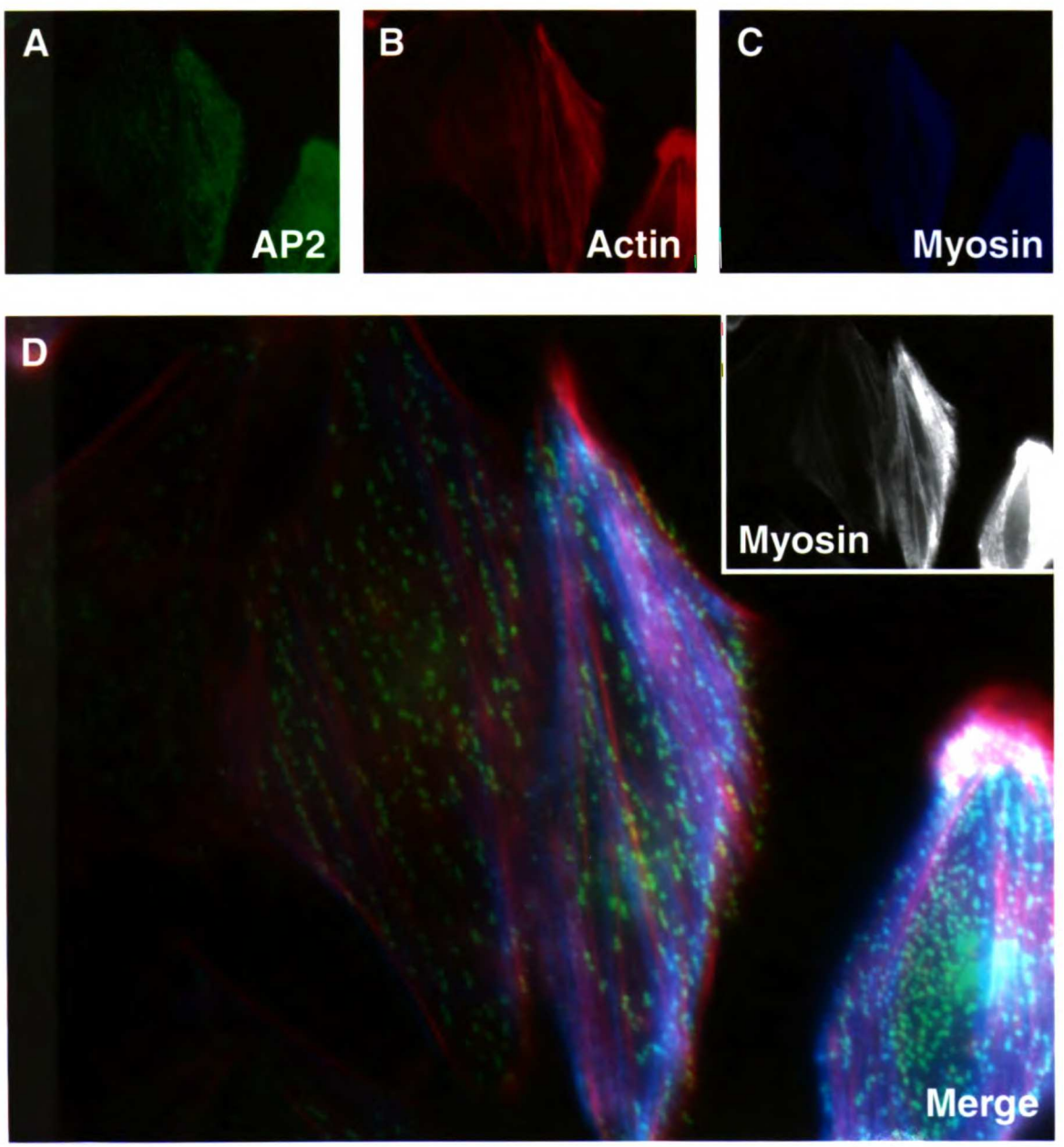


Figure 3.6. **Triple staining of AP2, actin, and myosin.**

Cells were grown on coverslips and processed for immunofluorescence. Cells were incubated with mAb AP.6 and anti-myosin rabbit serum followed by FITC-conjugated donkey anti-mouse (B) and Cy5-conjugated donkey anti-rabbit (C) secondary antibodies. Cells were then incubated with Alexa568-Phalloidin to label the actin cytoskeleton (A). Panel D shows the merged image of all three staining patterns. For visual ease, the Cy5 channel (myosin staining, identical to panel B) is shown without color in the inset of panel D.

Figure 3.7

Confocal images of AP2 and actin staining

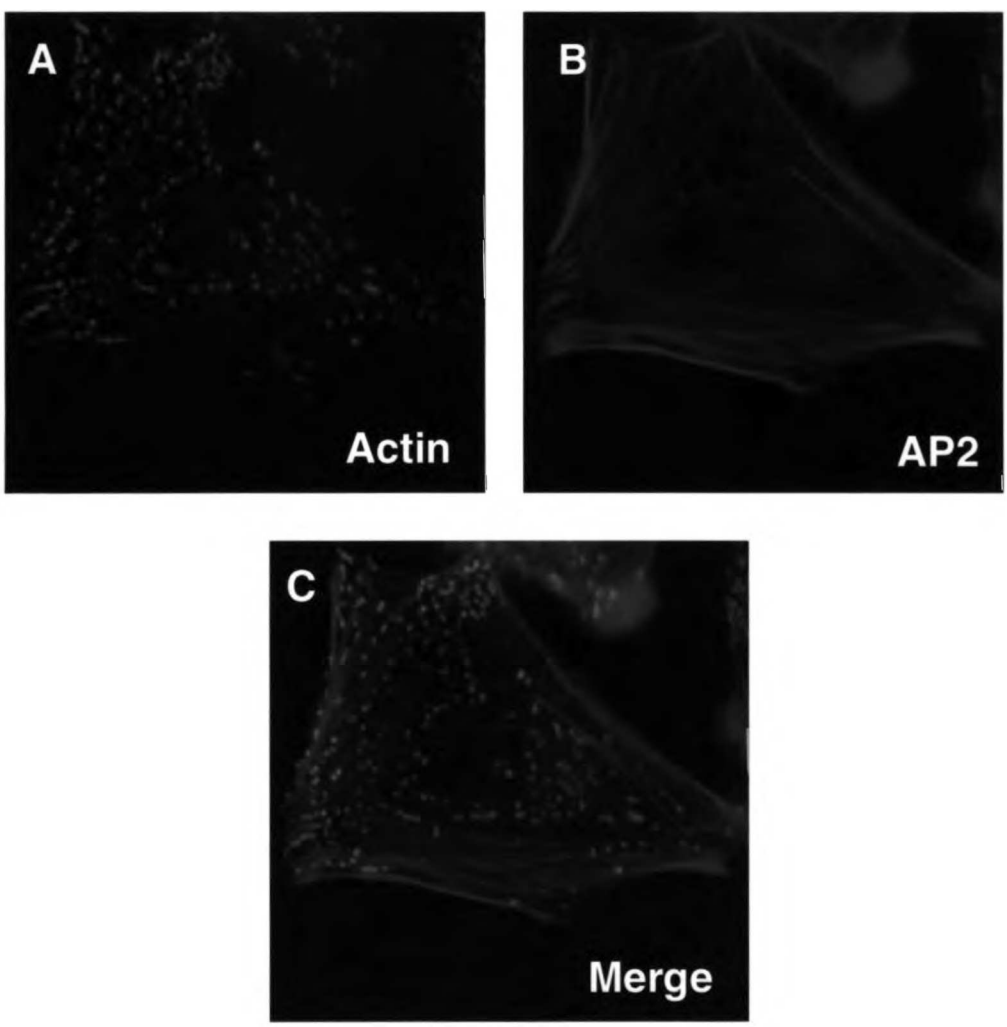
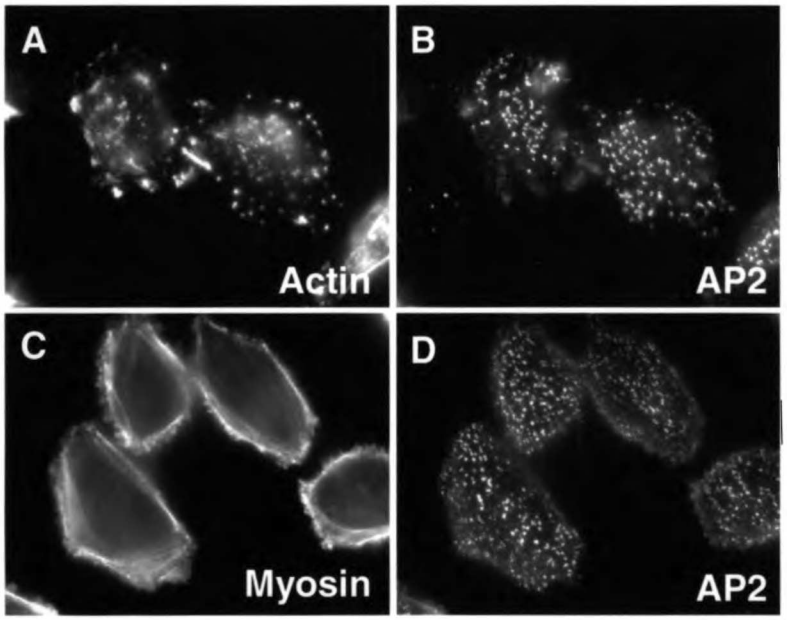


Figure 3.7. **Confocal images of AP2 and actin staining.**

Cells were grown on coverslips and processed for immunofluorescence. Cells were incubated with mAb AP.6 followed by RRX-conjugate goat anti-mouse secondary antibody (A). Cells were then incubated with Alexa488 Phalloidin to label the actin cytoskeleton (B). C shows the merged image of AP2 and actin staining. These cells were photographed using a confocal microscope.

Figure 3.8

Linear organization of AP2-coated vesicles is dependent upon the actin cytoskeleton



Cytochalasin D treatment

BDM treatment

Figure 3.8. **Linear organization AP2-coated vesicles is dependent upon the actin cytoskeleton.**

(A and B) Cells were grown on coverslips and treated with 5μM cytochalasin D for 40 minutes at 37°C prior to fixation and processing for immunofluorescence. Cells were incubated with mAb AP.6 followed by RRX-conjugated goat anti-mouse secondary antibody (B). Cells were then incubated with Alexa488-phalloidin to label the actin cytoskeleton (A). Cytochalasin D induces depolymerization of actin, as evidenced by the aggregation of actin in treated cells (compare A to Fig. 3.4 A).

(C and D) Cells were grown on coverslips and treated with 10mM BDM for 15 minutes at 37°C prior to fixation and processing for immunofluorescence. Cells were incubated with mAb AP.6 and anti-myosin rabbit serum followed by RRX-conjugated goat anti-mouse (D) and FITC-conjugated goat anti-rabbit (C) secondary antibodies. BDM inhibits myosin and causes myosin to dissociate from actin as indicated by the diffuse myosin staining pattern seen in C (compare to Fig. 3.4D).

colocalization of AP2-coated vesicles with actin (Fig. 3.4 and Fig. 3.7) suggests either that the interaction of AP2 with actin is mediated by other proteins which create a barrier between AP2 and actin or that actin acts by actually excluding AP2-coated vesicles from actin-occupied space. Further work is needed to investigate these possibilities.

Hub-induced redistribution of endosomes is not due to reorganization of actin or tubulin filaments

The data presented so far indicate that the organization of AP2-coated vesicles is directed by the actin cytoskeleton, resulting in the observed linear staining patterns of AP2. The data also indicate that clathrin mediates the influence of actin on the distribution of AP2-coated vesicles. Actin has been shown to play a role in RME, and it has also been shown that an intact microtubule network is necessary to maintain the organization of the recycling compartment (McGraw et al., 1993). Hub-induced redistribution of AP2-coated vesicles and perinuclear aggregation of sorting and recycling endosomes led us to wonder whether Hub expression disrupts the organization of either the actin or microtubule cytoskeleton. However, no alteration of the actin cytoskeleton was observed in HeLa-T7Hub cells (compare Fig. 3.9 A to Fig. 3.4 A). Likewise, Hub expression did not affect the organization of microtubules. The staining pattern for tubulin was identical in HeLa-T7Hub (Fig. 3.9 C) and control cells (Fig. 3.9 E). Thus Hub-induced disruption of AP2 and early endosome distribution can be

Figure 3.9

Hub expression does not alter the organization of the actin or microtubule cytoskeleton

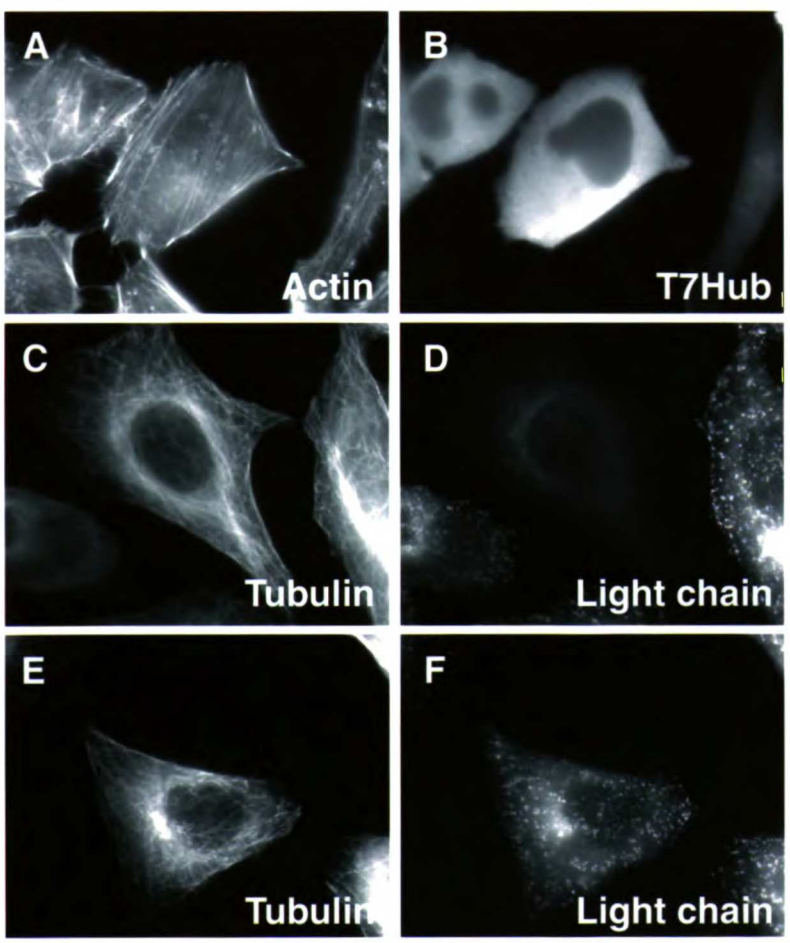


Figure 3.9. **Hub expression does not alter the organization of the actin or microtubule cytoskeleton.**

Cells were grown on coverslips, treated with doxycycline, and processed for immunofluorescence.

(A and B) HeLa-T7Hub cells were incubated with anti-T7 tag mAb followed by RRX-conjugated goat anti-mouse secondary antibody (B). Cells were then incubated with Alexa488-phalloidin to label the actin cytoskeleton (A).

(C-F) HeLa-T7Hub cells (C and D) and control cells (E and F) were incubated with anti-α-tubulin mAb and anti-clathrin light chain rabbit serum, followed by RRX-conjugated goat anti-mouse (C and E) and FITC-conjugated goat anti-rabbit (D and F) secondary antibodies. Light chain staining was performed to confirm Hub expression.

attributed to a direct effect of clathrin inhibition and is not a secondary effect of reorganization of cytoskeletal elements.

In light of these findings, we propose a model whereby actin filaments direct the spatial organization of AP2-coated vesicles in a clathrin-dependent fashion. There is evidence that the role of actin in RME is of an inhibitory nature (Fujimoto et al., 2000; Trifaró and Vitale, 1993) and that actin acts to maintain the peripheral distribution of endocytic patches of the plasma membrane (Gaidarov et al., 1999). Our data support this model and show that when actin's influence on AP2-coated vesicles is disrupted, early endosomes collapse into the perinuclear region. Based on this model, we suggest that the role of clathrin-coated buds on endosomes is to mediate the interaction of endocytic vesicles with elements of the cytoskeleton.

Hub inhibits transferrin receptor endocytosis with little impact on receptor recycling

In light of the redistribution of AP2-coated vesicles and the perinuclear aggregation of early endosomes in HeLa-T7Hub cells, we wanted to investigate whether Hub expression had any effect on the sorting and recycling functions of the early endosomal compartment. We used ^{125}I Tf to monitor the flow of material through the endocytic/recycling pathway. As expected, we observed a Hub-induced inhibition of Tf internalization. While control cells show a rapid internalization of ^{125}I Tf (Fig. 3.10 B, open triangles) accompanied by a disappearance of ^{125}I Tf from the cell surface (Fig. 3.10

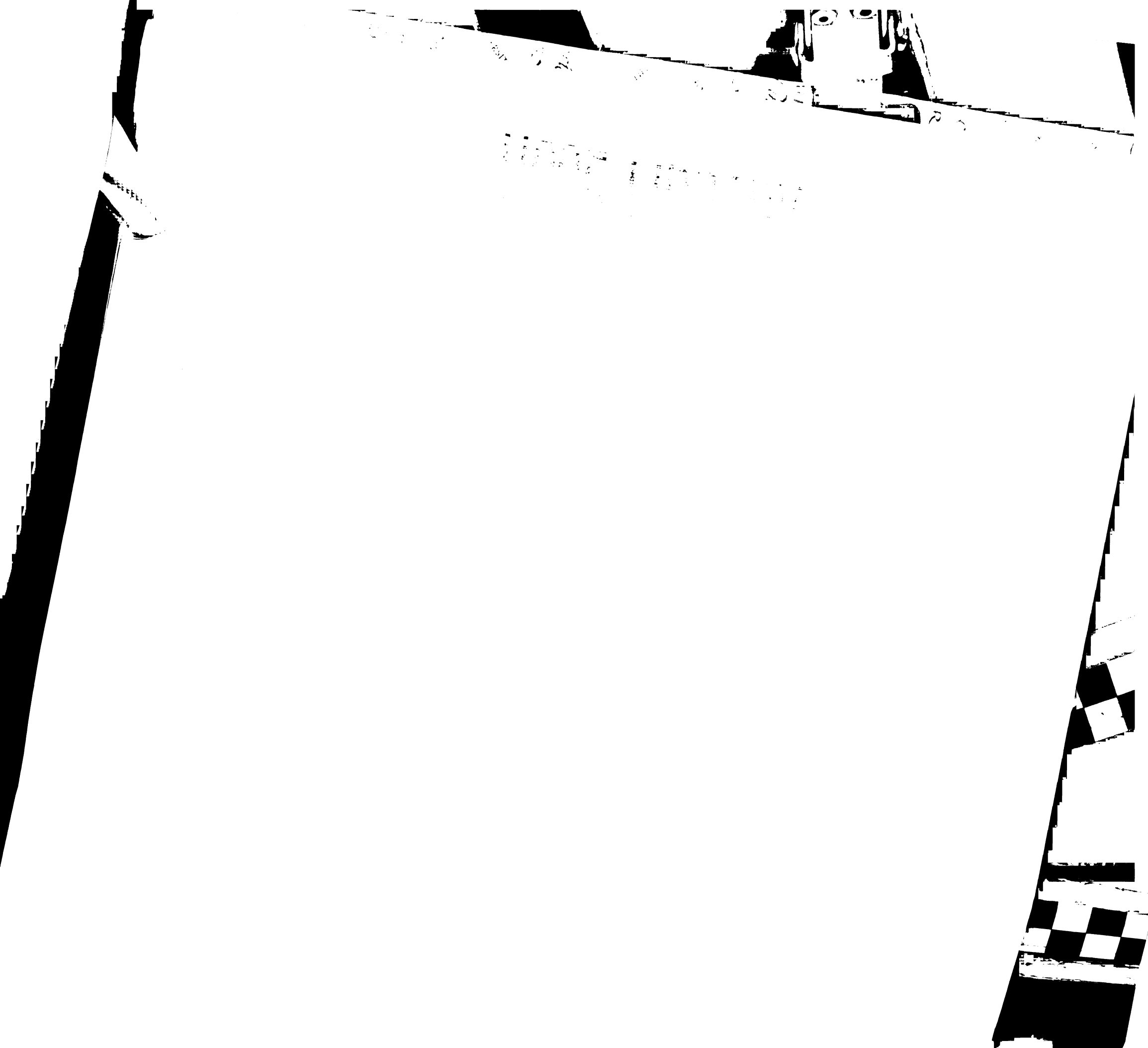


Figure 3.10

Effect of Hub on endocytosis and recycling of ^{125}I transferrin

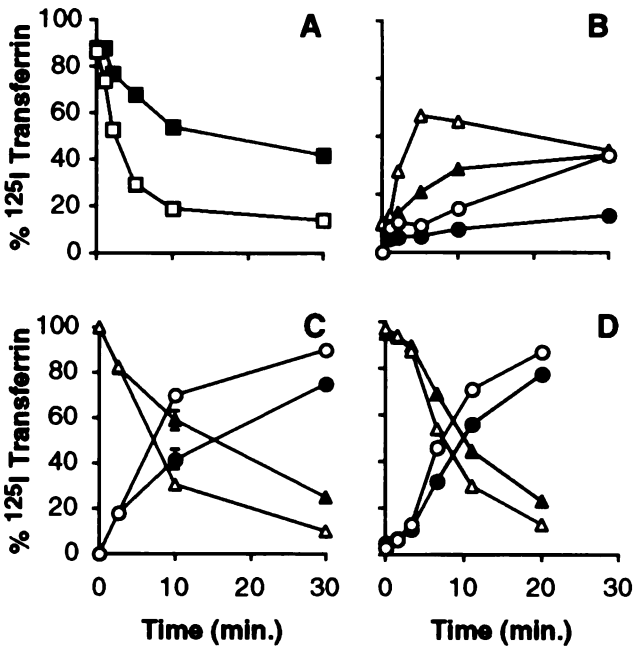


Figure 3.10. **Effect of Hub on endocytosis and recycling of ^{125}I transferrin.**

(A and B) HeLa-T7Hub cells (closed symbols) and control cells (open symbols) were grown in 12-well dishes and treated with doxycycline. Cells were serum starved and then incubated on ice in medium containing ^{125}I Tf. Following washing, cells were incubated in chase medium at 37°C for various times. At each time point acid (squares), lysate (triangles), and medium (circles) fractions were collected as described in the Materials and Methods, and the percent radioactivity in each fraction was determined. A and B show data from the same experiment. All points are the average of three wells.

(C and D) Cells were grown in 12-well dishes and treated with doxycycline. Cells were serum starved and then incubated at 37°C in medium containing ^{125}I Tf for 30 (C) or 60 (D) minutes. Surface ^{125}I Tf was removed by acid stripping, and cells were reincubated at 37°C in chase medium for various times. At each time point lysate and medium fractions were collected as described in the Materials and Methods, and the percent radioactivity in each fraction was determined. All points are the average of three wells. Symbols are as for A and B. Error bars are indicated on all graphs.

H

rep

if ceil

A, open squares) within 5 minutes, there is an obvious delay in the internalization of ^{125}I Tf in HeLa-T7Hub cells (Fig. 3.10 B, closed triangles). This delay is mirrored by a prolonged lifespan of ^{125}I Tf on the cell surface (Fig. 3.10 A, closed squares). By 10 minutes control cells begin to recycle ^{125}I Tf to the cell medium (Fig. 3.10 B, open circles), while there is little recycling in HeLa-T7Hub cells even after 20 minutes (Fig. 3.10 B, closed circles).

One explanation for the delay in Tf recycling in HeLa-T7Hub cells is that clathrin is directly involved in recycling of TfR and its ligand. However, a second possibility is that the delay in recycling is simply due to the initial delay in Tf internalization. We do not begin to observe substantial recycling in control cells until approximately 80% of the Tf has been endocytosed from the cell surface. We do not reach this level of Tf internalization in HeLa-T7Hub cells, and therefore the observed delay in recycling might indicate that Tf simply never progressed far enough along its intracellular route to recycle. To distinguish between these two possibilities, we allowed cells to internalize ^{125}I Tf to saturate the entire endocytic/recycling pathway prior to measuring recycling. Fig. 3.10 C shows the kinetics of Tf recycling in HeLa-T7Hub and control cells following 30 minutes of continuous ^{125}I Tf uptake. A slight delay is observed in Tf recycling in HeLa-T7Hub cells (closed symbols). While the delay is not extreme, it is consistent and reproducible. Interestingly, however, the delay in recycling becomes even less dramatic if cells are allowed to internalize ^{125}I Tf for 60 minutes prior to measuring recycling (Fig.

3.10 D). Thus the longer cells are allowed to internalize ^{125}I Tf, the less of an effect Hub has on Tf recycling. These data imply that the farther along its intracellular pathway Tf progresses, the less of a role clathrin plays in directing its trafficking. Furthermore, the effect of Hub on ^{125}I recycling seen in Fig. 3.10 C and D is not nearly as striking as the effect on Tf endocytosis seen in Fig. 3.10 A and B. These results indicate that while clathrin directly mediates RME at the plasma membrane, its role in receptor sorting and recycling is minimal.

These findings are further supported by immunofluorescent data. HeLa-T7Hub and control cells were pulsed with Alex488 Tf and then chased for various times. When cells were labeled with Alexa488-Tf we again observed a delay in Tf internalization (Fig. 3.11 E versus A). However, despite the inhibition of Tf endocytosis, by 30 minutes of chase the majority of labeled Tf recycles from both control and HeLa-T7Hub cells (Fig. 3.11 D and H). This result is striking because previous work has shown that recycling is, in fact, a slower process than endocytosis (Bleil and Bretscher, 1982; Hopkins and Trowbridge, 1983; Karin and Mintz, 1981; Mayor et al., 1993; Presley et al., 1993). That HeLa-T7Hub cells can, in essence, catch up to control cells despite the initial inhibition of Tf internalization further indicates that while inhibition of clathrin affects the kinetics of early, endocytic steps of TfR trafficking it does not affect later, recycling events.

To confirm our results we wanted to follow the trafficking of TfR directly without relying on labeled Tf. We used a cleavable biotin reagent to label cell surface proteins so

Figure 3.11

Endocytosed transferrin recycles with normal kinetics in HeLa-T7Hub cells

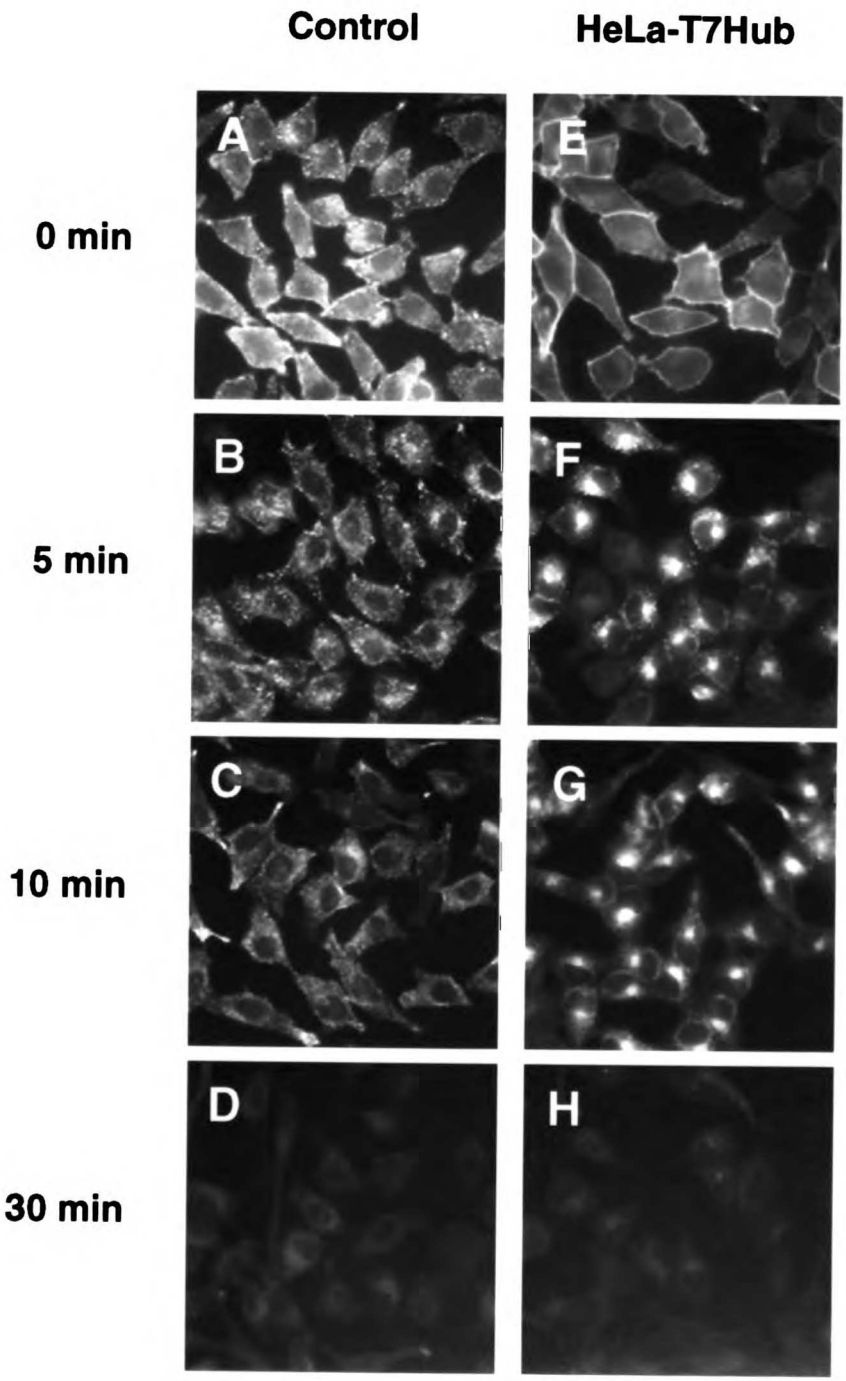


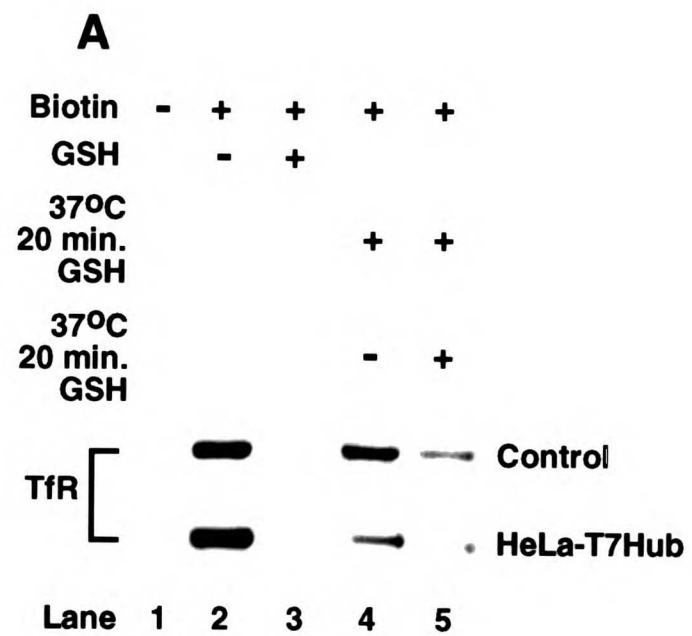
Figure 3.11. **Endocytosed transferrin recycles with normal kinetics in HeLa-T7Hub cells.**

HeLa-T7Hub cells (right) and control cells (left) were grown in coverslip-bottomed dishes and treated with doxycycline. Cells were incubated at 37°C for 3 minutes in medium containing Alexa488-Tf, followed by ice-cold washes. Cells were reincubated in chase medium at 37°C for 0 (A and E), 5 (B and F), 10 (C and G), or 30 (D and H) minutes. At each time point, cells were washed, fixed, and photographed.

that we could follow the progress of TfR through the endocytic and recycling pathway. Cells were incubated on ice with a disulfide-linked biotin label and then placed in medium at 37°C for 20 minutes, at which point surface biotin was removed by cleavage with glutathione (GSH). Cells were again incubated at 37°C for an additional 20 minutes, followed by another round of GSH stripping. In this manner we can follow the endocytosis and recycling of a pool of surface receptors by monitoring the gain or loss of biotinylated material at each step. Internalized receptors will be protected from GSH stripping, but will become resusceptible to stripping as they recycle to the plasma membrane. Fig. 3.12 A shows the levels of biotinylated TfR in HeLa-T7Hub and control cells following biotin labeling, incubation at 37°C, and GSH stripping. Lanes 2 and 3 show the total amount of biotinylated TfR following surface biotinylation without and with immediate GSH stripping, respectively. The increase in biotinylated material in lane 4 compared to lane 3 represents internalized TfR that is protected from GSH stripping. The decrease of biotinylated material in lane 5 compared to lane 4 represents TfR that has recycled to the plasma membrane and become resusceptible to GSH treatment. Fig. 3.12 B shows the percentage of TfR internalized and recycled in HeLa-T7Hub and control cells, based on the data shown in Fig. 3.12 A. As expected, HeLa-T7Hub cells internalize less TfR during the first 20 minute incubation period than control cells. While control cells internalized approximately 80% of biotinylated TfR during the first 20 minute incubation, HeLa-T7Hub cells internalized only 30%. TfR is endocytosed via

Figure 3.12

Hub inhibits endocytosis of transferrin receptor without affecting receptor recycling



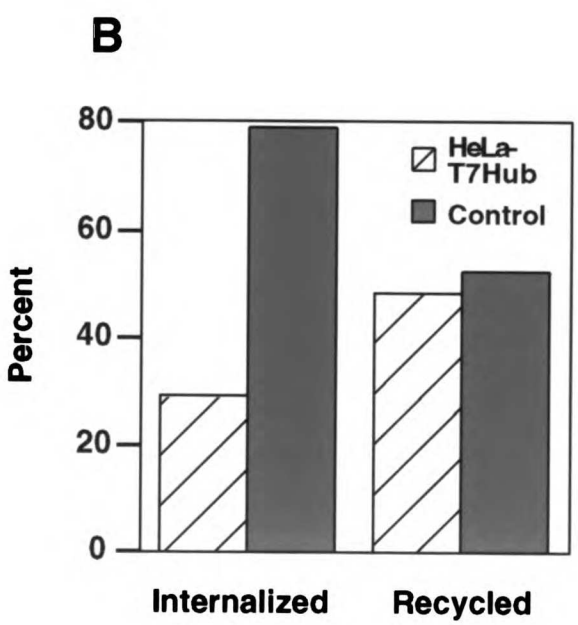


Figure 3.12. **Hub inhibits endocytosis of transferrin receptor without affecting receptor recycling.**

(A) HeLa-T7Hub cells and control cells treated with doxycycline were biotinylated on ice using a disulfide-linked, cleavable biotin reagent (lanes 2-5; lane 1 is an non-biotinylated control). To follow the internalization of TfR (lane 4), cells were incubated at 37°C for 20 minutes and then treated with GSH to remove residual surface biotin. To follow TfR recycling (lane 5), cells were incubated at 37°C for 20 minutes, stripped with GSH, then reincubated at 37°C for an additional 20 minutes and stripped again with GSH. TfR was immunoprecipitated, and immunoprecipitates were separated by SDS-PAGE and blotted with streptavidin-HRP. Lanes 2 and 3 indicate levels of total biotinylated TfR and GSH stripping efficiency, respectively.

(B) Bands from A were quantitated, and the percent TfR endocytosed and recycled was calculated. Percent endocytosed was calculated as the intensity of bands in lane 4 divided by the intensity of bands in lane 2. Percent recycled was calculated as the loss of intensity of bands in lane 5 compared to lane 4 divided by the intensity of bands in lane 4. For all calculations stripping inefficiency (intensity of bands in lane 3 divided by intensity of bands in lane 2, approximately 15%) was taken into account.

clathrin-coated pits, so this result was not surprising and confirms that Hub does, in fact, inhibit clathrin function. However, for receptors that were internalized by HeLa-T7Hub cells, there is hardly any delay in recycling to the plasma membrane. Both control and HeLa-T7Hub cells recycled approximately half (52% for control cells, 48% for HeLa-T7Hub cells) of internalized TfR during the second 20 minutes incubation. These results confirm that although clathrin is crucial for the timely endocytosis of TfR, it does not play a major role in TfR recycling.

Perinuclear aggregation of early endosomes does not inhibit sorting of Tf from LDL

Although perinuclear aggregation of endosomes in HeLa-T7Hub cells did not affect the kinetics of Tf recycling, we wanted to determine if inhibition of clathrin and reorganization of early endosomes has an effect on the endosomal sorting process. In contrast to Tf, LDL dissociates from its receptor in the sorting endosome (Davis et al., 1987) and progresses to the lysosome. Because Tf and LDL are both internalized via RME to sorting endosomes and then separate from one another, we can use these two ligands as markers to monitor receptor endocytosis, sorting, and recycling. HeLa-T7Hub and control cells were given a short pulse with Alexa488-Tf (Fig. 3.13 shown in green) and DiI-LDL (shown in red). At various chase times confocal images were taken to determine whether the two ligands colocalized to the same vesicles (indicated by yellow) or whether they had properly sorted from one another. In control cells some sorting

Figure 3.13

Transferrin and low density lipoprotein sort with normal kinetics in HeLa-T7Hub cells

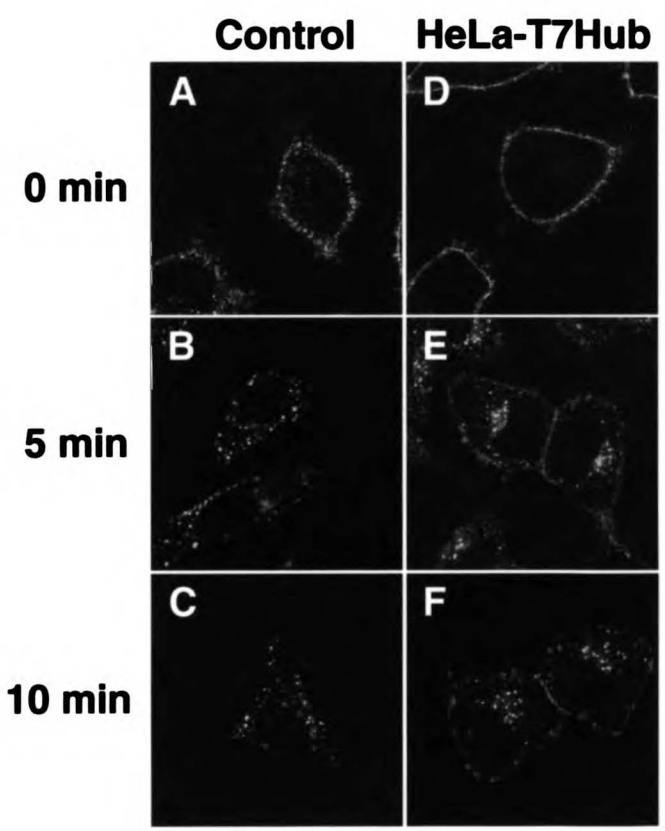


Figure 3.13. **Transferrin and low density lipoprotein sort with normal kinetics in HeLa-T7Hub cells.**

HeLa-T7Hub cells (right) and control cells (left) were grown in coverslip-bottomed dishes and treated with doxycycline. Cells were incubated at 37°C for 3 minutes in medium containing Alex488-Tf (green) and DiI-LDL (red), followed by ice-cold washes. Cells were then incubated in chase medium at 37°C for 0 (A and D), 5 (B and E), or 10 (C and F) minutes. At each time point cells were washed, fixed, and photographed using a confocal microscope.

occurs by 5 minutes of chase (Fig. 3.13 B), and by 10 minutes (C) almost all the Tf and LDL have sorted to distinctly separate vesicles. As expected, there is an inhibition of internalization of Tf and LDL in HeLa-T7Hub cells (Fig. 3.13 D), and residual plasma membrane staining is evident throughout the chase (E and F). In HeLa-T7Hub cells Tf and LDL proceed rapidly to the perinuclear aggregate (Fig. 3.13 E). For this reason we conclude that sorting endosomes are present in the perinuclear aggregate. However, despite the altered distribution of these Tf- and LDL-containing vesicles, the ligands do seem to sort from one another with kinetics similar to control cells. By 10 minutes of chase HeLa-T7Hub cells contain a substantial number of vesicles which stain only for Tf, despite the fact that these vesicles remain clustered in the perinuclear area (Fig. 3.13 F). This result indicates that proper sorting of Tf from LDL does occur in the presence of Hub and that recycling vesicles (which stain only for Tf) are also included in the perinuclear aggregate.

The appearance of singly stained green vesicles by 5-10 minutes of chase indicates that Tf does properly sort from LDL in HeLa-T7Hub cells. Interestingly, despite the appearance of Tf-containing vesicles, there is not a corresponding appearance of vesicles staining only for LDL, as is seen in control cells (Fig. 3.13 F versus C). The majority of LDL-containing vesicles in HeLa-T7Hub cells continue to costain for Tf, at least partially, throughout the 10 minute chase. This finding could indicate that LDL is not progressing to late endosomes/lysosomes or that the maturation of these latter

compartments is disrupted by the expression of Hub. An investigation into the effect of Hub on late endosomal pH and the trafficking of LDL is in progress.

Discussion

We set out to determine the role of clathrin-coated buds on endosomes using the dominant-negative clathrin inhibitor Hub to disrupt clathrin function. We discovered a novel effect of clathrin inhibition on the distribution of AP2-coated vesicles and early endosomes which suggests that the role of endosomal clathrin-coated buds is to mediate the cellular organization of endosomes. We observed that in control cells, AP2-coated vesicles are normally found in arrays which are collinear with actin filaments. This finding is consistent with a proposed role for actin in RME. These linear arrays can be disrupted by cytochalasin D treatment, which depolymerizes the actin cytoskeleton, or by BDM treatment, which inhibits myosin. Therefore, the data indicate that the actin cytoskeleton is responsible for establishing the organization of the AP2 arrays, and that the process is mediated by myosin. Most importantly, Hub expression disrupted the organization of AP2-coated vesicles in an identical fashion to cytochalasin D and BDM treatment, indicating that clathrin is also involved in mediating actin-influenced organization of AP2-coated vesicles. This is the first study to report an effect of clathrin inhibition on the spatial relationship between AP2-coated vesicles and actin. Based on this data we conclude that clathrin is capable of mediating an interaction between

clathrin-coated buds or vesicles and the actin cytoskeleton. Such a role for clathrin would explain the perinuclear aggregation of early endosomes that we observe in HeLa-T7Hub cells. In light of the evidence of a role for actin in maintaining the peripheral distribution of endocytic vesicles (Gaidarov et al., 1999), we propose a model whereby actin directs the organization of endosomes and maintains their disperse cytosolic distribution, and that in the absence of actin's influence, early endosomes aggregate in the perinuclear area. Here we show that clathrin inhibition results in inhibition of actin-influenced endosomal distribution.

Despite the collinearity that we found in the staining patterns of AP2 and actin, perfect colocalization of the two proteins was not observed. Whether this result indicates that actin actually excludes endocytic vesicles from certain regions or simply that the interaction between AP2 and actin is mediated by many layers of linking proteins is under investigation. In support of a link between clathrin coats and actin, the Hip1R protein has been shown to bind actin *in vitro* and to be enriched in CCV cellular fractions (Engqvist-Goldstein et al., 1999). Hip1R was recently shown to bind clathrin *in vivo* and to induce cross linking of both clathrin and actin (Engqvist-Goldstein et al., 2000). These findings make Hip1R an attractive candidate for the link between clathrin coats and the actin cytoskeleton.

Although we did not find evidence for a clathrin-mediated receptor recycling pathway, the suggestion that clathrin might mediate receptor sorting or recycling is not

ill-founded. The existence of clathrin-coated buds on endosomes enriched for Tf and TfR (Killisch et al., 1992; Stoorvogel et al., 1996; Whitney et al., 1995) and the requirement for clathrin in proper basolateral targeting of recycling TfR in polarized MDCK cells (Futter et al., 1998) are strongly suggestive of a role for clathrin in the post-endocytic trafficking of TfR. However, in accordance with prior kinetic studies (Damke et al., 1994; Jing et al., 1990; Mayor et al., 1993; McGraw and Maxfield, 1990) we were unable to detect a significant effect of clathrin inhibition on the sorting or recycling of Tf. We conclude that although clathrin plays a major role in the internalization of TfR, it is not required for efficient receptor sorting or recycling.

That Hub can so drastically alter the distribution of early endosomes without greatly affecting the kinetics of receptor sorting and recycling is surprising but not without precedent. The distribution of recycling endosomes varies between cell types (Apodaca et al., 1994; Daro et al., 1996; Ghosh et al., 1994; Hopkins et al., 1990; Marsh et al., 1995; McGraw et al., 1993; Tooze and Hollinshead, 1991), indicating that the overall organization of the recycling compartment is not crucial to its function. This fact is further illustrated by the observation that reorganization of the recycling compartment does not result in altered receptor recycling kinetics (Futter et al., 1998; Johnson et al., 1996; McGraw et al., 1993). Thus clathrin-coated buds on endosomes seem to play a strictly morphological role in maintaining the organization of the early endosomal compartment, though under conditions which inhibit clathrin function, trafficking

through this compartment occurs with normal kinetics. These results raise the issue as to why there is any organization of the early endosomal compartment if sorting and recycling can occur normally when the compartment is disrupted. It is likely that in the context of an organized tissue, there is a much greater need for polarized sorting and recycling functions than in the tissue culture cells used in these experiments. Therefore, clathrin's role in maintaining endosomal organization is probably of greater importance in living tissue.

The results presented here, in combination with prior work, point to a role for clathrin on endosomes that is characteristically distinct from its role at the plasma membrane and is quite different from the protein trafficking role of clathrin that is so well studied . While clathrin exerts a direct kinetic effect on the endocytosis of receptors at the plasma membrane, the role for clathrin-coated buds on endosomes appears to be of a morphological nature. Thus clathrin coats can perform two different cellular functions--controlling protein trafficking and mediating an interaction with actin. The data in this report suggest that at the plasma membrane clathrin actually plays both roles by directing RME and mediating the spatial relationship between AP2 and actin. These data highlight an important role for endosomal clathrin-coated buds in mediating actin-directed cellular distribution. Research is underway to better define the mechanics of how clathrin performs this function.

Chapter 3: Supplemental Data

The following data was produced as part of the work presented in Chapter 3 but will not be included in the manuscript submitted to *The Journal of Cell Biology*.

Anti-hook 1, hook 2, and hook 3 rabbit sera were provided by Dr. Helmut Krämer of the Department of Cell Biology and Neuroscience at the University of Texas Southwestern Medical Center.

While carrying out the research presented in this chapter, we included in our analysis an investigation into the effect of Hub expression on the cellular distribution of hook 1, hook 2, and hook 3. Hook proteins localize to endocytic vesicles and large vacuoles that are distinct from lysosomes (Krämer and Phistry, 1996; Krämer and Phistry, 1999). Recent data indicates that hook is involved in the maturation of multivesicular endosomes and delivery of cargo from multivesicular bodies to late endosomes and lysosomes (Sunio et al., 1999). The role for hook in these processes is thought to be of an inhibitory nature. Hook proteins associate with microtubules, though each hook protein seems to also associate with different organelles (personal communication from Dr. Helmut Krämer). Hook 3 has been observed in the cis Golgi (Dr. Krämer.) To see whether Hub expression affects the distribution of hook proteins, we stained cells with rabbit sera against hook 1, hook 2, and hook 3. Fig. 3.14 shows the staining patterns of the three proteins in control and HeLa-T7Hub cells. The staining pattern for hook 1 clearly demonstrates hook 1's association with microtubules (compare Fig. 3.14 A and B to Fig. 3.9 C and E). Hook 2 shows a staining pattern somewhat reflective of the microtubule cytoskeleton, particularly at the edges of cellular projections (see the upper right-hand portion of the cell in Fig. 3.14 E). The staining pattern for hook 3 does not appear to be coincident with microtubules, though as communicated by Dr. Krämer, hook 3 might be localized to the Golgi. In comparing Fig. 3.14 A and B, D and E, and G and H it is clear that Hub does not alter the distribution of any of the hook

proteins. This results leads us to conclude that Hub exerts its effect on components of the early endocytic pathway (as shown in this chapter) and not on late endosomes or lysosomes.

Figure 3.14

Hub does not alter the cellular distribution of Hook 1, 2, or 3

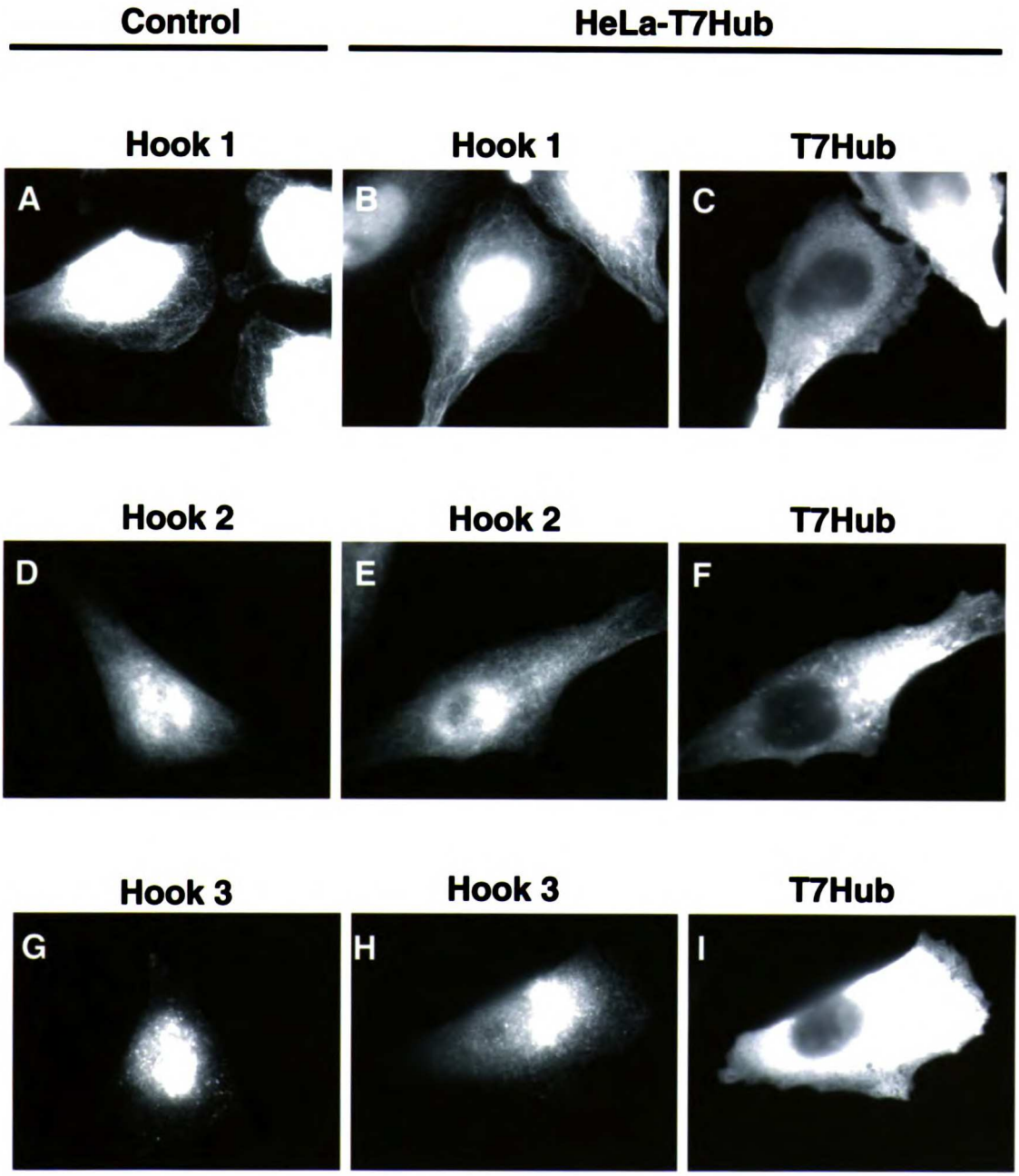


Figure 3.14. **Hub does not alter the cellular distribution of Hook 1, 2, or 3.** HeLa-T7Hub cells (middle and right columns) and control cells (left column) were grown on coverslips, treated with doxycycline, and processed for immunofluorescence. Cells were stained with rabbit serum against hook 1 (A and B), hook 2 (D and E), or hook 3 (G and H) followed by FITC-conjugated donkey anti-rabbit secondary antibody. HeLa-T7Hub cells were also stained with anti-T7 tag mAb followed by RRX-conjugated donkey anti-mouse secondary antibody to confirm Hub expression (C, F, and I).

Chapter 4

Discussion and Future Directions

Chapter 4 summarizes the findings of Chapters 2 and 3 and proposes future directions for the research. Preliminary results are presented.

The research presented in this dissertation was undertaken to investigate mechanisms by which viruses inhibit antigen presentation by class I major histocompatibility complex molecules. Antigen presentation by class I MHC molecules is a fundamental element of our immune response against viral infection. The ability of viruses to prevent antigen presentation has proven to be a fascinating area of investigation: it not only contributes to the establishment of persistent or latent viral infection, but also highlights the evolutionary forces which drive viruses and hosts to coevolve. This latter point is best illustrated by a host NK cell response against cells lacking class I MHC molecules and the ability of viruses to selectively downregulate class I molecules such that an NK cell attack can be avoided.

In studying the effects of viral proteins on the assembly of class I MHC molecules, it becomes clear that no step in the assembly pathway escapes viral interference. A key finding of the research presented here is that the adenovirus E19 protein binds TAP and inhibits class I molecule/TAP interaction. E19 is the first viral protein shown to affect the interaction of class I molecules with TAP and also the first viral protein discovered to affect class I MHC expression by two mechanisms: inhibition of class I molecule/TAP association and ER retention of class I molecules. Whether other viral proteins also affect the interaction of class I molecules with TAP or exert multiple effects on class I assembly and trafficking remains to be seen.

Another viral protein of interest for this body of work is the HIV Nef protein. To

study the effect of Nef on the expression of class I MHC molecules, it was necessary to develop tools and techniques to study class I MHC endocytosis and trafficking. Such tools and techniques were developed while investigating the role of clathrin-coated buds on early endosomes. While it is well established that clathrin mediates RME from the plasma membrane, the presence of clathrin-coated buds on endosomes left open the possibility that clathrin might also mediate later trafficking events such as receptor sorting and recycling. Prior studies used mutated transferrin receptors or indirect clathrin inhibitors to investigate the role of clathrin in receptor sorting and recycling. The research presented here is the first study to use a direct inhibitor of clathrin, the clathrin Hub, to study the effect of clathrin inhibition on these trafficking events. Despite our unique approach, our data are in agreement with earlier studies which concluded that clathrin does not mediate the sorting or recycling of transferrin receptor. However, using Hub we did uncover two novel effects of clathrin inhibition: redistribution of early endosomes into a perinuclear aggregate and disruption of the spatial relationship between AP2-coated vesicles and the actin cytoskeleton. We propose a model whereby the actin cytoskeleton helps maintain the peripheral distribution of endocytic vesicles. In the presence of Hub the spatial relationship between endocytic vesicles and actin filaments is disrupted. Thus actin is unable to exert an inhibitory effect on trafficking, and endocytic vesicles collapse into the perinuclear area. The HeLa-T7Hub cell line and the protocols developed during this investigation can now be applied to studying constitutive and HIV

Nef-induced endocytosis of class I MHC molecules.

Future Directions

Some questions remain with regard to this body of research. In the case of adenovirus E19, an obvious follow-up investigation is to determine whether E19 affects peptide loading of class I MHC molecules. Because E19 inhibits association of class I molecules with TAP, it is likely that the viral protein also inhibits peptide loading. Inhibition of peptide loading would explain why class I alleles which do not associate well with E19 still experience delayed maturation in $E19^+$ cells (Beier et al., 1994). A common technique for determining the state of assembly of class I molecules is to take advantage of the fact that empty class I dimers (consisting of heavy chain and β_2m without peptide) are inherently unstable (Townsend et al., 1990). Empty class I dimers dissociate when incubated overnight at 4°C or 1-2 hours at 37°C, whereas peptide-loaded class I molecules remain assembled. Because recognition of class I molecules by mAb W6/32 is dependent upon heavy chain association with β_2m, W6/32 immunoprecipitation can be used to determine whether class I dimers are loaded with peptide. When this technique was used to determine the assembly state of class I molecules in $E19^+$ cells, W6/32 immunoprecipitated equivalent amounts of class I molecules before and after lysate incubation. This result seems to indicate that peptide loading of class I molecules is not inhibited by E19. However, E19 remains bound to class I molecules during lysate

incubation. Because the viral protein associates with the class I peptide binding groove it may stabilize empty dimers, complicating interpretations of the results. While there are monoclonal antibodies which specifically recognize peptide-loaded class I molecules (Bresnahan et al., 1997), the association of E19 with empty class I dimers might complicate their use as well. The best approach would entail dissociating E19 from class I molecules without disturbing the class I assembly state and then assaying whether the class I molecules contain bound peptide. Such an approach has yet to be developed.

Investigation of the function of clathrin-coated buds on endosomes yielded interesting results. The data clearly show that expression of Hub has two effects on endosomes: redistribution of early endosomes and disruption of the spatial relationship between AP2-coated vesicles and the actin cytoskeleton. While we propose a model whereby actin inhibits the collapse of endocytic vesicles into the perinuclear region, there are a few experiments which should be performed to solidify this hypothesis. One phenomenon which we have been unable to observe is the induction of a perinuclear aggregate of endosomes in Hub-negative cells as a result of actin inhibition. Two approaches were used to attempt to recreate the effect of Hub expression: disruption of the actin cytoskeleton by cytochalasin D and inhibition of myosin by BDM. Unfortunately, following both treatments, when cells were allowed to internalize fluorescently labeled transferrin, too little Tf was internalized to yield reliable results. The recreation of a perinuclear aggregate in Hub-negative cells will require the

development of an assay to inhibit actin while still allowing measurable Tf uptake. A related issue that should be investigated is whether the aggregation of endosomes in Hub-expressing cells is actin-dependent. Again, however, an assay must be developed which will allow for actin disruption or myosin inhibition without completely abolishing Tf uptake.

An additional protein which should be studied in Hub-expressing cells is Hip1R, a protein closely related to the huntingtin interacting protein 1 (Hip1). As discussed in the Introduction, Hip1R could serve as a link between CCV and the actin cytoskeleton and might be responsible for mediating the spatial relationship between AP2-coated vesicles and actin.

A final area which should be investigated is the effect of Hub expression on the acidification of the endocytic pathway. Clathrin inhibition may result in deficient transport of proteins necessary to maintain the proper pH of endosomes and lysosomes. One such factor is the vacuolar membrane ATPase complex (V-ATPase) (Stevens and Forgac, 1997) which is known to associate with AP2 (Liu et al., 1994; Myers and Forgac, 1993). Inhibition of clathrin may disrupt the delivery of V-ATPase to endosomes and therefore might alter the pH of the endocytic pathway. Altered pH may be partially responsible for the observed effects of Hub expression on early endosomes distribution. A variety of pH-sensitive endocytic probes are commercially available (e.g., Molecular Probes), and both immunofluorescence- and FACS-based approaches can be used to

determine the pH of different endocytic compartments.

The tools and reagents developed during the course of this research can be used to study constitutive and HIV Nef-induced endocytosis of class I MHC molecules. Class I endocytic pathways vary with cell type and class I allele under normal and Nef^+ conditions. Endocytic pathways also vary with regard to whether the process is spontaneous or induced. Therefore, a unified approach must be developed which takes into account cell- and allele-specific differences and is consistent with regard to the use of antibodies or crosslinking agents. Some of the questions which remain to be answered include:

- Is class I endocytosis spontaneous?
- Is class I endocytosis clathrin-mediated?
- Are endocytosed class I molecules recycled or degraded?
- How does class I endocytosis vary with cell type and class I allele?
- Do viral proteins such as HIV Nef and KSHV K3 and K5 utilize constitutive class I endocytic pathways or access novel trafficking pathways?

One approach to study class I endocytosis is to label cell surface proteins with a cleavable biotin reagent and monitor the amount of recoverable biotinylated class I molecules following incubation at 37°C, (presented in Chapter 3). The benefit of this approach is that it does not depend upon the use of antibodies or crosslinking agents

which might induce class I endocytosis. This assay has been performed on the B lymphoblastoid cell line JY (Fig. 4.1). Cell surface proteins were labeled with a disulfide-linked, cleavable biotin reagent, cells were incubated at 37°C for 30 minutes, and biotin was stripped from the cell surface by reduction with GSH. The class I heavy chain band in lane 4 of Fig. 4.1 represents material that was endocytosed and is protected from GSH stripping. This result shows that biotinylation can be used to monitor class I endocytosis and that endocytosis is slow but spontaneous in JY cells. Another useful approach to study class I endocytosis is to label cell surface class I molecules with anti-class I antibodies, incubate cells to allow for class I endocytosis, and then monitor the level of antibody on the cell surface by FACS analysis. This protocol has the advantage that endocytosis can be assessed at multiple time points with ease, allowing one to study the kinetics of class I endocytosis in greater detail. The complexity of the biotinylation assay makes it difficult to monitor multiple time points. When using a FACS-based approach certain controls should be included. First, samples should be done in duplicate so that one sample can be permeabilized prior to staining with secondary antibody. This step will assure that loss of surface staining is due to internalization and not antibody dissociation from the cell surface. Second, Fab fragments should be used alongside whole antibody to confirm that endocytosis is not the result of antibody crosslinking.

A biotinylation protocol can also be used to determine whether class I MHC molecules are recycled or degraded following endocytosis. After cells have been allowed

Figure 4.1

Spontaneous endocytosis of class I MHC molecules in JY cells

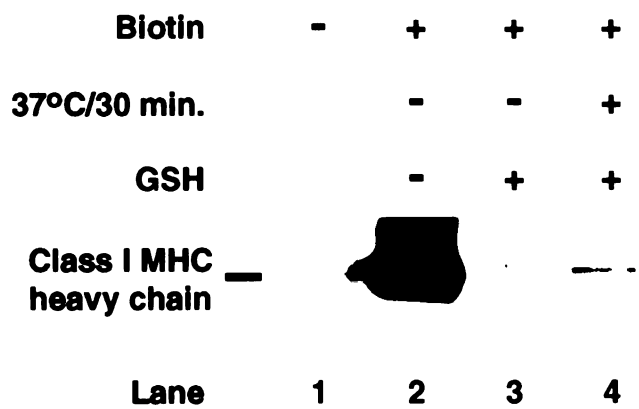


Figure 4.1. **Spontaneous endocytosis of class I MHC molecules in JY cells.**

JY cells (1 x 10^7 per sample) were biotinylated on ice using a disulfide-linked, cleavable biotin reagent (lanes 2-4; lane 1 is an non-biotinylated control). To follow the internalization of class I MHC molecules (lane 4), cells were incubated at 37°C for 30 minutes and then treated with GSH to remove residual surface biotin. Class I molecules were immunoprecipitated with mAb W6/32, and immunoprecipitates were separated by SDS-PAGE and blotted with streptavidin-HRP. Lanes 2 and 3 indicate the total level of biotinylated class I MHC heavy chain and GSH stripping efficiency, respectively. The migration position of the class I MHC heavy chain is indicated at the left.

to internalize biotin-labeled class I molecules and surface biotin has been removed, cells can be reincubated at 37°C. The expected result is a reduction in the level of class I molecules protected from GSH stripping, either because the molecules recycled to the plasma membrane and became resusceptible to GSH treatment or because they were degraded. To distinguish between these two possibilities, the assay can be done in the presence of lysosomal inhibitors (e.g., chloroquine or NH_4Cl). If class I molecules are recycled, the presence of lysosomal inhibitors will not affect the results of the experiment. If class I molecules are degraded, treatment with lysosomal inhibitors will result in equivalent levels of class I molecules detected prior to and following the second incubation step. Primaquine can also be used in this assay to determine whether class I molecules recycle. Treatment with primaquine has been shown to inhibit MHC molecule recycling [Reid, Nauter, 1990). If class I MHC molecules recycle, primaquine treatment will result in equivalent levels of class I molecules detected prior to and following the second incubation step. This concept is illustrated in Fig. 4.2.

To determine the role of clathrin in class I endocytosis and/or recycling, the assays just described can be performed on Hub-expressing cells alongside Hub-negative cells. Using the pJM601-T7Hub construct, any tetracycline-inducible cell line can be transfected and induced to express Hub. Currently tetracycline-responsive cell lines are limited, though they can be made fairly easily with commercially available reagents (e.g., Clontech, Palo Alto, CA). The T7Hub construct has also been cloned in the pCDM8

Figure 4.2

Conceptual representation of class I MHC molecule recycling or degradation using a biotinylation protocol

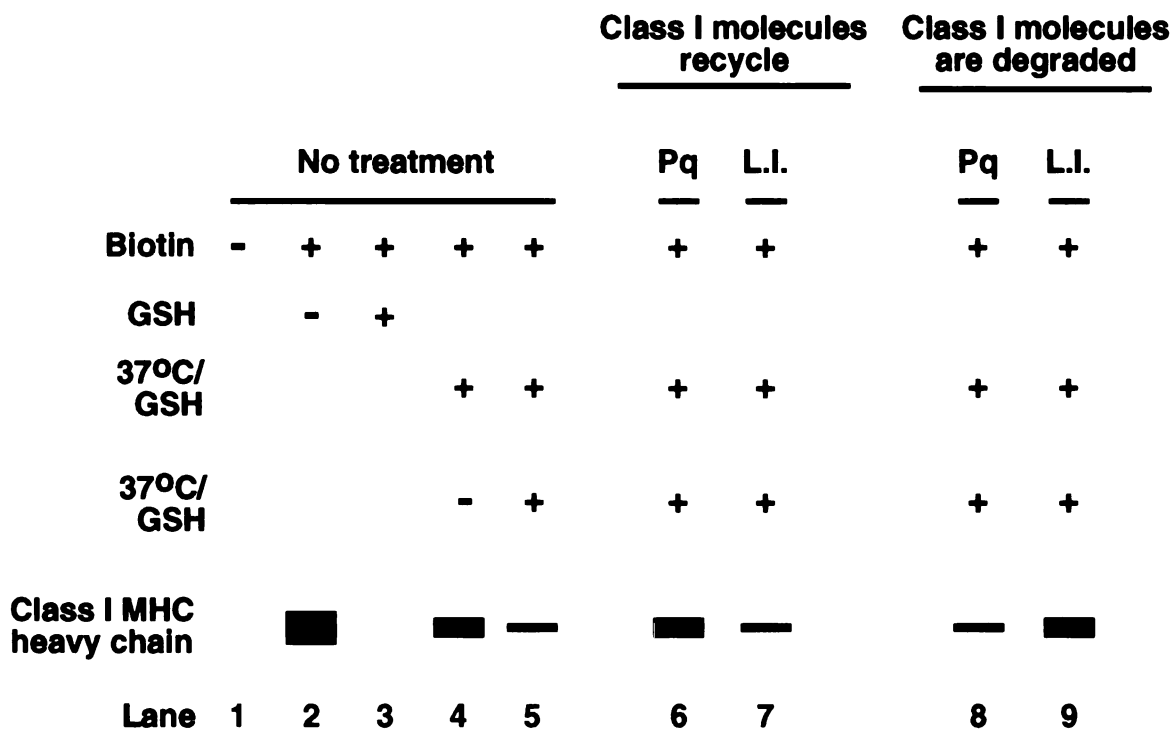


Figure 4.2. **Conceptual representation of class I MHC molecule recycling or degradation using a biotinylation protocol.**

The biotinylation protocol outlined in Chapter 3 (Fig. 3.10) can be used to monitor the endocytosis of class I MHC molecules. It can also be used to determine whether class I molecules recycle or are degraded following endocytosis. The sample data shown here illustrate the expected results for cells in which class I molecules recycle (lanes 6 and 7) and cells in which class I molecules are degraded (lanes 8 and 9). (Details of the experimental protocol are described in Chapter 3). Lane 2 indicates the total amount of biotinylated class I heavy chain at the start of the experiment. Lane 4 indicates the amount of biotinylated heavy chain following incubation at 37°C and GSH stripping. This band represents class I molecules that were endocytosed and are resistant to GSH. Lane 5 indicates the amount of biotinylated heavy chain following a second incubation at 37°C and GSH stripping. The decrease in biotinylated heavy chain in lane 5 versus lane 4 could be due to class I recycling, which would render class I molecules resusceptible to GSH treatment. The decrease in band size could also be due to class I heavy chain degradation. To distinguish between these two possibilities the assay can be done in the presence of primaquine (Pq), and inhibitor of recycling, or lysosomal inhibitors (L.I.). If class I molecules recycle (lanes 6 and 7) treatment with Pq will prevent class I molecules from becoming resusceptible to GSH stripping, and the class I band size following the second incubation/stripping step (lane 6) will be equivalent to the band in lane 4.

Lysosomal inhibitors will have no effect on the resulting band size (lane 7), and the class I heavy chain band will be equivalent to the band in lane 5. If class I molecules are degraded (lanes 8 and 9), Pq treatment will have no effect on the resulting band size (lane 8), but lysosomal inhibitors will prevent the reduction in class I heavy chain band size (lane 9) yielding a band equivalent to the band in lane 4.

vector (Liu et al., 1998) which can be used to transiently transfect cells. Both techniques yield high levels of Hub expression. Because caveolae are implicated in class I endocytosis in fibroblasts, caveolae inhibitors should also be analyzed for their effect on class I trafficking. Methyl-β-cytodextrin, nystatin, and filipin can all be used to inhibit caveolae-mediated trafficking events.

The procedures outlined above can be used to study cell-and allele-specific differences in class I endocytosis and to study the effect of viral proteins on class I trafficking. Various cell types expressing different class I alleles can be assayed, both with and without Hub and in the presence and absence of viral proteins. In this manner it can be determined whether viruses utilize constitutive endocytic pathways to induce class I MHC endocytosis and also whether they utilize clathrin-mediated pathways.

An aspect of HIV Nef-induced class I downregulation that remains to be investigated is whether PACS-1-meidated TGN accumulation of class I molecules is clathrin-dependent. PACS-1 has been shown to link furin to AP1 (Wan et al., 1998), suggesting that PACS-1-mediated trafficking is clathrin-dependent. Immunofluorescence can be used to study the accumulation of class I molecules in the TGN in Nef$^+$ cells. Surface class I molecules can be labeled on ice with mAb W6/32 and then incubated at 37°C to allow endocytosis. Cells are then fixed, permeabilized, and stained with labeled anti-mouse secondary antibody. When this technique was used to follow class I endocytosis in Nef$^+$ melanoma cells, class I molecules were observed in the TGN (Piguet

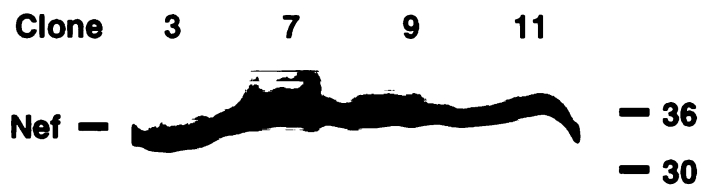
et al., 2000). This experiment can be repeated in the presence of Hub to determine whether accumulation of class I molecules in the TGN is clathrin-mediated.

As a first approach to studying Nef-induced class I downregulation, a FACS-based assay was used to measure surface levels of class I MHC molecules on wildtype and Nef$^+$ HeLa cells which express a consensus HIV1 Nef sequence (Shugars et al., 1993). Consensus Nef contains the myrisylation signal, proline-based repeat, and acidic cluster necessary for Nef-induced class I MHC endocytosis. Surprisingly, however, no difference was seen in levels of class I surface expression in HeLa-Nef cells compared to the parental HeLa cell line (Fig. 4.3 B). While the expected result was a decline in class I surface expression in the presence of Nef (Schwartz et al., 1996), Nef$^+$ clones actually stained higher for surface class I molecules. The lack of Nef-induced downregulation could be HeLa-cell specific. Alternatively, the amount of W6/32 antibody used may not have been saturating. A personal communication from Vincent Piguet suggested that levels of Nef expression may not have been sufficient. Piguet reports that extremely high levels of Nef expression are necessary to observe class I downregulation. Further work is necessary to determine whether HeLa-Nef cells express insufficient levels of Nef to induce class I endocytosis. These HeLa-Nef cells have not yet been assayed for accumulation of class I molecules in the TGN.

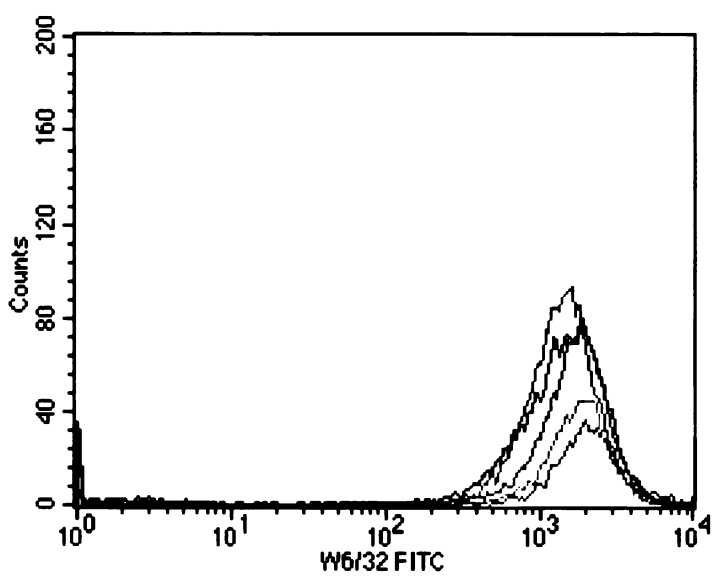
Figure 4.3

Surface expresison of class I MHC molecules on HeLa-Nef cells

A



B



Cell line	Color	Goemetric Mean
Clone 3	red	1582
Clone 7	orange	1475
Clone 9	blue	1439
Clone 11	turquoise	1148
HeLa	black	1023

Figure 4.3. **Surface expression of class I MHC molecules on HeLa-Nef cells.** HeLa-Nef cells were generated using retrovirus-mediated gene transfer. Briefly, the amphotrophic Phoenix packaging cell line was transfected with pLConsNefSN, which encodes a consensus HIV1 Nef sequence (Shugars et al., 1993), using a calcium phosphate transfection protocol. Viral supernatant was collected and used to infect HeLa cells. Positive clones were selected in G418.

(A) Four different HeLa-Nef clones (numbered 3, 7, 9, and 11) were screened for Nef expression. Cells were lysed in 1% NP40 lysis buffer, and lysates were separated by SDS-PAGE and blotted for Nef using mAb EH1, supplied by Dr. James Hoxie at the University of Pennsylvania. The migration position of Nef is indicated at the left, and the positions of molecular weight marker proteins (in kilodaltons) are indicated at the right. Uninfected HeLa cells show no EH1-reactive bands (data not shown).

(B) HeLa-Nef clones 3, 7, 9, and 11 were assayed for surface levels of class I MHC molecules. Cells were incubated on ice with mAb W6/32 for 30 minutes. Cells were then washed and incubated with FITC-conjugated goat anti-mouse secondary antibody. Surface levels of class I MHC molecules were measured using a Becton Dickinson FACScan instrument.

Conclusion

The spatial and temporal control of cellular protein distribution directly impacts the ability of proteins to perform their intended functions. Therefore, understanding intracellular trafficking pathways of proteins is paramount to fully understanding the roles that different proteins play within the cell. This body of work has focused on two different areas of membrane traffic: the assembly and trafficking of class I MHC molecules and ways that viruses perturb these processes, and the role of clathrin-coated buds on endosomes.

While we know a great deal about the assembly and trafficking of class I MHC molecules, some details remain elusive, such as the order of events in class I assembly, the fate of class I molecules at the cell surface, and the role of class I endocytosis and recycling in antigen presentation. By fully understanding the cell biology of antigen presentation, we not only gain a better insight into the role class I molecules play in the immune response, but we also gain insight into the myriad strategies viruses use to prevent antigen recognition. To this end, an important development of this research was the creation of tools and reagents to study the effect of viral proteins on class I antigen presentation. Clinically persistent and latent viral infections pose a significant threat to immunosuppressed patients such as organ transplant recipients and individuals with immunodeficiencies. With further research in this area, we will hopefully be able to prevent viral immune evasion and gain a therapeutic edge over these problematic

pathogens.

The mechanics of clathrin-mediated membrane traffic have been well studied. However, the full host of protein trafficking processes in which clathrin is involved has not yet been established. The discovery of new adaptor proteins (Dell'Angelica et al., 1998; Dell'Angelica et al., 1999; Hirst et al., 1999; Simpson et al., 1997) and novel classes of clathrin-coated buds (e.g., endosomal) suggest that there may still be much to learn about the many roles of clathrin. Here we have shown that expression of the clathrin Hub molecule induces a redistribution of endosomes and disrupts the spatial relationship between AP2-coated vesicles and the actin cytoskeleton. More work is needed to further define the role of clathrin in mediating endosomal distribution, though it is likely this phenomenon is important for maintaining polarity of protein sorting in the context of organized tissue.

Chapter 5

Experimental Procedures and Reagents

Vaccinia virus protocols

These protocols are based on Section 16.6 of *Current Protocols in Molecular Biology* (Ausubel et al., 2000) and on the advice of Drs. Jonathan Yewdell and Jack Bennink of the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Vaccinia virus safety precautions

Before beginning work with vaccinia virus, contact the Centers for Disease Control in Atlanta, GA to obtain their guidelines for working with vaccinia virus and their recommendations for vaccination. Vaccination can be arranged through UCSF employee health services.

- Always wear gloves, lab coat, and safety goggles when working with vaccinia virus. DO NOT get the virus in your eyes.
- Carry out all steps in a BSL-2 biosafety hood.
- Keep a biohazard trash bag and a liquid waste container in the hood. The liquid waste container should contain enough bleach to result in 10% bleach when the container is full. Discard all materials directly into these waste containers--do not take contaminated materials out of the hood. Seal the biohazard bag in the hood and autoclave immediately. Liquid waste should be sealed and allowed to soak in bleach overnight. The liquid can then be disposed of in the sink.

- When done in the hood, wash with 10% bleach and treat with UV light for 30 minutes.
- When sonicating vaccinia stocks, use a sealed tube of virus and a cuphorn sonicator. A probe sonicator will produce airborne viral particles.

Vaccinia virus stock production

Materials

1. Ten 175 cm^2 flasks of confluent cells. HeLa cells work well. Drs. Yewdell and Bennink use143B (TK^-) cells. CB1 cells can also be used.

2. An aliquot of the vaccinia virus construct of interest. Aim for 0.1-0.5 plaque forming units (pfu) per cell (approximately 1 x 10^7 pfu per 150 cm^2 flask).

3. PBS containing 0.1% BSA.

4. DMEM with 10% FCS.

All materials should be kept sterile.

Procedure

1. Prepare approximately 1 x 10^8 pfu of virus in 100 mL PBS/0.1% BSA.

2. Remove medium from each flask and add 10 mL of viral solution.

3. Incubate at 37°C/6% CO_2 for two hours, rocking every 20 minutes to keep cells moist.

4. Remove the viral inoculum and add 25 mL DMEM/10% FCS to each flask.

5. Incubate at 37°C/6% CO_2 for 48 hours. Cells should show marked pathology at this point (see Fig. 5.1).

6. Collect medium and cells. It may be necessary to trypsinize the cells. Pool medium and cells in two 250 mL centrifuge tubes. Pellet the cells and discard the supernatant.

7. Resuspend both cell pellets together in 10 mL PBS/0.1% BSA.

8. Freeze/thaw the sample three times and sonicate if necessary to break up clumps. Use

a cuphorn sonicator. Do not use a probe sonicator, as this will produce airborne viral particles.

9. Total yield should be about 2 x 10^{10} pfu. Dilute to 1 x 10^{8} pfu/mL with PBS/0.1%BSA and store 1 mL aliquots at -85°C.

Figure 5.1

Cytopathic effect of vaccinia virus infection on HeLa cells

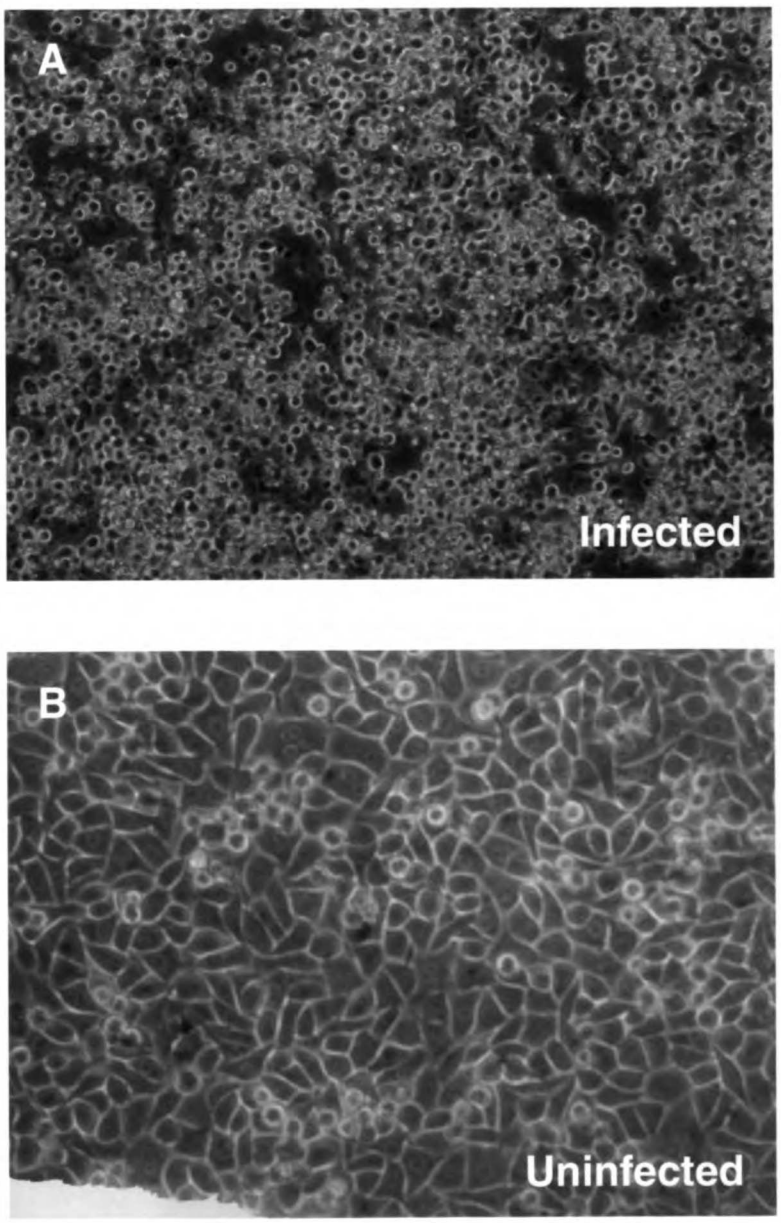


Figure 5.1. **Cytopathic effect of vaccinia virus infection on HeLa cells.**

HeLa cells were infected with E19-Vac as described in the Materials and Methods section of Chapter 2, except that the infection was allowed to proceed for 6 hours to accentuate the cytopathic effects. (A) shows infected cells 6 hours post-infection. The rounding of cells and the presence of debris are indicative of viral infection. (B) is an uninfected control. Some rounding of cells is seen due to overcrowding.

Plaque assay to determine viral titer

Materials

1. Two 6-well plates of HeLa cells, just reaching confluency.

2. Vaccinia virus stock to be assayed.

3. DMEM with 10% FCS.

4. Crystal violet, Sigma catalog # C6158. Prepare 0.1% (w/v) in 20% ethanol (in water).

Procedure

1. Make nine 10-fold serial dilutions of the virus stock in DMEM/10%FCS as follows: Prepare nine 15 mL tubes each containing 4.5 mL medium. Number the tubes 10^{-1}, 10^{-2}, 10^{-3}, ..., 10^{-9}. To the first tube (10^{-1}) add 0.5 mL virus stock. Mix well. Using a fresh pipet, transfer 0.5 mL of the 10^{-1} dilution to the second tube (10^{-2}). Continue in this fashion until you have prepared all nine dilutions.

2. Remove medium from cells. To one row of three wells add 0.5 mL of the 10^{-7} dilution to each well. To a second row of wells add 0.5 mL of the 10^{-8} dilution to each well. To a third row of wells add 0.5 mL of the 10^{-9} dilution to each well. Leave the fourth row of wells uninfected as a control. Place the plates at 37°C for two hours, rocking every 20 minutes to keep the cells moist.

3. Remove the viral inoculum and add 2 mL fresh medium to each well. Incubate at 37°C/6% CO_2 for 48 hours.

4. Remove medium from the wells. Add 0.5 mL of crystal violet solution to each well

and incubate for 5 minutes at room temperature.

5. Remove crystal violet and allow cells to dry completely. It may help to gently blow air over the cells to dry them. The crystal violet residue will have a chalky appearance once dry.

6. Count the plaques in each well. Plaques will appear as 1 to 2 mm areas of reduced staining due to retraction, rounding, and detachment of infected cells. Most accurate results are obtained from wells with 20-80 plaques. Average the results of three wells.

7. Calculate the viral titer by multiplying the average number of plaques per one row of wells by the dilution factor for that row (e.g. 10^7, 10^8, or 10^9). This number is the number of plaque forming units in 0.5 mL of virus stock. Double this number to obtain pfu/mL.

Infection with vaccinia virus

Materials

1. Cells to be infected. For adherent cells the cells should be just reaching confluency.
2. Vaccinia virus construct, 10-30 pfu/cell.
3. Cell medium appropriate for the cell line to be infected.

Procedure

1. Remove medium from cells.
2. Infect cells with 10-30 pfu/cell.

   For adherent cells, prepare the virus in just enough PBS/0.1%BSA to cover the cells. Incubate the cells at 37°C/6% CO_2 for 45 minutes to 2 hours, rocking every 20 minutes to keep the cells moist. Remove the viral inoculum and add fresh medium. Incubate the cells at 37°C/6% CO_2 for 6-48 hours (time determined by the level of protein expression and the health of the cells.)

   For suspension cells follow the same procedure except the cells will be in suspension. Inoculate the cells at 5 x 10^6 cells/mL, and after removing the inoculum suspend the cells at 1 x 10^6 cells/mL for the remainder of the infection. For examples of pfu's and infection times for HeLa and LCL cell lines, see the Materials and Methods section of Chapter 2.

3. Continue with the experiment as necessary. Cells can be radiolabeled, lysed, etc. as needed.

^{125}I transferrin endocytosis and recycling assay

Materials

1. Cells--12 well plates, 85% confluent.
2. ^{125}I Tf, NEN catalog #NEX212.
3. Holo (iron-loaded) transferrin, human, Sigma catalog #T4132.
4. Acid stripping buffer: 50 mM MES, pH 5; 0.5 M NaCl. Ice cold.
5. Lysis buffer: 1% Triton X 100, 0.1 M NaOH.

Terms: "Pulse" uptake/recycling refers to following one pool of ^{125}I Tf from the plasma membrane, through the endocytic/recycling pathway, back to the cell medium. To do a pulse experiment, ^{125}I Tf is loaded onto cell surface TfR on ice, and excess Tf is washed away prior to warming the cells to 37°C. In this way, only the pool of Tf initially bound to surface TfR will be monitored during the experiment. The appearance of ^{125}I Tf in the chase medium is indicative of ^{125}I Tf recycling. "Continuous" uptake refers to studying the internalization of ^{125}I Tf when there is excess ^{125}I Tf in the cell medium. Continuous uptake allows the entire endocytic/recycling pathway to become saturated with ^{125}I Tf. When studying continuous uptake, it is impossible to simultaneously study recycling because there is always excess ^{125}I Tf in the medium. However, following continuous uptake cells can be incubated in ^{125}I Tf-free chase medium, and the appearance of ^{125}I Tf in the chase medium will be indicative of ^{125}I Tf recycling.

Procedure

1. Serum starve cells in serum-free medium, 37°C, 60 minutes.

2. For continuous uptake, add 0.5 mL serum-free medium containing ^{125}I Tf (0.1 μCi, approximately 0.2 μg/mL) to each well and proceed to step 3. For a pulse uptake, surface label cells with ^{125}I Tf for 60 minutes on ice (0.1 μCi, approximately 0.2 μg/mL, per well), then wash 3 times in ice-cold PBS and add 0.5 mL 37°C serum-free medium.

    *Appropriate control samples should be included: 1) unlabeled cells (no ^{125}I Tf) to measure overall background, 2) cells which are surfaced labeled but kept on ice for the remainder of the experiment to measure background internalization, and 3) cells which are surfaced labeled but kept on ice for the remainder of the experiment and then acid stripped to measure acid stripping efficiency.

3. Float 12-well plates on a 37°C water bath.

4. At [X] minutes, place plates on ice. Remove medium and wash twice with 0.5 mL ice-cold PBS. Collect the medium and both PBS washes (1.5 mL total volume) in a tube labeled "medium fraction."

    *The medium fraction is only relevant when doing pulse uptake/recycling. When measuring continuous uptake the medium always contains excess ^{125}I Tf.

5. Acid strip on ice.

Acid stripping procedure: Add 0.5 mL stripping buffer to each well, wait 1 minute, remove. Add another 0.5 mL stripping buffer, wait 3 minutes, remove. Wash three times with 0.5 mL ice-cold PBS: a fast rinse, a 3 minute incubation, another fast rinse. Collect both acid washes and all three PBS washes (2.5 mL total volume) in a tube labeled "acid fraction."

6. To measure recycling, add 0.5 mL 37°C chase medium containing10% FCS plus 0.5 mg/mL unlabeled Tf. Return plate to 37°C water bath. If not measuring recycling, proceed to step 8.

7. At [X] minutes, repeat steps 4 and 5, then proceed to step 8

8. Lyse in 1 mL lysis buffer. Collect lysate in a tube labeled "lysate fraction."

9. Count 0.75 mL of each sample in a gamma counter.

Calculating TfR endocytosis and recycling

1. Adjust readings from the gamma counter for the total volume of each sample: divide counts by 0.75 to determine counts per mL, then multiply by the total number of mL in each sample (i.e. 2.5 mL for the acid fraction).

2. For each set of samples, calculate the total counts recovered in the medium, acid, and lysate fractions and represent the three fractions as a percentage of the total. The total counts recovered from each sample should be similar in value to the total counts

recovered from control samples which were labeled with ^{125}I Tf but kept on ice.

Notes

- Wear lab coat, gloves, and safety goggles. Use appropriate lead shields to shield yourself and others from radiation. Keep an ^{125}I-calbirated radiation monitor outside the lead shields to monitor radiation.
- Keep a dry waste bag and a liquid waste container, also protected with lead shields. Waste should be disposed of immediately.
- Samples should be done in triplicate and averaged. Use a separate 12-well plate for each incubation time so that the plate can easily be removed from the water bath without disturbing other samples. A convenient method is to grow cells only in the outer two columns of 3 wells, control cells on one side and cells of interest on the other. In this manner, one plate is used for each time point, and the samples are done in triplicate.
- Acid incubation removes iron from Tf. Neutral PBS incubations remove apo (iron-free) Tf from TfR. Therefore the 3 minute PBS incubation is a crucial step of the acid stripping procedure and is not just a "wash."
- "[X] minutes" indicates that the incubation time should be chosen depending upon the objective of the experiment. The $t_{1/2}$ of Tf endocytosis is approximately 5-10 minutes, while the $t_{1/2}$ for recycling is approximately 15-20 minutes.

Biotinylation of transferrin receptor to study receptor endocytosis and recycling

This protocol is based on that of Bretscher and Lutter, 1988.

Materials

1. Cells--2.5 x 10^6 cells per sample.

2. EZ-Link NHS-SS-Biotin, Pierce catalog #21331.

3. Ca/Mg-free PBS (henceforth referred to as PBS) with and without 0.04% EDTA, available from the UCSF Cell Culture Facility.

4. Falcon 12 x 17 mm polystyrene round-bottom tubes with snap cap, Fisher catalog #149592A.

5. Glutathione (GSH), reduced form, Sigma catalog #G-6529.

6. mAb H.684, against TfR, Zymed catalog #13-6800.

7. Streptavidin HRP, Zymed catalog #43-8323.

8. 5x non-reducing sample buffer: 250 mM Tris HCl, pH 6.8, 10% SDS, 25% glycerol, pinch of bromophenol blue.

9. GSH stripping buffer:

Prepare	**Final concentration in 10 mL**
155 mg GSH (0.5 mmol)	50 mM GSH
Dissolve GSH in 8.6 mL ice-cold H_2O	
Add 0.15 mL NaCl	75 mM NaCl
Add 0.1 mL 0.1 M EDTA	1 mM EDTA
Just before use add 75 µL 10M NaOH	75 mM NaOH
and 1 mL 10% BSA	1% BSA

Procedure

1. Detach adherent cells by washing twice with 37°C PBS with 0.04% EDTA and then incubating in 37°C PBS with 0.04% EDTA for 20-30 minutes. (See note below on detaching cells). Following step 1, samples are kept cold, on ice, unless otherwise indicated.

2. Spin down cells (1000 rpm, 5 minutes) and wash in cold PBS. This wash can be done in a 15 mL conical tube.

3. Biotinylate cells in 0.5 mg/mL biotin in PBS, rocking at 4^0C for 30 minutes This step can be performed in the same 15 mL tube used to wash the cells.

4. Wash cells 3 times in cold PBS. Aliquot samples into Falcon snap cap tubes. All subsequent steps are performed in these tubes.

5. The following table outlines the procedure for measuring TfR endocytosis and recycling, where sample 1 is a measure of GSH stripping inefficiency, sample 3 is a measure of TfR internalization, and sample 5 is a measure of TfR recycling. Samples 2, 4, and 6 are controls for the total amount of biotinylated material throughout the procedure.

To see total biotinylation and measure stripping efficiency (samples 1 and 2)	To measure TfR internalization (samples 3 and 4)	To measure TfR recycling (samples 5 and 6)
Treat sample 1 with GSH stripping buffer; mock treat sample 2 with PBS.	Incubate in serum-free medium with 1% BSA, 37°C, 20 min. Wash 3 times in cold PBS.	Incubate in serum-free medium with 1% BSA, 37°C, 20 min. Wash 3 times in cold PBS.
Lyse cells in 1% NP40 lysis buffer (5 x 10^6 cells/mL).	Treat sample 3 with GSH stripping buffer; mock treat sample 4 with PBS.	Treat sample 5 with GSH stripping buffer; mock treat sample 6 with PBS.
	Lyse cells in 1% NP40 lysis buffer (5 x 10^6 cells/mL).	Incubate in serum-free medium with 1% BSA, 37°C, 20min. Wash 3 times in cold PBS.
		Treat sample 5 with GSH stripping buffer; mock treat sample 6 with PBS.
		Lyse cells in 1% NP40 lysis buffer (5 x 10^6 cells/mL).

The protocol for GSH stripping is as follows:

a. Incubate cells in 1 mL stripping buffer rocking at 4°C for 25 minutes.

b. Spin down cells, remove buffer, and incubate in a fresh mL of stripping buffer for another 25 minutes.

c. Wash 3 times in cold PBS.

(To mock treat cells simply substitute cold PBS for GSH stripping buffer.)

6. Save 80 µL of lysate and to it add 20µL 5x sample buffer (can be reducing or non-

reducing).

7. From the remaining lysate immunoprecipitate TfR using 5 μg mAb H.684. Following immunoprecipitation, resuspend beads in non-reducing sample buffer.

8. Incubate samples in a boiling water bath for 10 minutes.

9. Run immunoprecipitates on an 8% SDS-PAGE gel and transfer to nitrocellulose (400 mA, 3 hours). Block nitrocellulose and blot with streptavidin HRP (1:5,000 dilution). Lysates can be separated by SDS-PAGE and blotted for any protein (e.g., TfR, class I MHC heavy chain) as an additional control for the total amount of material in each sample.

10. View samples by ECL.

11. Quantitate TfR bands using NIH Image.

Calculating TfR endocytosis and recycling

Ideally, sample 1 will yield no TfR band, and samples 2, 4, and 6 will yield equal bands. However, to normalize the values of the bands, the band intensity of sample 3 should be divided by the intensity of sample 4 (this value is referred to as 3/4), and the band intensity of sample 5 should be divided by the intensity of sample 6 (this value is referred to as 5/6). Stripping inefficiency is determined by dividing the band intensity of sample 1 by the intensity of sample 2 (this value is referred to as 1/2 and is usually about 0.15). The increase in value of 3/4 compared to 1/2 represents TfR endocytosed in the first 20 minute incubation. The decrease in value from 3/4 to 5/6 indicates TfR that

recycled during the second 20 minute incubation. To account for stripping inefficiency, adjust the value of 3 vs. 4 and 5 vs. 6 before normalizing (i.e. if stripping efficiency is 85%, and sample 3 is 75% of sample 4, then the 25% decline between 4 and 3 is only 85% of the true decline, and the adjusted value of sample 3 should be 71% of the value for sample 4.)

Notes

1. Store biotin in a dessicator at -20°C and warm to room temperature (approximately 1 hour) before opening to reduce condensation.

- Store GSH in a dessicator at 4°C.
- Do not use trypsin to detach cells, as TfR is trypsin-sensitive.
- It may take some time for cells to detach in 37°C PBS with 0.04% EDTA (30 minutes or more.) Be patient--wait for the cells to completely round up. Bang the petri dish on the table top to speed things along. DO NOT force cells off the petri dish by blasting them with high speed liquid from a pipet aid. Although hitting the cells with high speed liquid will get them off the plate, it will damage their plasma membrane and they will not be healthy for the experiment. After detaching the cells, count them using trypan blue to ensure that they are healthy and that you didn't remove them from the petri dish too forcefully.
- Pierce recommends biotinylating as many as 2.5×10^7 cells/mL. I typically use 2×10^6 cells/mL.

- A decrease in the total number of cells is often observed during the 37°C incubation steps. The use of serum-free medium helps minimize this reduction.
- Include a non-biotinylated control the first time the procedure is performed to ensure low background--simply remove an aliquot of cells from the 15 mL conical tube prior to biotinylating.
- The GSH stripping step precludes using the Pierce Micro BSA kit to measure protein concentration. Therefore blotting lysate is the only way to ensure equal protein content between samples.
- Reducing sample buffer will cleave the S-S bond in the linker arm of the biotin.
- TfR runs at 180-190 kD on a non-reducing gel, 90-95 kD on a reducing gel.

Maintenance of HeLa-T7Hub cells and Hub induction

The creation of HeLaT7Hub cells is described in the Materials and Methods section of Chapter 3. A vector map for the pJM601 plasmid is shown in Fig. 5.2.

HeLa-T7Hub cells are maintained in DMEM containing 10% FCS, 2 mM glutamine, 20 mM HEPES, 100 μg/mL streptomycin, 100 units/mL penicillin, 0.2 mg/mL G418, and 0.4 mg/mL hygromycin.

To induce Hub expression, cells are treated for 48 hours with 2 μg/mL doxycycline (Sigma catalog #D9891). Doxycycline is not stable to freeze-thawing. Therefore, a 2 mg/mL stock (1000x) should be made in sterile H_2O and aliquoted into single use amounts (50 μL is convenient). Thaw an aliquot at the time of use and discard the unused portion.

When thawing a new tube of HeLa-T7Hub cells, I have noticed that it takes approximately 10 days of growth before cells respond well to doxycycline treatment. If Hub induction is weak just after thawing cells, allow the cells to grow for a week or two and see if Hub expression improves.

Figure 5.2

Map of vector pJM601

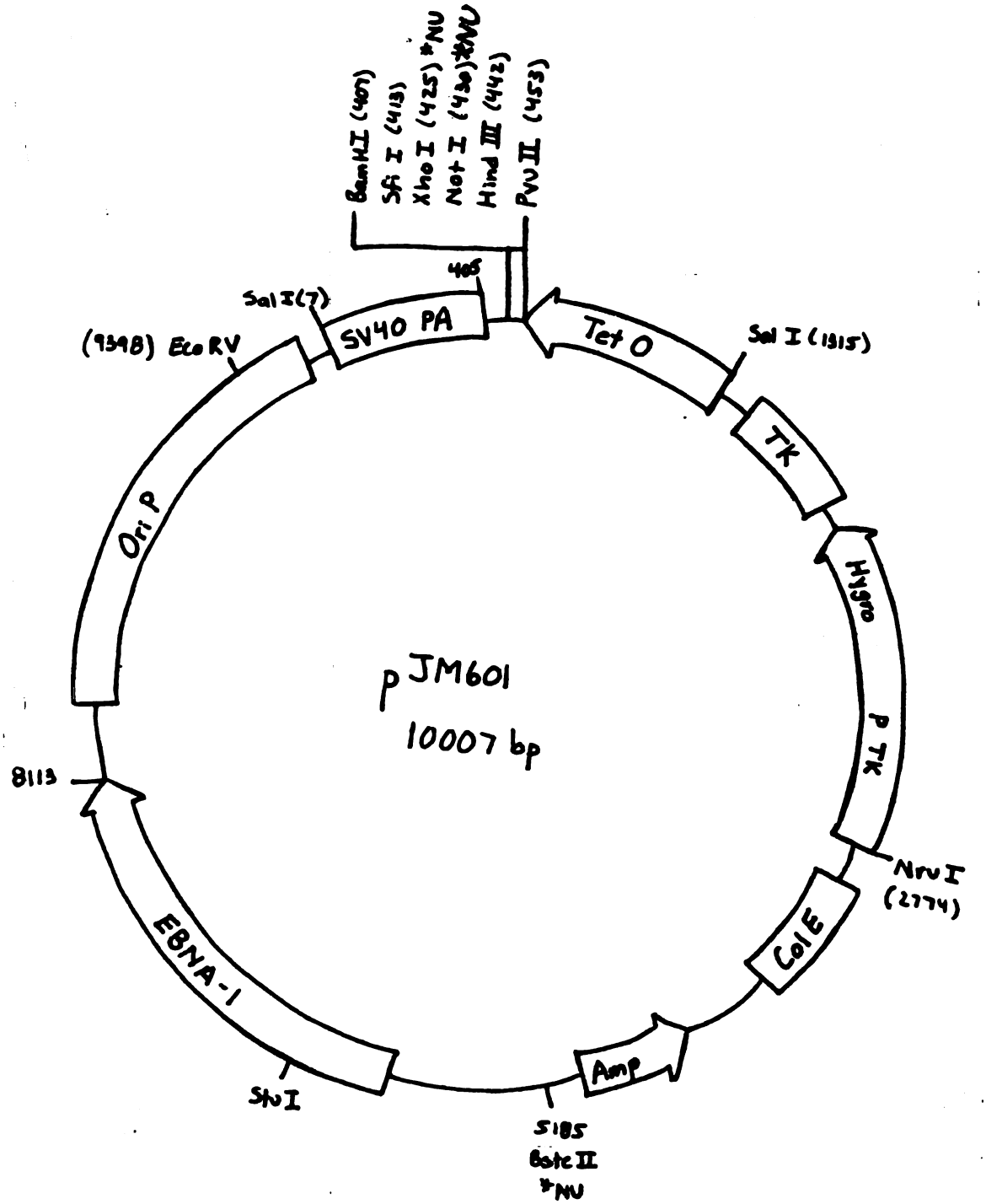


Figure 5.2. **Map of plasmid pJM601.**

pJM601 was created by Dr. John Moran at the University of Michigan Medical School. The vector was created by replacing the CMV promoter of pCEP4 (Invitrogen, Carlsbad, CA) with the tetracycline operator sequence (TetO). TetO was cloned into the *SalI*/*Pvu*II site, leaving the *Pvu*II site in tact. *NU indicates that a restriction site is not unique, probably because the site exists in the TetO insert. The creation of pJM601-T7Hub is described in the Materials and Methods section of Chapter 3.

Table 5.1. Class I HLA haplotypes of cell lines.

Cell Line	Class I HLA Haplotype	Reference
JY	A2, B7, C7	
LCL 721	A1/A2, B5/B8, C1	(Kavathas et al., 1980)
LCL 721.221	A-, B-, and C-negative	(Shimizu and DeMars, 1989)
LCL 721.220	A- and B-negative, some C	(Greenwood et al., 1994)
LCL 721.174	A1/A2, B5/B8, C1	(Spies et al., 1992)
HeLa	A68, B1503, C1203	(Yee et al., 1985)

Table 5.2. HeLa cell transfectants.

Cell Line	Description
HeLa-T7Hub	Transfected with pJM601-T7Hub. Express Hub under control of the tetracycline operator sequence.
HeLa-pJM601	Transfected with the pJM601 vector as a control for HeLa-T7Hub cells.
HeLa-Nef	Constitutively express HIV1 consensus Nef.

Table 5.3. Antibody descriptions.

Antibody/Antigen	Use/Concentration	Source/Reference
AP.6, anti-AP2 mAb	**IF:** 10 μg/mL	Lab stock; ATCC (Chin et al., 1989)
BBM.1, anti-β_2m mAb	**IP:** 2 μg per sample. **Western blot:** 1:2500	Lab stock; ATCC (Brodsky et al., 1979)
EH1, anti HIV1 Nef mAb	**Western blot:** 1:1000	James Hoxie, University of Pennsylvania
H68.4, anti-human TfR mAb	**IP:** 5 μg per sample. **Western blot:** 1:1000	Zymed Laboratories, South San Francisco, CA
HC10, anti-free class I heavy chain mAb	**IP:** 2 μg per sample. **Western blot:** 1:3000	Lab stock (Stam et al., 1986)
Anti-Hook1, 2, and 3 rabbit sera	**IF:** 1:400	Helmut Krämer, University of Texas Southwestern Medical Center.
L01.1, anti-human TfR mAb	**IF:** 1:1000	BD Biosciences, San Jose, CA
Anti-clathrin light chain consensus rabbit serum	**IF:** 1:1000	Lab stock (Acton and Brodsky, 1990)
Anti-non-muscle myosin rabbit serum	**IF:** 1:25	Biomedical Technologies, Inc., Stoughton, MA
P129-143, anti-E19 rabbit serum	**Western blot:** 1:1000	Bill Wold, St. Louis University School of Medicine (Hermiston et al., 1993)
R.gp48N, anti-tapasin rabbit serum	**IP:** 2 μL per sample. **Western blot:** 1:1000	Peter Cresswell, Yale University School of Medicine (Sadasivan et al., 1996)

IF = Immunofluorescence

IP = Immunoprecipitation, "per sample" = 10^6-10^7 cells.

Uses listed are not exhaustive--they represent antibody uses for this dissertation.

Table 5.3. Antibody descriptions continued

R.RING4C, anti-TAP1 rabbit serum	**IP:** 7.5 μL per sample. **Western blot:** 1:1000	Peter Cresswell, Yale University School of Medicine (Ortmann et al., 1994)
Anti-T7 tag mAb Biotinylated anti-T7 tag mAb	**IF:** 1:5000 **Western blot:** 1:5000 **IP:** 5 μg per sample. **IF:** 1:2000	Novagen, Madison, WI
anti-TAP2 rabbit serum	**IP:** 1.5 μL per sample. **Western blot:** 1:2000	Lab stock (Bresnahan et al., 1997)
Anti-α-tubulin mAb	**IF:** 1:700	Sigma, St. Louis, MO
TW1.3, anti-E19 mAb	**IP:** 2.5 μL per sample. **Does not blot.**	Jonathan Yewdell, Laboratory of Viral Diseases, NIAID, NIH (Cox et al., 1991)
UCSF#2, anti-HLA-A2 rabbit serum (reacts with all alleles tested)	**Western blot:** 1:2000 **Does not IP.**	Lab stock (Bresnahan et al., 1997)
W6/32, anti-class I dimer mAb	**IP:** 2 μg per sample. **FACS:** titrate 1-5 μg/mL (per approx. 10^6 cells) to determine saturation. **Does not blot.**	Lab stock; ATCC (Barnstable et al., 1978)

IF = Immunofluorescence

IP = Immunoprecipitation, "per sample" = 10^6-10^7 cells.

Uses listed are not exhaustive--they represent antibody uses for this dissertation.

References

Abdel Motal, U.M., X. Zhou, A. Joki, A.R. Siddiqi, B.R. Srinivasa, K. Stenvall, J. Dahmén, and M. Jondal. 1993. Major histocompatibility complex class I-binding peptides are recycled to the cell surface after internalization. *Eur. J. Immunol.* 23:3224-3229.

Acton, S., and F.M. Brodsky. 1990. Predominance of clathrin light chain LC_b correlates with the presence of a regulated secretory pathway. *J. Cell Biol.* 111:1419-1426.

Adrian, T., M. Becker, J.C. Hierholzer, and R. Wigand. 1989a. Molecular epidemiology and restriction site mapping of adenovirus 7 genome types. *Arch. Virol.* 106:73-84.

Adrian, T., B. Best, J.C. Hierholzer, and R. Wigand. 1989b. Molecular epidemiology and restriction site mapping of adenovirus type 3 genome types. *J. Clin. Microbiol.* 27:1329-1334.

Ahn, K., A. Angulo, P. Ghazal, P.A. Peterson, Y. Yang, and K. Früh. 1996a. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc. Natl. Acad. Sci. USA.* 93:10990-10995.

Ahn, K., A. Bruhler, B. Galocha, T.R. Jones, E.J.H.J. Wiertz, H.L. Ploegh, P.A. Peterson, Y. Yang, and K. Früh. 1997. The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity.* 6:613-621.

Ahn, K., T. Meyer, S. Uebel, P. Sempé, H. Djaballah, Y. Yang, P.A. Peterson, K. Früh, and R. Tampé. 1996b. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus protein ICP47. *EMBO J.* 15:3247-3255.

Alcami, A., and U.H. Koszinowski. 2000. Viral mechanisms of immune evasion. *Immunol. Today.* 21:447-455.

Altschuler, Y., S.-H. Liu, L. Katz, K. Tang, S. Hardy, F.M. Brodsky, G. Apodaca, and K.E. Mostov. 1999. ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* 147:7-12.

Anderson, H.A., Y. Chen, and L.C. Norkin. 1998. MHC class I molecules are enriched in caveolae but do not enter with simian virus 40. *J. Gen. Virol.* 79:1469-1477.

Anderson, R.G.W., M.S. Brown, and J.L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* 10:351-364.

Anderson, R.G.W., B. M.S., U. Beisiegel, and J.L. Goldstein. 1982. Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. *J. Cell Biol.* 93:523-531.

Andersson, M., A. McMichael, and P.A. Peterson. 1987. Reduced allorecognition of adenovirus-2 infected cells. *J. Immunol.* 138:3960-3966.

Andersson, M., S. Pääbo, T. Nilsson, and P.A. Peterson. 1985. Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell.* 43:215-222.

Androlewicz, M.J. 1999. The role of tapasin in MHC class I antigen assembly. *Immunol. Res.* 20:79-88.

Androlewicz, M.J., K.S. Anderson, and P. Cresswell. 1993. Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc. Natl. Acad. Sci. USA.* 90:9130-9134.

Androlewicz, M.J., B. Ortmann, P.M. Van Endert, T. Spies, and P. Cresswell. 1994. Characteristics of peptide and major histocompatibility complex class I/beta 2-microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc. Natl. Acad. Sci. USA.* 91:12716.

Apodaca, G., L.A. Katz, and K.E. Mostov. 1994. Receptor-mediated transcytosis of IgA in MDCK cells is via apical recycling endosomes. *J. Cell Biol.* 125:67-86.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 2000. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Bangia, N., P.J. Lehner, E.A. Hughes, M. Surman, and P. Cresswell. 1999. The N-terminal region of tapasin is required to stabilize the MHC class I loading complex. *Eur. J. Immunol.* 29:1858-1870.

Barnden, M.J., A.W. Purcell, J.J. Gorman, and J. McCluskey. 2000. Tapasin-mediated retention and optimization of peptide ligands during the assembly of class I molecules. *J. Immunol.* 165:322-330.

Barnes, P.D., and J.E. Grundy. 1992. Down-regulation of the class I HLA heterodimer and β_2-microglobulin on the surface of cells infected with cytomegalovirus. *J. Gen. Virol.* 73:2395-2403.

Barnstable, C.J., W.F. Bodmer, G. Brown, G. Galfre, C. Milstein, A.F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens--new tools for genetic analysis. *Cell.* 14:9-20.

Basu, S.K., J.L. Goldstein, R.G.W. Anderson, and M.S. Brown. 1981. Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts. *Cell.* 24:493-502.

Baumeister, W., J. Walz, F. Zühl, and E. Seemüller. 1998. The proteasome: paradigm of a self-compartmentalizing protease. *Cell.* 92:367-380.

Beck, S., and B.G. Barrell. 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature.* 331:269-272.

Beersma, M.F.C., M.J.E. Bijlmakers, and H. Ploegh. 1993. Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains. *J. Immunol.* 151:4455-4464.

Beier, D.C., J.H. Cox, D.R. Vining, P. Cresswell, and V.H. Englehard. 1994. Association of human class I MHC alleles with the adenovirus E3/19K protein. *J. Immunol.* 152:3862-3871.

Beninga, J., K.L. Rock, and A.L. Goldberg. 1998. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J. Biol. Chem.* 273:18734-18742.

Bennett, E.M., J.W. Yewdell, J.R. Bennink, and F.M. Brodsky. 1999. Cutting edge: Adenovirus has two mechanisms for affecting class I MHC expression. *J. Immunol.*:5049-5052.

Benson, R.E., A. Sanfridson, J.S. Ottinger, C. Doyle, and B.R. Cullen. 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med.* 177:1561-1566.

Bergeron, J.J., M.B. Brenner, D.Y. Thomas, and D.B. Williams. 1994. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.* 19:124-128.

Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells [see comments]. *N. Engl. J. Med.* 320:1731-1735.

Bleil, J.D., and M.S. Bretscher. 1982. Transferrin receptor and its recycling in HeLa cells. *EMBO J.* 1:351-355.

Borrego, F., M. Ulbrecht, E.H. Weiss, J.E. Coligan, and A.G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187:813-818.

Braud, V.M., D.S.J. Allan, D. Wilson, and A.J. McMichael. 1997. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* 8:1-10.

Bresnahan, P.A., L.D. Barber, and F.M. Brodsky. 1997. Localization of class I histocompatibility molecule assembly by subfractionation of the early secretory pathway. *Hum. Immunol.* 53:129-139.

Bresnahan, P.A., W. Yonemoto, S. Ferrell, D. Williams-Herman, R. Geleziunas, and W.C. Greene. 1998. A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. *Curr. Biol.* 8:1235-1238.

Bretscher, M.S., and R. Lutter. 1988. A new method for detecting endocytosed proteins. *EMBO J.* 7:4087-4092.

Britt, W.J., and C.A. Alfold. 1996. Cytomegalovirus. *In* Fields Virology. B.N. Fields, D.M. Knipe, and P.M. Howley, editors. Lippincott-Raven, New York.

Brodsky, F.M., W.F. Bodmer, and P. Parham. 1979. Characterization of a monoclonal anti-b2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. *Eur. J. Immunol.* 9:536-545.

Brown, M.S., R.G.W. Anderson, S.K. Basu, and J.L. Goldstein. 1982. Recycling of cell surface receptors: observations from the LDLR system. *C.S.H.S.* 46:713-721.

Browne, H., G. Smith, S. Beck, and T. Minson. 1990. A complex between the MHC class I homologue encoded by human cytomegalovirus and β_2 microglobulin. *Nature.* 347:770-772.

Burgert, H.-G., and S. Kvist. 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. *Cell.* 41:987-997.

Burgert, H.-G., and S. Kvist. 1987. The E3/19K protein of adenovirus type 2 binds to the domains of histocompatibility antigens required for CTL recognition. *EMBO J.* 6:2019-2026.

Burgert, H.-G., J.L. Maryanski, and S. Kvist. 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proc. Natl. Acad. Sci. USA.* 84:1356-1360.

Carpenter, G., and S. Cohen. 1976. ^{125}I-labeled human epidermal growth factor. *J. Cell Biol.* 71:159-171.

Carreno, B.M., J.C. Solheim, M. Harris, I. Stroynowski, J.M. Connolly, and T.H. Hansen. 1995. TAP associates with a unique class I conformation, whereas calnexin associates with multiple forms in mouse and man. *J. Immunol.* 155:4726-4733.

Chang, M.P., W.G. Mallet, K.E. Mostov, and F.M. Brodsky. 1993. Adaptor self-aggregation, adaptor-receptor recognition and binding of α-adaptin subunits to the plasma membrane contribute to recruitment of adaptor (AP2) components of clathrin-coated pits. *EMBO J.* 12:2169-2180.

Chin, D.J., R.M. Straubinger, S. Acton, I. Näthke, and F.M. Brodsky. 1989. 100-kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 86:9289-9293.

Chiu, I., D.M. Davis, and J.L. Strominger. 1999. Trafficking of spontaneously endocytosed MHC proteins. *Proc. Natl. Acad. Sci. USA.* 96:13944-13949.

Cohen, G.B., R.T. Gandhi, D.M. Davis, O. Mandelboim, B.K. Chen, J.L. Strominger, and D. Baltimore. 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity.* 10:661-671.

Collawn, J.F., M. Stangel, L.A. Kuhn, V. Esekogwu, S. Jing, I.S. Trowbridge, and J.A. Tainer. 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell.* 63:1061-1072.

Collins, K.L., B.K. Chen, S.A. Kalams, B.D. Walker, and D. Baltimore. 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature.* 391?:397-401.

Colonna, M., G. Borsellino, M. Falco, G.B. Ferrara, and J.L. Strominger. 1993. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc. Natl. Acad. Sci. USA.* 90:12000-12004.

Copeman, J., N. Bangia, J.C. Cross, and P. Cresswell. 1998. Elucidation of the genetic basis of the antigen presentation defects in the mutant cell line .220 reveals polymorphism and alternative splicing of the tapasin gene. *Eur. J. Immunol.* 28:3783-3791.

Coscoy, L., and D. Ganem. 2000. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc. Natl. Acad. Sci. USA.* 97:8051-8056.

Cox, J.H., J.R. Bennink, and J.W. Yewdell. 1991. Retention of adenovirus E19 glycoprotein in the endoplasmic reticulum is essential to its ability to block antigen presentation. *J. Exp. Med.* 174:1629-1637.

Cox, J.H., J.W. Yewdell, L.C. Eisenlohr, P.R. Johnson, and J.R. Bennink. 1990. Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. *Science*. 247:715-718.

Cresswell, P., B. Arunachalam, N. Bangia, T. Dick, G. Diedrich, E. Hughes, and M. Maric. 1999a. Thiol oxidation and reduction in MHC-restricted antigen processing and presentation. *Immunol. Res.* 19:191-200.

Cresswell, P., N. Bangia, T. Dick, and G. Diedrich. 1999b. The nature of the MHC class I peptide loading complex. *Immunol. Rev.* 172:21-28.

Dalgleish, A.G., P.C. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves, and R.A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature*. 312:763-767.

Damke, H., T. Baba, A.M. van der Bliek, and S.L. Schmid. 1995. Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J. Cell Biol.* 131:69-80.

Damke, H., T. Baba, D.E. Warnock, and S.L. Schmid. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* 127:915-934.

Daniel, S., V. Brusic, S. Caillat-Zucman, N. Petrovsky, L. Harrison, D. Riganelli, F. Sinigaglia, F. Gallazzi, J. Hammer, and P.M. van Endert. 1998. Relationship between peptide selectivities of human transporters associated with antigen processing and HLA class I molecules. *J. Immunol.* 161:617-624.

Daro, E., P. Van Der Sluijs, T. Galli, and I. Mellman. 1996. Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. *Proc. Natl. Acad. Sci. USA*. 93:9559-9564.

Dasgupta, J.D., S. Watkins, H. Slayter, and E.J. Yunis. 1988. Receptor-like nature of class I HLA: endocytosis via coated pits. *J. Immunol.* 141:2577-2580.

Dautry-Varsat, A., A. Ciechanover, and H.F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA*. 80:2258-2262.

Davis, C.G., J.L. Foldstein, T.C. Südhof, R.G.W. Anderson, D.W. Russell, and M.S. Brown. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature*. 326:760-765.

Davis, D.M., H.T. Reyburn, L. Pazmany, I. Chiu, O. Mandelboim, and J.L. Strominger. 1997. Impaired spontaneous endocytosis of HLA-G. *Eur. J. Immunol.* 27:2714-2719.

Degen, E., M.F. Cohen-Doyle, and D.B. Williams. 1992. Efficient dissociation of the p88 chaperone from major histocompatibility complex class I molecules requires both beta 2-microglobulin and peptide. *J. Exp. Med.* 175:1653-1661.

Degen, E., and D.B. Williams. 1991. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell Biol.* 112:1099-1115.

Dell'Angelica, E.C., J. Klumperman, W. Stoorvogel, and J.S. Bonifacino. 1998. Association of the AP-3 complex with clathrin. *Science*. 280:431-434.

Dell'Angelica, E.C., C. Mullins, and J.S. Bonifacino. 1999. AP-4, a novel protein complex related to clathrin adaptors. *J. Biol. Chem.* 274:7278-7285.

Demaria, S., R. Schwab, S.R.S. Gottesman, and Y. Bushkin. 1994. Soluble β2-microglobulin-free class I heavy chains are released from the surface of activated and leukemia cells by a metalloprotease. *J. Biol. Chem.* 269:6689-6694.

Drubin, D.G., K.G. Miller, and D. Botstein. 1988. Yeast actin-binding proteins: evidence for a role in morphogenesis. *J. Cell Biol.* 107:2551-2561.

Dunn, K.W., T.E. McGraw, and F.R. Maxfield. 1989. Iterative fractionation of recycling receptors from lysosomally destined ligands in an early sorting endosome. *J. Cell Biol.* 109:3303-3314.

Elliott, J.G., J.D. Oliver, and S. High. 1997. The thiol-dependent reductase ERp57 interacts specifically with N-glycosylated integral membrane proteins [published erratum appears in J Biol Chem 1997 Aug 8;272(32):20312]. *J. Biol. Chem.* 272:13849-13855.

Elliott, T. 1997. Transporter associated with antigen processing. *Adv. Immunol.* 65:47-109.

Elliott, T., A. Willis, V. Cerundolo, and A. Townsend. 1995. Processing of major histocompatibility class I- restricted antigens in the endoplasmic reticulum. *J. Exp. Med.* 181:1481-1491.

Engqvist-Goldstein, A.E., M.M. Kessels, V.S. Chopra, M.R. Hayden, and D.G. Drubin. 1999. An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles. *J. Cell Biol.* 147:1503-1518.

Engqvist-Goldstein, A.E.Y., R.A. Warren, M.M. Kessels, J.E. Heuser, J.H. Keen, and D.G. Drubin. 2000. The actin-binding protein Hip1R binds to clathrin and functions together with clathrin in the early stags of endocytosis. *Mol. Biol. Cell.* 11:147a.

Fahnestock, M.L., J.L. Johnson, R.M. Feldman, J.M. Neveu, W.S. Lane, and P.J. Bjorkman. 1995. The MHC class I homolog encoded by human cytomegalovirus binds endogenous peptides. *Immunity.* 3:583-590.

Farmery, M.R., S. Allen, A.J. Allen, and N.J. Bulleid. 2000. The role of ERp57 in disulfide bond formation during the assembly of major histocompatibility complex class I in a synchronized semipermeabilized cell translation system. *J. Biol. Chem.* 275:14933-14938.

Farrell, H.E., M.A. Degli-Esposti, and N.J. Davis-Poynter. 1999. Cytomegalovirus evasion of natural killer cell responses. *Immunol. Rev.* 168:187-197.

Farrell, H.E., H. Vally, D.M. Lynch, P. Fleming, G.R. Shellam, A.A. Scalzo, and N.J. Davis-Poynter. 1997. Inhibition of natural killer cells by cytomegalovirus MHC class I homologue *in vivo*. *Nature.* 386:510-514.

Fehling, H.J., W. Swat, C. Laplace, R. Kuhn, K. Rajewsky, U. Müller, and H. Von Boehmer. 1994. MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science.* 265:1234-1237.

Ferrari, D.M., and H.D. Söling. 1999. The protein disulphide-isomerase family: unravelling a string of folds. *Biochem. J.* 339:1-10.

Feuerbach, D., and H.-G. Burgert. 1993. Novel proteins associated with MHC class I antigens in cells expressing adenovirus protein E3/19K. *EMBO J.* 12:3153-3161.

Feuerbach, D., S. Etteldorf, C. Ebanau-Jehle, J.-P. Abastado, D. Madden, and H.-G. Burgert. 1994. Identification of amino acids within the MHC molecule important for the interaction with the adenovirus protein E3/19K. *J. Immunol.* 153:1626-1636.

Flomenberg, P., E. Gutierrez, and K. Hogan. 1994. Identification of class I MHC regions which bind to the adenovirus E3-19k protein. *Mol. Immunol.* 31:1277-1284.

Flynn, G.C., T.G. Chappell, and J.E. Rothman. 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science.* 245:385-390.

Foti, M., A. Mangasarian, V. Piguet, D.P. Lew, K.-H. Krause, D. Trono, and J.-L. Carpentier. 1997. Nef-mediated clathrin-coated pit formation. *J. Cell Biol.* 139:37-47.

Fox, F.C., and M. Das. 1979. Internalization and processing of the EGF receptor in the induction of DNA synthesis in cultured fibroblasts: the endocytic activation hypothesis. *J. Supramol. Struc.* 10:199-214.

Früh, K., K. Anh, H. Djaballah, P. Sempé, P.M. van Endert, R. Tampé, P.A. Peterson, and Y. Yang. 1995. A viral inhibitor of peptide transporters for antigen presentation. *Nature.* 375:415-418.

Früh, K., A. Gruhler, R.M. Krishna, and G.J. Schoenhals. 1999. A comparison of viral immune escape strategies targeting the MHC class I assembly pathway. *Immunol. Rev.* 168:157-166.

Fujimoto, L.M., R. Roth, J.E. Heuser, and S.L. Schmid. 2000. Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic.* 1:161-171.

Futter, C.E., A. Gibson, E.H. Allchin, S. Maxwell, L.J. Ruddock, G. Odorizzi, D. Domingo, I.S. Trowbridge, and C.R. Hopkins. 1998. In polarized MDCK cells basolateral vesicles arise from clathrin-γ-adaptin-coated domains on endosomal tubules. *J. Cell Biol.* 141:611-623.

Gaidarov, I., F. Santini, R.A. Warren, and J.H. Keen. 1999. Spatial control of coated-pit dynamics in living cells. *Nat. Cell Biol.* 1:1-7.

Gall, S.L., L. Erdtmann, S. Benichou, C. Berlioz-Torrent, L. Liu, R. Benarous, J.-M. Heard, and O. Schwartz. 1998. Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity.* 8:483-495.

Galvin, K., S. Krishna, F. Ponchel, M. Frohlich, D.E. Cummings, R. Carlson, J.R. Wands, K.J. Isselbacher, S. Pillai, and M. Ozturk. 1992. The major histocompatibility complex class I antigen-binding protein p88 is the product of the calnexin gene. *Proc. Natl. Acad. Sci. USA.* 89:8452-8456.

Garcia, J.V., and A.D. Miller. 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by Nef. *Nature.* 350:508-511.
Gething, M.J., and J. Sambrook. 1992. Protein folding in the cell. *Nature.* 355:33-45.

Geuze, H.J., J.W. Slot, and A.L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. *J. Cell Biol.* 104:1715-1723.

Geuze, H.J., J.W. Slot, G.J.A.M. Strous, J.F. Lodish, and A.L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell.* 32:277-287.

Ghosh, R.N., D.L. Gelman, and F.R. Maxfield. 1994. Quantification of low density lipoprotein and transferrin endocytic sorting in HEp2 cells using confocal microscopy. *J. Cell Sci.* 107:2177-2189.

Ghosh, R.N., and F.R. Maxfield. 1995. Evidence for nonvectorial, retrograde transferrin trafficking in the early endosomes of HEp2 cells. *J. Cell Biol.* 128:549-561.

Gilbert, M.J., S.R. Riddell, B. Plachter, and P.D. Greenberg. 1996. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature.* 383:720-722.

Ginsberg, H.S., U. Lundholm-Beauchamp, R.L. Horswood, B. Pernis, W.S.M. Wold, R.M. Chanock, and G.A. Prince. 1989. Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. USA.* 86:3823-3827.

Glynne, R., S.H. Powis, S. Beck, A. Kelly, L.-A. Kerr, and J. Trowsdale. 1991. A proteasome-related gene between the two ABC transporter loci in the class II region of the human MHC. *Nature*. 353:357-360.

Gooding, L.R., L.W. Elmore, A.E. Tollefson, H.A. Brady, and W.S.M. Wold. 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell*. 53:341-346.

Gooding, L.R., T.S. Ranheim, A.E. Tollefson, L. Aquino, P. Duerksen-Hughes, T.M. Horton, and W.S.M. Wold. 1991. The 10,400- and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all mouse cell lines against lysis by tumor necrosis factor. *J. Virol.* 65:4114-4123.

Grandea, A.G., 3rd, P.J. Lehner, P. Cresswell, and T. Spies. 1997. Regulation of MHC class I heterodimer stability and interaction with TAP by tapasin. *Immunogenetics*. 46:477-483.

Grandea, I.A.G., M.J. Androlewicz, R.S. Athwal, D.E. Geraghty, and T. Spies. 1995. Dependence of peptide binding by MHC Class I molecules on their interaction with TAP. *Science*. 270:105-108.

Greenberg, M., L. DeTulleo, I. Rapoport, J. Skowronski, and T. Kirchhausen. 1998a. A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. *Curr. Biol.* 8:1239-1242.

Greenberg, M.E., S. Bronson, M. Lock, M. Neumann, G.N. Pavlakis, and J. Skowronski. 1997. Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. *EMBO J.* 16:6964-6976.

Greenberg, M.E., A.J. Iafrate, and J. Skowronski. 1998b. The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. *EMBO J.* 17:2777-2789.

Greene, B., S.-H. Liu, A. Wilde, and F.M. Brodsky. 2000. Complete reconstitution of clathrin basket formation with recombinant protein fragments: Adaptor control of clathrin self-assembly. *Traffic*. 1:69-75.

Greenwood, R., Y. Shimizu, G.S. Sekhon, and R. DeMars. 1994. Novel allele-specific, post-translational reduction in HLA class I surface expression in a mutant human B cell line. *J. Immunol.* 153:5525-5536.

Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. *J. Cell Biol.* 109:2703-2720.

Gromme, M., F.G. Uytdehaag, H. Janssen, J. Calafat, R.S. van Binnendijk, M.J. Kenter, A. Tulp, D. Verwoerd, and J. Neefjes. 1999. Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci. USA.* 96:10326-10331.

Gruhler, A., P.A. Peterson, and K. Früh. 2000. Human cytomegalovirus immediate early glycoprotein US3 retains MHC class I molecules by transient association. *Traffic.* 1:318-325.

Gur, H., T.D. Geppert, and P.E. Lipsky. 1997. Structural analysis of class I MHC molecules: the cytoplasmic domain is not required for cytoskeletal association, aggregation and internalization. *Mol. Immunol.* 24:125-132.

Haas, I.G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature.* 306:387-389.

Hammond, C., I. Braakman, and A. Helenius. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA.* 91:913-917.

Harris, M.R., Y.Y. Yu, C.S. Kindle, T.H. Hansen, and J.C. Solheim. 1998. Calreticulin and calnexin interact with different protein and glycan determinants during the assembly of MHC class I. *J. Immunol.* 160:5404-5409.

Hedman, K., K.L. Goldenthal, A.V. Rutherford, I. Pastan, and M.C. Willingham. 1987. Comparison of the intracellular pathways of transferrin recycling and vesicular stomatitis virus membrane glycoprotein exocytosis by ultrastructural double-label cytochemistry. *J. Histochem. Cytochem.* 35:233-243.

Hengel, H., T. Flohr, G.J. Hämmerling, U.H. Koszinowski, and F. Momburg. 1996. Human cytomegalovirus inhibits peptide translocation into the endoplasmic reticulum for MHC class I assembly. *J. Gen. Virol.* 77:2287-2296.

Hengel, H., J.-O. Koopmann, T. Flohr, W. Muranyi, E. Goulmy, G.J. Hämmerling, and F. Momburg. 1997. A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter. *Immunity*. 6:623-632.

Hermiston, T.W., R.A. Tripp, T. Sparer, L.R. Gooding, and W.S.M. Wold. 1993. Deletion mutation analysis of the adenovirus type 2 E3-gp19K protein: identification of sequences within the endoplasmic reticulum lumenal domain that are required for class I antigen binding and protection from adenovirus-specific cytotoxic T lymphocytes. *J. Virol.* 67:5289-5298.

Higgins, C.F. 1992. ABC transporters: From microorganisms to man. *Annu. Rev. Cell Biol.* 8:67-113.

High, S., F.J. Lecomte, S.J. Russell, B.M. Abell, and J.D. Oliver. 2000. Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Lett.* 476:38-41.

Hill, A., P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh, and D. Johnson. 1995. Herpes simplex virus turns off the TAP to evade host immunity. *Nature*. 375:411-413.

Hill, A.B., B.C. Barnett, A.J. McMichael, and D.J. McGeoch. 1994. HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2. *J. Immunol.* 152:2736-2741.

Hirano, N., F. Shibasaki, R. Sakai, T. Tanaka, J. Nishida, Y. Yazaki, T. Takenawa, and H. Hirai. 1995. Molecular cloning of the human glucose-regulated protein ERp57/GRP58, a thiol-dependent reductase. Identification of its secretory form and inducible expression by the oncogenic transformation. *Eur. J. Biochem.* 234:336-342.

Hirst, J., N.A. Bright, B. Rous, and M.S. Robinson. 1999. Characterization of a fourth adaptor-related protein complex. *Mol. Biol. Cell.* 10:2787-2802.

Hochman, J.H., H. Jiang, L. Matyus, M. Edidin, and B. Pernis. 1991. Endocytosis and dissociation of class I MHC molecules labeled with fluorescent β-2 microglobulin. *J. Immunol.* 146:1862-1867.

Hopkins, C.R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell.* 35:321-330.

Hopkins, C.R., A. Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. *Nature*. 346:335-338.

Hopkins, C.R., A. Gibson, M. Shipman, D.K. Strickland, and I.S. Trowbridge. 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. *J. Cell Biol.* 125:1265-1274.

Hopkins, C.R., and I.S. Trowbridge. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97:508-521.

Horio, M., M.M. Gottesman, and I. Pastan. 1988. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc. Natl. Acad. Sci. USA*. 85:3580-3584.

Horwitz, M.S. 1996. Adenoviruses. *In* Fields Virology. B.N. Fields, D.M. Knipe, and P.M. Howley, editors. Lippincott-Raven, New York.

Huet, C., J.F. Ash, and S.J. Singer. 1980. The antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. *Cell*. 21:429-438.

Hughes, E.A., and P. Cresswell. 1998. The thiol oxidoreductase ERp57 is a component of the MHC class I peptide-loading complex. *Curr. Biol.* 8:709-712.

Hughes, E.A., C. Hammond, and P. Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. *Proc. Natl. Acad. Sci. USA*. 94:1896-1901.

Hurtley, S.M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:277-307.

Ishido, S., C. Wang, B.S. Lee, G.B. Cohen, and J.U. Jung. 2000. Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J. Virol.* 74:5300-5309.

Jackson, M., R., T. Nilsson, and P.A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* 9:3153-3162.

Jeffries, W.A. 1990. E3/19K from adenovirus 2 is an immunosubversive protein that binds to a structural motif regulating the intracellular transport of major histocompatibility complex class I proteins. *J. Exp. Med.* 172:1653-1664.

Jennings, S.R., P.L. Rice, E.D. Kloszewski, R.W. Anderson, D.L. Thompson, and S.S. Tevethia. 1985. Effect of herpes simplex virus types 1 and 2 on surface expression of class I major histocompatibility complex antigens on infected cells. *J. Virol.* 56:757-766.

Jing, S., T. Spencer, K. Miller, C. Hopkins, and I.S. Trowbridge. 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. *J. Cell Biol.* 110:283-294.

Johnson, A.O., R.N. Ghosh, K.W. Dunn, R. Garippa, J. Park, S. Mayor, F.R. Maxfield, and T.E. McGraw. 1996. Transferrin receptor containing the SDYQRL motif of TGN38 causes a reorganization of the recycling compartment but is not targeted to the TGN. *J. Cell Biol.* 135:1749-1762.

Johnson, L.S., K.W. Dunn, B. Pytowski, and T.E. McGraw. 1993. Endosome acidification and receptor trafficking: bafilomycin A_1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Mol. Biol. Cell.* 4:1251-1266.

Johnson, L.S., J.F. Presley, J.C. Park, and T.E. McGraw. 1994. Slowed receptor trafficking in mutant CHO lines of the *end1* and *end2* complementation groups. *J. Cell. Phys.* 158:29-38.

Jondal, M., R. Schirmbeck, and J. Reimann. 1996. MHC class I-restricted CTL responses to exogenous antigens. *Immunity.* 5:295-302.

Jones, T.R., L.K. Hanson, L. Sun, J.S. Slater, R.M. Stenberg, and A.E. Campbell. 1995. Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J. Virol.* 69:4830-4841.

Jones, T.R., and L. Sun. 1997. Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains. *J. Virol.* 71:2970-2979.

Jones, T.R., E.J.H.J. Wiertz, L. Sun, K.N. Fish, J.A. Nelson, and H.L. Ploegh. 1996. Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc. Natl. Acad. Sci. USA.* 93:11327-11333.

Kahn-Perles, B., J. Salamero, and O. Jouans. 1994. Biogenesis of MHC class I antigen: involvement of multiple chaperone molecules. *Eur. J. Cell Biol.* 64:176-185.

Kaplan, J. 1980. Evidence for reutilization of surface receptors for α-macroglobulin•protease complexes in rabbit alveolar macrophages. *Cell.* 19:197-205.

Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245-3252.

Kärre, K. 1995. Express yourself or die: peptides, MHC molecules, and NK cells [comment]. *Science.* 267:978-979.

Kavathas, P., F.H. Bach, and R. DeMars. 1980. Gamma ray-induced loss of expression of HLA and glyoxalase I alleles in lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 77:4251-4255.

Kelly, A., S.H. Powis, R. Glynne, E. Radley, S. Beck, and J. Trowsdale. 1991. Second proteasome-related gene in the human MHC class II region. *Nature.* 353:667-668.

Kerkau, T., I. Bacik, J.R. Bennink, J.W. Yewdell, T. Hunig, A. Schimpl, and U. Schubert. 1997. The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. *J. Exp. Med.* 185:1295-1305.

Kestler, H.W.d., D.J. Ringler, K. Mori, D.L. Panicali, P.K. Sehgal, M.D. Daniel, and R.C. Desrosiers. 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell.* 65:651-662.

Killisch, I., P. Steinlein, K. Römisch, R. Hollinshead, H. Beug, and G. Griffiths. 1992. Characterization of early and late endocytic compartments of the transferrin cycle. *J. Cell Sci.* 103:211-232.

Kirchhausen, T. 2000. Clathrin. *Annu. Rev. Biochem.* 69:699-727.

Klausner, R.D., G. Ashwell, J. van Renswoude, J.B. Harford, and K.R. Bridges. 1983a. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA*. 80:2263-2266.

Klausner, R.D., J. van Renswoude, G. Ashwell, C. Kempf, A.N. Schechter, A. Dean, and K.R. Bridges. 1983b. Receptor-mediated endocytosis of transferrin in K562 cells. *J. Biol. Chem.* 258:4715-4724.

Koelle, D.M., M.A. Tigges, R.L. Burke, F.W. Symington, S.R. Riddell, H. Abbo, and L. Corey. 1993. Herpes simplex virus infection of human fibroblasts and keratinocytes inhibits recognition by cloned CD8+ cytotoxic T lymphocytes. *J. Clin. Invest.* 91:961-968.

Krämer, H., and M. Phistry. 1996. Mutations in the Drosophila hook gene inhibit endocytosis of the boss transmembrane ligand into multivesicular bodies. *J. Cell Biol.* 133:1205-1215.

Krämer, H., and M. Phistry. 1999. Genetic analysis of hook, a gene required for endocytic trafficking in drosophila. *Genetics*. 151:675-684.

Krause, K.-H., and M. Michalak. 1997. Calreticulin. *Cell*. 88:439-443.

Kubler, E., and H. Riezman. 1993. Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J.* 12:2855-2862.

Kuchler, K., R.E. Sterne, and J. Thorner. 1989. *Saccharomyces cerevisae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. *EMBO J.* 8:3973-3984.

Kvist, A., L. Ostberg, H. Persson, L. Philipson, and P.A. Peterson. 1978. Molecular association between transplantation antigens and cell surface antigen in adenovirus-transformed cell line. *Proc. Natl. Acad. Sci. USA*. 75:5674-5678.

Lama, J., A. Mangasarian, and D. Trono. 1999. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. *Curr. Biol.* 9:622-631.

Lamaze, C., L.M. Fujimoto, H.L. Yin, and S.L. Schmid. 1997. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J. Biol. Chem.* 272:20332-20335.

Lanier, L.L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359-393.

Lankat-Buttgereit, B., and R. Tampé. 1999. The transporter associated with antigen processing TAP: structure and function. *FEBS Lett.* 464:108-112.

Le Bouteiller, P., and A. Blaschitz. 1999. The functionality of HLA-G is emerging. *Immunol. Rev.* 167:233-244.

Le Gall, S., F. Buseyne, A. Trocha, B.D. Walker, J.M. Heard, and O. Schwartz. 2000. Distinct trafficking pathways mediate Nef-induced and clathrin- dependent major histocompatibility complex class I down-regulation. *J. Virol.* 74:9256-9266.

Le Gall, S., L. Erdtmann, S. Benichou, C. Berlioz-Torrent, L. Liu, R. Benarous, J.-M. Heard, and L. Schwartz. 1998. Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity.* 8:483-495.

Lehner, P.J., J.T. Karttunen, G.W. Wilkinson, and P. Cresswell. 1997. The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation. *Proc. Natl. Acad. Sci. USA.* 94:6904-6909.

Lehner, P.J., M.J. Surman, and P. Cresswell. 1998. Soluble tapasin restores MHC class I expression and function in the tapasin-negative cell line .220. *Immunity.* 8:221-231.

Lehner, P.J., and J. Trowsdale. 1998. Antigen presentation: coming out gracefully. *Curr. Biol.* 8:R605-R608.

Leibson, P.J. 1998. Cytotoxic lymphocyte recognition of HLA-E: utilizing a nonclassical window to peer into classical MHC. *Immunity.* 9:289-294.

Levitskaya, J., M. Coram, V. Levitsky, S. Imreh, P.M. Steigerwald-Mullen, G. Klein, M.G. Kurilla, and M.G. Masucci. 1995. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature*. 375:685-688.

Levitskaya, J., A. Sharipo, A. Leonchiks, A. Ciechanover, and M.G. Masucci. 1997. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proc. Natl. Acad. Sci. USA*. 94:12616-12621.

Lewis, J.W., A. Sewell, D. Price, and T. Elliott. 1998. HLA-A*0201 presents TAP-dependent peptide epitopes to cytotoxic T lymphocytes in the absence of tapasin. *Eur. J. Immunol.* 28:3214-3220.

Li, S., K.M. Paulsson, S. Chen, H.O. Sjögren, and P. Wang. 2000. Tapasin is required for efficient peptide binding to transporter associated with antigen processing. *J. Biol. Chem.* 275:1581-1586.

Li, S., H.-O. Sjögren, U. Hellman, R.F. Pettersson, and P. Wang. 1997. Cloning and functional characterization of a subunit of the transporter associated with antigen processing. *Proc. Natl. Acad. Sci. USA*. 94:8708-8713.

Lindquist, J.A., O.N. Jensen, M. Mann, and G.J. Hämmerling. 1998. ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. *EMBO J.* 17:2186-2195.

Liu, Q., Y. Feng, and M. Forgac. 1994. Activity and in vitro reassembly of the coated vesicle (H+)-ATPase requires the 50-kDa subunit of the clathrin assembly complex AP-2. *J Biol Chem.* 269:31592-31597.

Liu, S.-H., M.S. Marks, and F.M. Brodsky. 1998. A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. *J. Cell Biol.* 140:1023-1037.

Liu, S.-H., M.L. Wong, C.S. Craik, and F.M. Brodsky. 1995. Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. *Cell.* 83:257-267.

Lu, X., H. Yu, S.H. Liu, F.M. Brodsky, and B.M. Peterlin. 1998. Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4. *Immunity*. 8:647-656.

Machold, R.P., E.J.H.J. Wiertz, T.R. Jones, and H.L. Ploegh. 1997. The HCMV gene products US11 and US2 differ in their ability to attack allelic forms of murine major histocompatibility complex (MHC) class I heavy chains. *J. Exp. Med.* 185:363-366.

Machy, P., J. Barbet, and L.D. Leserman. 1982. Differential endocytosis of T and B lymphocyte surface molecules evaluated with antibody-bearing fluorescent liposomes containing methotrexate. *Proc. Natl. Acad. Sci. USA*. 79:4148-4152.

Machy, P., A. Truneh, D. Gennaro, and S. Hoffstein. 1987a. Endocytosis and de novo expression of major histocompatibility complex encoded class I molecules: kinetic and ultrastructural studies. *Eur. J. Cell Biol.* 45:126-136.

Machy, P., A. Truneh, D. Gennaro, and S. Hoffstein. 1987b. Major histocompatibility complex class I molecules internalized via coated pits in T lymphocytes. *Nature*. 328:724-726.

Maloy, W.L. 1987. Comparison of the primary structure of class I molecules. *Immunol. Res.* 6:11-29.

Mangasarian, A., M. Foti, C. Aiken, D. Chin, J.-L. Carpentier, and D. Trono. 1997. The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. *Immunity*. 6:67-77.

Mangasarian, A., V. Piguet, J.K. Wang, Y.L. Chen, and D. Trono. 1999. Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. *J. Virol.* 73:1964-1973.

Margolese, L., G.L. Waneck, C.K. Suzuki, E. Degen, R.A. Flavell, and D.B. Williams. 1993. Identification of the region on the class I histocompatibility molecule that interacts with the molecular chaperone, p88 (calnexin, IP90). *J. Biol. Chem.* 268:17959-17966.

Marsh, E.W., P.L. Leopold, N.L. Jones, and F.R. Maxfield. 1995. Oligomerized transferrin receptors are selectively retained by a lumenal sorting signal in a long-lived endocytic recycling compartment. *J. Cell Biol.* 129:1509-1522.

Marsh, M., G. Griffiths, G.E. Dean, I. Mellman, and A. Helenius. 1986. Three-dimensional structure of endosomes in BHK-21 cells. *Proc. Natl. Acad. Sci. USA.* 83:2899-2903.

Mayor, S., J.F. Presley, and F.R. Maxfield. 1993. Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J. Cell Biol.* 121:1257-1269.

McGraw, T.E., K.W. Dunn, and F.R. Maxfield. 1993. Isolation of a temperature-sensitive variant Chinese hamster ovary cell line with a morphologically altered endocytic recycling compartment. *J. Cell. Phys.* 155:579-594.

McGraw, T.E., and F.R. Maxfield. 1990. Human transferrin receptor internalization is partially dependent upon an aromatic amino acid in the cytoplasmic domain. *Cell Reg.* 1:369-377.

Miller, D.M., and D.D. Sedmak. 1999. Viral effects on antigen processing. *Curr. Opin. Immunol.* 11:94-99.

Morin, J.E., M.D. Lubeck, J.E. Barton, A.J. Conley, A.R. Davis, and P.P. Hung. 1987. Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. Natl. Acad. Sci. USA.* 84:4626-4630.

Morrice, N.A., and S. Powis, J. 1998. A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. *Curr. Biol.* 8:713-716.

Mukherjee, S., R.N. Ghosh, and F.R. Maxfield. 1997. Endocytosis. *Phys. Rev.* 77:759-803.

Myers, M., and M. Forgac. 1993. The coated vesicle vacuolar (H^+)-ATPase associates with and is phosphorylated by the 50-kDa polypeptide of the clathrin assembly protein AP-2. *J. Biol. Chem.* 268:9184-9186.

Neefjes, J.J., F. Momburg, and G.J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science.* 261:769.

Neisig, A., R. Wubbolts, X. Zang, C. Melief, and J. Neefjes. 1996. Allele-specific differences in the interaction of MHC class I molecules with transporters associated with antigen processing. *J. Immunol.* 156:3196-3206.

Neumann, L., and R. Tampé. 1999. Kinetic analysis of peptide binding to the TAP transport complex: evidence for structural rearrangements induced by substrate binding. *J. Mol. Biol.* 294:1203-1213.

Nilsson, T., M. Jackson, and P.A. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell.* 58:707-718.

Nössner, E., and P. Parham. 1995. Species specific difference in chaperone interaction of human and mouse major histocompatibility complex class I molecules. *J. Exp. Med.* 181:327-337.

Odorizzi, G., A. Pearse, D. Domingo, I.S. Trowbridge, and C.R. Hopkins. 1996. Apical and basolateral endosomes of MDCK cells are interconnected and contain a polarized sorting mechanism. *J. Cell Biol.* 135:139-152.

Ohno, H., J. Stewart, M.-C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Galluser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science.* 269:1872-1875.

Oldridge, J., and M. Marsh. 1998. Nef--an adaptor adaptor? *Trends Cell Biol.* 8:302-305.

Oliver, J.D., H.L. Roderick, D.H. Llewellyn, and S. High. 1999. ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. *Mol. Biol. Cell.* 10:2573-2582.

Oliver, J.D., F.J. van der Wal, N.J. Bulleid, and S. High. 1997. Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science.* 275:86-88.

Omary, M.B., and I.S. Trowbridge. 1981. Biosynthesis of the human transferrin receptor in cultured cells. *J. Biol. Chem.* 24:12888-12892.

Ortiz-Navarrete, V., A. Seelig, M. Gernold, S. Frentzel, P.M. Kloetzel, and G.J. Hämmerling. 1991. Subunit of the '20S' proteasome (multicatalytic proteinase) encoded by the major histocompatibility complex. *Nature.* 353:662-664.

Ortmann, B., M.J. Androlewicz, and P. Cresswell. 1994. MHC class I/β2-microglobulin complexes associate with TAP transporters before peptide binding. *Nature.* 368:864-867.

Ortmann, B., J. Copeman, P.J. Lehner, B. Sadasivan, J.A. Herberg, A.G. Grandea, S.R. Riddell, R. Tampé, T. Spies, J. Trowsdale, and P. Cresswell. 1997. A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science.* 277:1306-1309.

Otsu, M., R. Urade, M. Kito, F. Omura, and M. Kikuchi. 1995. A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. *J. Biol. Chem.* 270:14958-14961.

Ou, W.J., P.H. Cameron, D.Y. Thomas, and J.J. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature.* 364:771-776.

Pääbo, S., F. Weber, T. Nilsson, W. Schaffner, and P.A. Peterson. 1986. Structural and functional dissection of an MHC class I antigen-binding adenovirus glycoprotein. *EMBO J.* 5:1921-1927.

Pamer, E., and P. Cresswell. 1998. Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* 16:323-358.

Pearse, B.M.F., and M.S. Robinson. 1990. Clathrin, adaptors and sorting. *Annu. Rev. Cell Biol.* 6:151-171.

Peh, C.A., S.R. Burrows, M. Barnden, R. Khanna, P. Cresswell, D.J. Moss, and J. McCluskey. 1998. HLA-B27-restricted antigen presentation in the absence of tapasin reveals polymorphism in mechanisms of HLA class I peptide loading. *Immunity.* 8:531-542.

Peter, F. 1998. HIV Nef: the mother of all evil? *Immunity.* 9:433-437.

Piguet, V., Y.L. Chen, A. Mangasarian, M. Foti, J.L. Carpentier, and D. Trono. 1998. Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. *EMBO J.* 17:2472-2481.

Piguet, V., L. Wan, C. Borel, A. Mangasarian, N. Demaurex, G. Thomas, and D. Trono. 2000. HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complexes. *Nat. Cell Biol.* 2:163-167.

Plemper, R.K., S. Böhmler, J. Bordallo, T. Sommer, and D.H. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature.* 388:891-895.

Ploegh, H.L. 1998. Viral strategies of immune evasion. *Science.* 280:248-253.

Pouyssegur, J., R.P.C. Shiu, and I. Pastan. 1977. Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. *Cell.* 11:213-244.

Powis, S.J. 1997. Major histocompatibility complex class I molecules interact with both subunits of the transporter associated with antigen processing, TAP1 and TAP2. *Eur. J. Immunol.* 27:2744-2747.

Presley, J.F., S. Mayor, K.W. Dunn, L.S. Johnson, T.E. McGraw, and F.R. Maxfield. 1993. The *end2* mutation in CHO cells slows the exit of transferrin receptors from the recycling compartment but bulk membrane recycling is unaffected. *J. Cell Biol.* 122:1231-1241.

Presley, J.F., S. Mayor, T.E. McGraw, K.W. Dunn, and F.R. Maxfield. 1997. Bafilomycin A_1 treatment retards transferrin receptor recycling more than bulk membrane recycling. *J. Biol. Chem.* 272:13929-13936.

Qualmann, B., M.M. Kessels, and R.B. Kelly. 2000. Molecular links between endocytosis and the actin cytoskeleton. *J. Cell Biol.* 150:F111-F116.

Rajagopalan, S., and M.B. Brenner. 1994. Calnexin retains unassembled major histocompatibility complex class I free heavy chains in the endoplasmic reticulum. *J. Exp. Med.* 180:407-412.

Rajagopalan, S., Y. Xu, and M.B. Brenner. 1994. Retention of unassembled components of integral membrane proteins by calnexin. *Science*. 263:387-390.

Rao, K., C. van Renswoude, C. Kempf, and R.D. Klausner. 1983. Separation of Fe^{+3} from transferrin in endocytosis. *FEBS Lett.* 160:213-216.

Raposo, G., H.M. van Santen, R. Leijendekker, H.J. Geuze, and H.L. Ploegh. 1995. Misfolded major histocompatibility complex class I molecules accumulate in an expanded ER-golgi intermediate compartment. *J. Cell Biol.* 131:1403-1419.

Reid, P.A., and C. Watts. 1990. Cycling of cell surface MHC glycoproteins through primaquine sensitive intracellular compartments. *Nature*. 346:655-657.

Riggs, N.L., H.M. Craig, M.W. Pandori, and J.C. Guatelli. 1999. The dileucine-based sorting motif in HIV-1 Nef is not required for down- regulation of class I MHC. *Virol.* 258:203-207.

Riordan, J.R., J.M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.L. Chou, and et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA [published erratum appears in Science 1989 Sep 29;245(4925):1437]. *Science*. 245:1066-1073.

Rock, K.L. 1996. A new foreign policy: MHC class I molecules monitor the outside world. *Immunol. Today*. 17:131-137.

Rock, K.L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A.L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and generation of peptides presented on MHC class I molecules. *Cell*. 78:761-771.

Roelse, J., M. Gromme, F. Momburg, G. Hammeling, and J. Neefjes. 1994. Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. *J. Exp. Med.* 180:1591-1597.

Roizman, B. 1996. Herpesviridae. *In* Fields Virology. B.N. Fields, D.M. Knipe, and P.M. Howley, editors. Lippincott-Raven, New York.

Rome, L.H. 1985. Curling receptors. *TIBS*. 10:151.

Ross, T.M., A.E. Oran, and B.R. Cullen. 1999. Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. *Curr. Biol.* 9:613-621.

Rothman, J.E., and F.T. Wieland. 1996. Protein sorting by transport vesicles. *Science.* 272:227-234.

Sadasivan, B., P.J. Lehner, B. Ortmann, T. Spies, and P. Cresswell. 1996. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity.* 5:103-114.

Sadasivan, B.K., A. Cariappa, G.L. Waneck, and P. Cresswell. 1995. Assembly, peptide loading, and transport of MHC class I molecules in a calnexin-negative cell line. *Cold Spring Harb. Symp. Quant. Biol.* 60:267-275.

Salisbury, J.L., J.S. Condeelis, and P. Satir. 1980. Role of coated vesicles, microfilaments, and calmodulin in receptor- mediated endocytosis by cultured B lymphoblastoid cells. *J. Cell. Biol.* 87:132-141.

Sandvig, K., and B. van Deurs. 1990. Selective modulation of the endocytic uptake of ricin and fluid phase markers without alteration in transferrin endocytosis. *J. Biol. Chem.* 265:6382-6388.

Sanfridson, A., B.R. Cullen, and C. Doyle. 1994. The simian immunodeficiency virus Nef protein promotes degradation of CD4 in human T cells. *J. Biol. Chem.* 269:3917-3920.

Sanfridson, A., S. Hester, and C. Doyle. 1997. Nef proteins encoded by human and simian immunodeficiency viruses induce the accumulation of endosomes and lysosomes in human T cells. *Proc. Natl. Acad. Sci. USA.* 94:873-878.

Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. *Science.* 271:1526-1533.

Schmid, S.L., M.A. McNiven, and P. De Camilli. 1998. Dynamin and its partners: a progress report. *Curr. Opin. Cell Biol.* 10:504-512.

Schoenhals, G.J., R.M. Krishna, A.G. Grandea, 3rd, T. Spies, P.A. Peterson, Y. Yang, and K. Früh. 1999. Retention of empty MHC class I molecules by tapasin is essential to reconstitute antigen presentation in invertebrate cells. *EMBO J.* 18:743-753.

Schust, D.J., D. Tortorella, J. Seebach, C. Phan, and H.L. Ploegh. 1998. Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. *J. Exp. Med.* 188:497-503.

Schwartz, A.L. 1995. Receptor cell biology: receptor-mediated endocytosis. *Ped. Res.* 38:835-843.

Schwartz, O., A. Dautry-Varsat, B. Goud, V. Maréchal, A. Subtil, J.-M. Heard, and O. Danos. 1995. Human immunodeficiency virus type 1 Nef induces accumulation of CD4 in early endosomes. *J. Virol.* 69:528-533.

Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J.-M. Heard. 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nature Med.* 2:338-342.

Scott, J.E., and J.R. Dawson. 1995. MHC class I expression and transport in a calnexin-deficient cell line. *J. Immunol.* 155:143-148.

Sege, K., L. Rask, and P.A. Peterson. 1981. Role of beta2-microglobulin in the intracellular processing of HLA antigens. *Biochemistry.* 20:4523-4530.

Sester, M., D. Feuerbach, R. Frank, T. Preckel, A. Gutermann, and H.G. Burgert. 2000. The amyloid precursor-like protein 2 associates with the major histocompatibility complex class I molecule K(d). *J. Biol. Chem.* 275:3645-3654.

Sheff, D.R., E.A. Daro, M. Hull, and I. Mellman. 1999. The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. *J. Cell Biol.* 145:123-139.

Shih, W., A. Gallusser, and T. Kirchhausen. 1995. A clathrin-binding site in the hinge of the β2 chain of mammalian AP-2 complexes. *J. Biol. Chem.* 270:31083-31090.

Shimizu, Y., and R. DeMars. 1989. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J. Immunol.* 142:3320-3328.

Shimizu, Y., D.E. Geraghty, B.H. Koller, H.T. Orr, and R. DeMars. 1988. Transfer and expression of three cloned human non-HLA-A,B,C class I major histocompatibility complex genes in mutant lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 85:227-231.

Shisler, J., C. Yang, B. Walter, C.F. Ware, and L.R. Gooding. 1997. The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface Fas (CD95) and resistance to Fas-induced apoptosis. *J. Virol.* 71:8299-8306.

Shugars, D.C., M.S. Smith, D.H. Glueck, P.V. Nantermet, F. Seillier-Moiseiwitsch, and R. Swanstrom. 1993. Analysis of human immunodeficiency virus type 1 Nef gene sequences present in vivo. *J. Virol.* 67:4639-4650.

Signäs, C., M.G. Katze, H. Persson, and L. Philipson. 1982. An adenovirus glycoprotein binds heavy chains of class I transplantation antigens from man and mouse. *Nature.* 299:175-178.

Sijts, A.J., and E.G. Pamer. 1997. Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes. *J. Exp. Med.* 185:1403-1411.

Simpson, F., A.A. Peden, L. Christopoulou, and M.S. Robinson. 1997. Characterization of the adaptor-related protein complex, AP3. *J. Cell Biol.* 137:835-845.

Smith, G.L., J.Z. Levin, P. Palese, and B. Moss. 1987. Synthesis and cellular location of ten influenza polypeptides individually expressed by recombinant vaccinia viruses. *Virol.* 160:336-345.

Snider, M.D., and O.C. Rogers. 1985. Intracellular movement of cell surface receptors after endocytosis: resialylation of asialo-transferrin receptor in human erythroleukemia cells. *J. Cell Biol.* 100:826-834.

Snyder, H.L., J.W. Yewdell, and J.R. Bennink. 1994. Trimming of antigenic peptides in an early secretory compartment. *J. Exp. Med.* 180:2389-2394.

Solheim, J.C. 1999. Class I MHC molecules: assembly and antigen presentation. *Immunol. Rev.* 172:11-19.

Solheim, J.C., M. Harris, R., C.S. Kindle, and T.H. Hansen. 1997. Prominence of β_2-microglobulin, class I heavy chain conformation, and tapasin in the interactions of class I heavy chain with calreticulin and transporter associated with antigen processing. *J. Immunol.* 158:2236-2241.

Spies, T., V. Cerundolo, M. Colonna, P. Cresswell, A. Townsend, and R. DeMars. 1992. Presentation of viral antigen by MHC class I molecules is dependent on a putative peptide transporter heterodimer. *Nature*. 355:644-646.

Springer, S., A. Spang, and R. Schekman. 1999. A primer on vesicle budding. *Cell.* 97:145-148.

Srivastava, P.K., H. Udono, N.E. Blachere, and Z. Li. 1994. Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics*. 39:93-98.

Srivastava, S.P., N.Q. Chen, Y.X. Liu, and J.L. Holtzman. 1991. Purification and characterization of a new isozyme of thiol:protein-disulfide oxidoreductase from rat hepatic microsomes. Relationship of this isozyme to cytosolic phosphatidylinositol-specific phospholipase C form 1A. *J. Biol. Chem.* 266:20337-20344.

Stam, N.J., H. Spits, and H.L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137:2299-2306.

Stang, E., J. Kartenbeck, and R.G. Parton. 1997. Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. *Mol. Biol. Cell.* 8:4-57.

Stevens, T.H., and M. Forgac. 1997. Structure, function and regulation of the vacuolar (H+)-ATPase. *Annu. Rev. Cell Dev. Biol.* 13:779-808.

Stoorvogel, W., H.J. Geuze, J.M. Griffith, and G.J. Strous. 1988. The pathways of endocytosed transferrin and secretory protein are connected in the trans-Golgi reticulum. *J. Cell Biol.* 106:1821-1829.

Stoorvogel, W., V. Oorschot, and H.J. Geuze. 1996. A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell Biol.* 132:21-33.

Subtil, A., and A. Dautry-Varsat. 1997. Microtubule depolymerization inhibits clathrin coated-pit internalization in non-adherent cell lines while interleukin 2 endocytosis is not affected. *J. Cell Sci.* 110:2441-2447.

Suh, W.K., M.F. Cohen-Doyle, K. Fruh, K. Wang, P.A. Peterson, and D.B. Williams. 1994. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science.* 264:1322-1326.

Sunio, A., A.B. Metcalf, and H. Krämer. 1999. Genetic dissection of endocytic trafficking in Drosophila using a horseradish peroxidase-bride of sevenless chimera: hook is required for normal maturation of multivesicular endosomes. *Mol. Biol. Cell.* 10:847-859.

Taunton, J., B.A. Rowning, M.L. Coughlin, M. Wu, R.T. Moon, T.J. Mitchison, and C.A. Larabell. 2000. Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. *J. Cell Biol.* 148:519-530.

Tenney, D.J., and A.M. Colberg-Poley. 1991. Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection. *J. Virol.* 65:6724-6734.

Tomasec, P., V.M. Braud, C. Rickards, M.B. Powell, B.P. McSharry, S. Gadola, V. Cerundolo, L.K. Borysiewicz, A.J. McMichael, and G.W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science.* 287:1031.

Tomazin, R., J. Boname, N.R. Hegde, D.M. Lewinsohn, Y. Altschuler, T.R. Jones, P. Cresswell, J.A. Nelson, S.R. Riddell, and D.C. Johnson. 1999. Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nat. Med.* 5:1039-1043.

Tomazin, R., A.B. Hill, P. Jugovic, I. York, P. van Endert, H.L. Ploegh, D.W. Andrews, and D.C. Johnson. 1996. Stable binding or the herpes simplex virus ICP47 protein to the peptide binding site of TAP. *EMBO J.* 15:3256-3266.

Tooze, J., and M. Hollinshead. 1991. Tubular early endosomal networks in AtT20 and other cells. *J. Cell Biol.* 115:635-653.

Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed in vitro. *Cell.* 62:285-295.

Trejo, J., Y. Altschuler, H.W. Fu, K.E. Mostov, and S.R. Coughlin. 2000. Protease-activated receptor-1 down-regulation. A mutant HeLa cell line suggests novel requirements for PAR1 phosphorylation and recruitment to clathrin-coated pits. *J. Biol. Chem.* 275:31255-31265.

Trifaró, J.M., and M.L. Vitale. 1993. Cytoskeleton dynamics during neurotransmitter release. *Trends Neurosci.* 16:466-472.

Trischler, M., W. Stoorvogel, and O. Ullrich. 1999. Biochemical analysis of distinct rab5- and rab11-positive endosomes along the transferrin pathway. *J. Cell Sci.* 112:4773-4783.

Tse, D.B., C. C.R., J. McDowell, and B. Pernis. 1986. Recycling of class I MHC antigens: dynamics of internalization, acidification, and ligand-degradation in murine T lymphoblasts. *J. Mol. Cell. Immunol.* 2:315-329.

Tse, D.B., and B. Pernis. 1984. Spontaneous internalization of class I major histocompatibility complex molecules in T lymphoid cells. *J. Exp. Med.* 159:193-207.

Uebel, S., W. Kraas, S. Kienle, K.H. Wiesmüller, G. Jung, and R. Tampé. 1997. Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. *Proc. Natl. Acad. Sci. USA.* 94:8976-8981.

Uebel, S., T.H. Meyer, W. Kraas, S. Kienle, G. Jung, K.H. Wiesmüller, and R. Tampé. 1995. Requirements for peptide binding to the human transporter associated with antigen processing revealed by peptide scans and complex peptide libraries. *J. Biol. Chem.* 270:18512-18516.

Ulbrecht, M., S. Martinozzi, M. Grzeschik, H. Hengel, J.W. Ellwart, M. Pla, and E.H. Weiss. 2000. Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J. Immunol.* 164:5019-5022.

Urade, R., and M. Kito. 1992. Inhibition by acidic phospholipids of protein degradation by ER-60 protease, a novel cysteine protease, of endoplasmic reticulum. *FEBS Lett.* 312:83-86.

Urade, R., T. Oda, H. Ito, T. Moriyama, S. Utsumi, and M. Kito. 1997. Functions of characteristic Cys-Gly-His-Cys (CGHC) and Gln-Glu-Asp-Leu (QEDL) motifs of microsomal ER-60 protease. *J. Biochem.* 122:834-842.

Van der Wal, F.J., J.D. Oliver, and S. High. 1998. The transient association of ERp57 with N-glycosylated proteins is regulated by glucose trimming. *Eur. J. Biochem.* 256:51-59.

van Endert, P.M., R. Tampé, T.H. Meyer, R. Tisch, J.F. Bach, and H.O. McDevitt. 1994. A sequential model for peptide binding and transport by the transporters associated with antigen processing. *Immunity.* 1:491-500.

Van Kaer, L., P.G. Ashton-Rickardt, M. Eichelberger, M. Gaczynska, K. Nagashima, K.L. Rock, A.L. Goldberg, P.C. Doherty, and S. Tonegawa. 1994. Altered peptidase and viral-specific T cell response in *LMP2* mutant mice. *Immunity.* 1:533-541.

Van Leuven, F., J.-J. Cassiman, and H. Van Den Berghe. 1980. Primary amines inhibit recycling of α_2M receptors in fibroblasts. *Cell.* 20:37-43.

Vassilakos, A., M.F. Cohen-Doyle, P.A. Peterson, M.R. Jackson, and D.B. Williams. 1996. The molecular chaperone calnexin facilitates folding and assembly of class I histocompatibility molecules. *EMBO J.* 15:1495-1506.

Vega, M.A., and J.L. Strominger. 1989. Constitutive endocytosis of HLA class I antigens requires a specific portion of the intracytoplasmic tail that shares structural features with other endocytosed molecules. *Proc. Natl. Acad. Sci. USA.* 86:2688-2692.

Vergés, M., R.J. Havel, and K.E. Mostov. 1999. A tubular endosomal fraction from rat liver: biochemical evidence of receptor sorting by default. *Proc. Natl. Acad. Sci. USA.* 96:10146-10151.

Vigers, G.P., R.A. Crowther, and B.M.F. Pearse. 1986. Location of the 100 kd-50 kd accessory proteins in clathrin coats. *EMBO J.* 5:2079-2085.

Vinitsky, A., L.C. Anton, H.L. Snyder, M. Orlowski, J.R. Bennink, and J.W. Yewdell. 1997. The generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors. *J. Immunol.* 159:554-564.

Wainwright, S.D., P.A. Biro, and C.H. Holmes. 2000. HLA-F is a predominantly empty, intracellular, TAP-associated MHC class Ib protein with a restricted expression pattern. *J. Immunol.* 164:319-328.

Wan, L., S.S. Molloy, L. Thomas, G. Liu, Y. Xiang, S.L. Rybak, and G. Thomas. 1998. PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. *Cell.* 94:205-216.

Warburton, R.J., M. Matsui, S.L. Rowland-Jones, M.C. Gammon, G.E. Katzenstein, T. Wei, M. Edidin, H.J. Zweerink, A.J. McMichael, and J.A. Frelinger. 1994. Mutation of the alpha 2 domain disulfide bridge of the class I molecule HLA-A*0201. Effect on maturation and peptide presentation. *Hum. Immunol.* 39:261-271.

Warren, A.P., D.H. Ducroq, P.J. Lehner, and L.K. Borysiewicz. 1994. Human cytomegalovirus-infected cells have unstable assembly of major histocompatibility complex class I complexes and are resistant to lysis by cytotoxic T lymphocytes. *J. Virol.* 68:2822-2829.

Wendland, B., S.D. Emr, and H. Riezman. 1998. Protein traffic in the yeast endocytic and vacuolar protein sorting pathways. *Curr. Opin. Cell Biol.* 10:513-522.

Wesp, A., L. Hicke, J. Palecek, R. Lombardi, T. Aust, A.L. Munn, and H. Riezman. 1997. End4p/Sla2p interacts with actin-associated proteins for endocytosis in Saccharomyces cerevisiae. *Mol. Biol. Cell.* 8:2291-2306.

Whitby, D., and C. Boshoff. 1998. Kaposi's sarcoma herpesvirus as a new paradigm for virus-induced oncogenesis. *Curr. Opin. Oncol.* 10:405-412.

Whitney, J.A., M. Gomez, D. Sheff, T.E. Kreis, and I. Mellman. 1995. Cytoplasmic coat proteins involved in endosome function. *Cell.* 83:703-713.

Wiertz, E.J.H.J., T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, and H.L. Ploegh. 1996a. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell.* 84:169-119.

Wiertz, E.J.H.J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996b. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*. 384:432-438.

Williams, D.B. 1995. The Merck Frosst Award Lecture 1994. Calnexin: a molecular chaperone with a taste for carbohydrate. *Biochem. Cell Biol.* 73:123-132.

Williams, D.B., S.J. Sweidler, and G.W. Hart. 1985. Intracellular transport of membrane glycoproteins: two closely related histocompatibility antigens differ in their rates of transit to the cell surface. *J. Cell Biol.* 101:725-734.

Williams, J.L., J. Garcia, D. Harrich, L. Pearson, F. Wu, and R. Gaynor. 1990. Lymphoid specific gene expression of the adenovirus early region 3 promoter is mediated by NF-kappa B binding motifs. *EMBO J.* 9:4435-4442.

Willingham, M.C., F.R. Maxfield, and I.H. Pastan. 1979. α_2Macroglobulin binding to the plasma membrane of cultured fibroblasts. *J. Cell Biol.* 82:614-625.

Wilson, C.M., M.R. Farmery, and N.J. Bulleid. 2000. Pivotal role of calnexin and mannose trimming in regulating the endoplasmic reticulum-associated degradation of major histocompatibility complex class I heavy chain. *J. Biol. Chem.* 275:21224-21232.

Wold, W.S.M., and L.R. Gooding. 1991. Region E3 of adenovirus: a cassette of genes involved in host immunosurveillance and virus-cell interactions. *Virol.* 184:1-8.

Woods, J.W., M. Doriaux, and M.G. Farquhar. 1986. Transferrin receptors recycle to cis and middle as well as trans-Golgi cisternae in Ig-secreting myeloma cells. *J. Cell Biol.* 103:277-286.

Yamashiro, D.J., and F.R. Maxfield. 1987. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. *J. Cell Biol.* 105:2723-2733.

Yamashiro, D.J., B. Tycko, S.R. Fluss, and F.R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-golgi compartment in the recycling pathway. *Cell.* 37:789-800.

Yamashita, Y., K. Shimokata, S. Mizuno, H. Yamaguchi, and Y. Nishiyama. 1993. Down-regulation of the surface expression of class I MCH antigens by human cytomegalovirus. *Virol.* 193:727-736.

Yamashita, Y., K. Shimokata, S. Saga, S. Mizuno, T. Tsurumi, and Y. Nishama. 1994. Rapid degradation of the heavy chain of class I major histocompatibility complex antigens in the endoplasmic reticulum of human cytomegalovirus-infected cells. *J. Virol.* 68:7933-7943.

Yang, M., S. Omura, J.S. Bonifacino, and A.M. Weissman. 1998. Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. *J. Exp. Med.* 187:835-846.

Yee, C., I. Krishnan-Hewlett, C.C. Baker, R. Schlegel, and P.M. Howley. 1985. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. *Am. J. Pathol.* 119:361-366.

York, I.A., C. Roop, D.W. Andrews, S.R. Riddell, F.L. Graham, and D.C. Johnson. 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell.* 77:525-535.

Zapun, A., N.J. Darby, D.C. Tessier, M. Michalak, J.J. Bergeron, and D.Y. Thomas. 1998. Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. *J. Biol. Chem.* 273:6009-6012.

Zhang, Q., and R.D. Salter. 1998. Distinct patterns of folding and interactions with calnexin and calreticulin in human class I MHC proteins with altered N-glycosylation. *J. Immunol.* 160:831-837.

Zhang, Q., M. Tector, and R.D. Salter. 1995. Calnexin recognizes carbohydrate and protein determinants of class I major histocompatibility complex molecules. *J. Biol. Chem.* 270:3944-3948.

Appendix

Frances M. Brodsky
Lawrence Lem
Alejandra Solache
Elizabeth M. Bennett

Human pathogen subversion of antigen presentation

Authors' addresses
Frances M. Brodsky[1, 2], Lawrence Lem[1], Alejandra Solache[1], Elizabeth M. Bennett[1, 2],
[1]The G. W. Hooper Foundation, Department of Microbiology and Immunology, University of California, San Francisco, California, USA.
[2]Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, University of California, San Francisco, California, USA.

Correspondence to:
Frances M. Brodsky
The G.W. Hooper Foundation
Department of Microbiology and Immunology
University of California
San Francisco CA 94143-0552
USA
Fax: 1 415 476 6185
e-mail: fmarbro@itsa.ucsf.edu

Acknowledgements
Research reviewed here was supported by grants AR20684, AI39152, GM57657 and GM38093 from the National Institutes of Health.

Immunological Reviews 1999
Vol. 168. 199–215
Printed in Denmark.
Immunological Reviews
ISSN 0105-2896

Summary: Many pathogens have co-evolved with their human hosts to develop strategies for immune evasion that involve disruption of the intracellular pathways by which antigens are bound by class I and class II molecules of the major histocompatibility complex (MHC) for presentation to T cells. Here the molecular events in these pathways are reviewed and pathogen interference is documented for viruses, extracellular and intracellular bacteria and intracellular parasites. In addition to a general review, data from our studies of adenovirus, *Chlamydia trachomatis* and *Coxiella burnetii* are summarized. Adenovirus E19 is the first viral gene product described that affects class I MHC molecule expression by two separate mechanisms, intracellular retention of the class I heavy chain by direct binding and by binding to the TAP transporter involved in class I peptide loading. *Coxiella* and *Chlamydia* both affect peptide presentation by class II MHC molecules as a result of their residence in endocytic compartments, although the properties of the parasitophorous vacuoles they form are quite different. These examples of active interference with antigen presentation by viral gene products and passive interference by rickettsiae and bacteria are typical of the strategies used by these different classes of pathogens, which need to evade different types of immune responses. Pathogen–host co-evolution is evident in these subversion tactics for which the pathogen crime seems tailored to fit the immune system punishment.

Introduction

During pathogen infection, host class I or class II molecules, encoded by the major histocompatibility complex (MHC), bind pathogen-derived peptides. In association with MHC molecules, these peptides are displayed on the cell surface, a process that alerts T lymphocytes to the presence of the pathogen and stimulates the development of a pathogen-specific immune response. The intracellular pathways that lead to the production of peptides and their binding to MHC molecules are collectively known as antigen processing and presentation. Interference with these pathways is an effective strategy for pathogen evasion of an immune response, and pathogens capable of such disruption tend to establish latent or chronic infections. Human pathogens with this phenotype (Table 1) include human immunodeficiency virus (HIV), adenovirus, and the herpes viruses cytomegalovirus (CMV), Epstein–Barr virus (EBV), herpes

simplex virus (HSV) and varicella-zoster virus (VZV). Infection by these organisms interferes with the class I antigen presentation pathway required for stimulation of anti viral cytotoxic T lymphocytes (CTL). The bacterial pathogens *Chlamydia trachomatis*, *Mycobacterium tuberculosis* and *Helicobacter pylori*, as well as the rickettsia *Coxiella burnetii*, also cause chronic infections in humans. Infection by these microbes interferes with the class II antigen presentation pathway, necessary for production of antibodies against these organisms and for stimulation of an inflammatory response.

Antigen processing and presentation by class I and class II MHC molecules involve the secretory and endocytic pathways, respectively. The complexity of these pathways provides numerous steps which can be affected directly by pathogen gene products or indirectly by the presence of pathogens which physically alter the function of the secretory and endocytic pathways. Characterization of the disruption mechanisms used by pathogens has revealed previously undefined steps in antigen presentation. Here we review the cellular pathways of antigen processing and presentation and the interaction of human pathogens with these pathways. Understanding the interplay between the cell biology of pathogen behavior and MHC expression is essential for developing therapeutic strategies against immune evasion.

MHC structure, polymorphism and pathogen/host co-evolution

The structure of class I and class II MHC molecules dictates that acquisition of a peptide is required for completion of folding. Therefore, peptide-binding for both molecules takes place during their biosynthetic assembly. Both class I and class II molecules have a peptide binding site, located between two α helices on the surface of the molecule, which, if empty, results in molecular instability. The protein sequence of these α-helices is variable between different allelic forms of class I and class II molecules and determines the sequence requirements (or motifs) for peptides that can be accommodated in the binding site (1). Class I and class II MHC molecules represent the most polymorphic genetic system in most species of vertebrates. In humans, there are more than 80–100 alleles at each of the *HLA-A, B* and *DRB1* loci of the MHC (2).

Due to structural constraints, each allelic form of MHC molecule can only bind a limited number of peptides generated from the proteins of any particular pathogen for presentation to T cells (3). Thus, allelic variation within a population and heterozygosity at MHC loci in an individual confers an advantage towards surviving infectious disease. Furthermore, selective pressure from infectious disease favors allelic variability within a population and it has been hypothesized that the evolution of MHC polymorphism is pathogen driven. Conversely, there is also considerable evidence that variation in pathogen proteins is driven by a need to escape from the immune response, through genetic alteration of epitopes recognized either by antibodies or by T-cell receptors (TCRs). Mutation away from peptide sequences that can be bound by the products of particular MHC alleles has been well documented for HIV, EBV and hepatitis C virus, among others (4–6). Thus MHC allelic diversity and pathogen mutation are products of interacting evolutionary selection.

Immune escape by inhibition of antigen presentation can be as harmful to the individual as epitope mutation, by allowing the pathogen to evade immune detection. However, since these escape mechanisms deal with aspects of MHC molecule assembly that are more general and less dependent on allelic variation, they are more insidious at a population level. Pathogen escape mechanisms that target processes unique to the host cell are less problematic for the pathogen than epitope variation, which is limited by the necessity of retaining pathogen protein function. The genetic origins of pathogen gene products that interfere with the fundamental steps of antigen processing and presentation are not established, but a reasonable hypothesis is that many of these pathogen proteins arise from acquisition of host genetic material. Therefore, studying mechanisms of pathogen immune subversion can reveal novel host proteins and elucidate the processes in which they operate.

Immune responses stimulated by MHC molecule antigen presentation

In the absence of infection, class I and class II molecules are stably expressed on cell surfaces by binding peptides derived from normal intracellular and extracellular proteins, respectively. This chronic display of self-protein-derived peptides does not stimulate T-cell activation because T cells with the potential to respond to self peptides are deleted during thymic development. During pathogen infection, however, pathogen proteins contribute to the pool of peptides that are presented by MHC molecules. When these pathogen-derived peptides appear on the cell surface, antigen-specific T cells are stimulated. Class I MHC molecules are expressed on all nucleated cells and bind peptides primarily from proteins which are degraded in the cytosol or signal peptides generated in the secretory pathway, thereby monitoring the intracellular environment. Thus class I molecules tend to alert T cells to infection by presenting peptides from viral proteins and from intracellular bacteria

Table 1. Human pathogens that interfere with antigen presentation

Pathogen	Gene product	MHC class affected	Function/phenotype (reference)
HIV[a,b]	Nef	class I	downregulation of class I (52)
		class II	downregulation of CD4 (52)
			proton pump binding[d] (78)
	Vpu	class I	degradation (60)
		class II	blocks CD4 expression (52)
Adenovirus[b]	E3/19K	class I	ER retention (53)
			TAP binding/tapasin inhibitor (55)
HSV	ICP47	class I	TAP binding (64)
			blocks TAP peptide binding
	unknown	class II	blocks neuronal expression (125)
CMV[b]	pp65	class I	antigen phosphorylation (62)
			inhibition of proteolysis
	US2	class I	reverse translocation (57)
			degradation
	US3	class I	ER retention (30)
	US6	class I	TAP binding (30)
			blocks peptide import
	US11	class I	reverse translocation (58)
			degradation
	UL18	class I	NK-cell decoy (49)
	ND	class II	blocks γ-interferon signaling (124)
EBV	EBNA1	class I	Gly-Ala repeat blocks its own
			proteasome processing (61)
VZV[b]	ND	class I	unknown (128)
HPV	ND	class I	undetectable viral protein level
	E5	class II	proton pump binding[d] (122)
	E6	class II	AP1 binding[d] (123)
measles virus	ND	class II	mixed effects (126)
C. trachomatis	ND	class II	downregulation (111, 112)
M. tuberculosis[b]	ND	class II	mixed effects (117, 118)
			blocks endosome acidification (119)
			blocks lysosome fusion (99)
	ND	CD1b[c]	downregulation (120)
H. pylori	Vac A	class II	disrupts late endosomes (96)
C. burnetii	ND	class II	distorts loading compartment (109)
S. typhimurium	SPI-II	class II	phagosome alteration (95)
Yersinia	Yops	class II	phagocytosis inhibition (95)
E. coli	LT	class II	inhibits phagocytic processing (90)
V. cholerae	CT	class II	inhibits phagocytic processing (90)
L. amazonensis	unknown	class II	degradation (121)

[a]Abbreviations: HIV, human immunodeficiency virus; CMV, cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein–Barr virus; HPV, human papilloma virus; VZV, varicella-zoster virus; ND, no data available; LT, heat labile enterotoxin; CT, cholera toxin.

[b]These pathogens have mechanisms for interfering with immune cell interactions, in addition to their effects on antigen presentation, a number of which are described in this volume of *Immunological Reviews*. For example, adenovirus inhibits apoptosis (53), CMV synthesizes chemokine homologs (50), HIV causes helper T-cell destruction (54), VZV infects T cells (128) and *M. tuberculosis* inhibits macrophage activation by γ-interferon (J. Ernst, personal communication).

[c]This molecule is a non-classical class I molecule, encoded outside the MHC, that presents glycolipid mycobacterial antigen to T cells following transport through the endocytic pathway, along the route trafficked by classical class II MHC molecules (92).

[d] These gene products have not been shown to have an effect on class II MHC function; however, they bind to proton pump subunits or the AP1 molecule, which have critical functions in the endocytic pathway for class II molecule maturation and cell surface expression.

which enter the cytoplasm. In addition to presenting peptides, class I molecules engage CD8 molecules present on CTL. TCR recognition of a peptide–class I complex, accompanied by class I–CD8 engagement, triggers cytotoxic activity against the antigen-presenting cell, resulting in elimination of a cell that harbors virus or intracellular bacteria. The ubiquitous tissue expression of class I molecules allows intracellular infection to be detected on all nucleated cell types, as long as pathogen peptides have access to the class I presentation pathway and responding cells have access to the site of infection. Disruption of the class I presentation pathway by pathogens can abrogate a CTL response.

The constitutive expression of class II MHC molecules is limited to B cells, monocytes, macrophages and dendritic cells. Class II molecule expression can be induced on other cell types by γ-interferon (γIFN). The pattern of constitutive tissue

expression is appropriate to the immunological role of class II MHC molecules, which is to present peptide antigens to CD4-positive T cells. These cells are known as helper T (Th) cells since they secrete cytokine mediators required for stimulation of humoral, cytotoxic and inflammatory immune responses. Release of appropriate cytokines by subpopulations of helper T cells is critical for stimulation of different pathways needed to eradicate infectious organisms (7, 8). Th1 cells, dependent on interleukin (IL)-12 for development, boost the cytotoxic and inflammatory response required for clearance of virus-infected cells and cells infected with intracellular bacteria. Th1 cells also stimulate B-cell production of complement-fixing antibodies and antibodies recognized by macrophage Fc receptors. Therefore, the Th1 response is needed also for elimination of extracellular bacteria which are targeted by these antibody-mediated mechanisms, as well as for antibody-mediated clearance of virus particles and neutralization of virus infection. Th2 cells, dependent on IL-4 for development, stimulate the production of antibodies, such as IgE, which are critical for the immune response against extracellular parasites. Stimulation of Th responses depends on antigen presentation by class II MHC molecules, which display peptides derived from extracellular proteins and are capable of warning helper T cells of the presence of extracellular pathogens, to solicit the extracellular immune responses required. Helper T cells also contribute to the eradication of the obligate intracellular pathogens, discussed below, but whether this is due to Th1 stimulation of CTL, antibody production, other inflammatory responses or combined effector mechanisms is not fully established (9–14). It is clear however that both bacteria and viruses which have strategies for disrupting the class II presentation pathway interfere with Th responses needed for an effective immune response against them.

Antigen presentation by class I MHC molecules

Class I molecules are composed of a polymorphic α-chain, encoded within the MHC, and the invariant subunit β_2-microglobulin (β_2m), encoded outside the MHC. In humans, there are three loci encoding α-chains, *HLA-A, B* and C. The genes are co-dominantly expressed, but expression levels of HLA-C gene products are generally tenfold less than A and B due to mRNA instability (15). Assembly of class I molecules takes place in the endoplasmic reticulum (ER) (Fig. 1). Early steps of this assembly process rely on ER chaperones that aid in the correct folding and assembly of many macromolecular protein complexes. These include calnexin and calreticulin, both calcium-dependent lectins with chaperone activity (reviewed in (16) and (17)), and ERp57, a thiol reductase with proteolytic activity (18, 19), which, like calnexin and calreticulin, associates with nascent glycoproteins in a carbohydrate-dependent fashion (20). After co-translational translocation, class I α-chain first associates with calnexin. The α-chain then forms a non-covalent dimer with β_2m, and calnexin is replaced by calreticulin. ERp57 joins the complex, although the timing of its interaction is unclear. Class I heterodimers, associated with calreticulin and ERp57, form a larger assembly complex with tapasin and the transporter associated with antigen processing (TAP) (21, 22). TAP, formed from subunits TAP1 and TAP2, and tapasin are encoded by the MHC and are unique to the class I assembly pathway. The sequential steps in build-up of the class I assembly complex are not yet fully defined, and the roles of the calcium-dependent lectins, ERp57 and even tapasin have not yet been fully elucidated (for a recent review see (23)). The timing of β_2m and ERp57 association with the class I assembly complex relative to the association and dissociation of calnexin is unclear and appears to differ between mouse and humans (22, 24–28). One study has suggested that β_2m binds TAP independently of the class I α-chain (29). Additionally, the role of tapasin in the association between class I molecules, calreticulin and ERp57 is controversial (22, 27). It is clear that tapasin is required for effective interaction between TAP and class I molecules (21), but tapasin may play a chaperone role as well (23, 30). All of these processes are sensitive to treatment of cells with castanospermine (21, 27, 28, 31), indicating that glycosylation of at least some of the components is important for their interaction.

It is the formation of trimeric complexes of class I heterodimers with peptide that releases the class I molecules from the assembly complex for transport to the plasma membrane via the Golgi apparatus and the trans-Golgi network (TGN). Formation of the class I assembly complex appears to be required for class I molecules to bind peptide, presumably keeping the class I peptide binding site in a peptide-receptive conformation and localizing class I molecules to the source of translocated peptide. TAP imports peptides generated on the cytoplasmic side of the ER into the ER lumen. Peptide import is ATP dependent (32), consistent with the fact that TAP is a member of a family of transporters with cytoplasmic ATP-binding domains (33, 34), and can be influenced by polymorphisms of TAP1 and TAP2 (35). A major source of TAP-transported peptides are the proteasome-based degradative systems, which degrade unfolded or ubiquitinated proteins in the cytoplasmic compartment of a cell. Two proteasome subunits are encoded in the MHC, LMP2 and LMP7. Their expression is regulated by γ IFN, and they compete with constitutively expressed proteasome

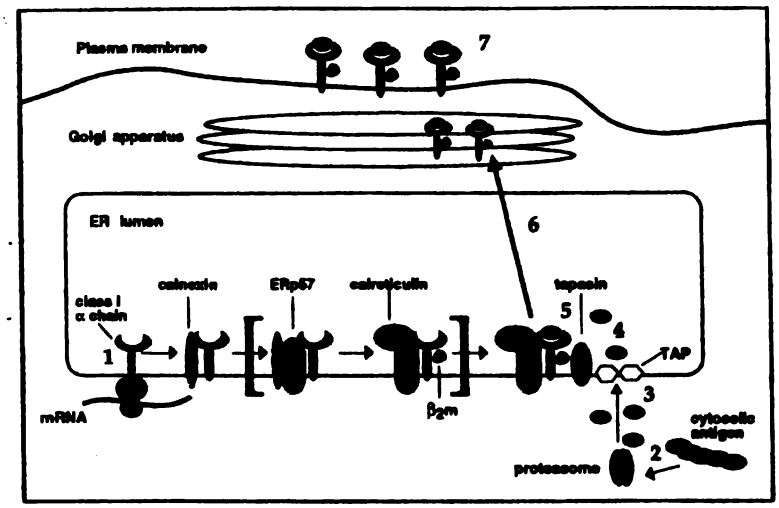


Fig. 1. Pathway for antigen presentation by class I MHC molecules and viral interference. Nascent class I α-chains are targeted to the ER lumen where they are bound by calnexin. As α-chains adopt a stable conformation, they bind β_2-microglobulin (β_2m), and calnexin is replaced by calreticulin. ERp57 aids in the folding of the class I complex, although whether it associates during or following class I α-chain chain association with calnexin and β_2m is undetermined (brackets indicate where order is uncertain). Tapasin bridges the multimeric complex (consisting of class I α-chain, β_2m, calreticulin and ERp57) to TAP, and peptide loading of the class I molecules occurs. Peptide-loaded class I molecules are then released to the cell surface. Steps of class I antigen presentation which have been shown to be inhibited by viruses are identified numerically (1–7).
Viral gene products which inhibit class I MHC antigen presentation act at the following steps:

1. Human cytomegalovirus (HCMV) US2 and US11 cause class I molecules to dislocate from the ER to the cytoplasm where they are degraded by the proteasome. Human immunodeficiency virus (HIV)-1 Vpu causes decay of nascent class I α-chains by an uncharacterized mechanism.
2. HCMV pp65 phosphorylates the HCMV 72K immediate early protein (IE), preventing IE antigen processing.
3. Herpes simplex virus ICP47 binds TAP, preventing TAP from binding peptide.
4. HCMV US6 binds TAP, preventing TAP from transporting peptide into the ER.
5. Adenovirus E19 inhibits the association of class I molecules with TAP.
6. Adenovirus E19 and HCMV US3 prevent or delay egress of class I molecules from the ER.
7. HIV Nef induces internalization of class I molecules from the cell surface.

subunits, influencing the proteolytic activity of proteasomes (36, 37). LMP2 and LMP7 are not absolutely required for the generation of peptides that bind class I molecules, because many class I-binding peptides are routinely generated by constitutively expressed proteasome subunits. However, by altering proteasome specificity, LMP2 and LMP7 influence the population of peptides generated. Genetic deletion of LMP2 and LMP7 correspondingly alters peptide-based CD8 T-cell selection in the thymus (38, 39). There is compelling evidence for other cytoplasmic proteolytic systems, in addition to proteasomes, which are capable of generating peptides that can be imported by TAP (40, 41). Class I molecules with appropriate hydrophobic binding sites, such as HLA-A2, can also bind signal peptides cleaved from translocated proteins, in a process that is not TAP dependent (42, 43). The non-classical class I molecule HLA-E, which is recognized by receptors on natural killer (NK) cells, exclusively binds signal peptides from classical class I molecules and inhibits NK activity against class I-expressing cells. However, HLA-E binding of signal peptides is both tapasin and TAP dependent (44). Finally, there is evidence that some trimming of peptides occurs in the ER (45–47), and it has been suggested that the exoprotease activity of ERp57 contributes to this function (23, 28).

Pathogenesis in the class I antigen presentation pathway

Viral gene products interfere with many steps in the assembly and surface expression of class I MHC molecules (Fig. 1, Table 1). The strategies of HSV, human and murine CMV, and HIV for interfering with class I MHC assembly and expression are

described in detail elsewhere in this volume (30, 48–52). Here these strategies are reviewed briefly, and recent data from our laboratory on the interference of adenovirus with class I antigen presentation is discussed. The pathogenesis of CMV, HSV, HIV and adenovirus is described in this volume (30, 48, 51, 53). All of these viruses can establish long-term and latent infections. Once an individual is infected with HSV or CMV, virus is never completely eradicated. HSV cycles between latency in neurons, which express only low levels of class I molecules, and outbreaks in epithelial cells, where active infection results in lesions (30). Unlike HSV, CMV does not cause recurrent problems for immunocompetent hosts. However, latent CMV can be reactivated and cause pathology in immunocompromised individuals, particularly in transplant recipients and AIDS patients. The so-called latent period of HIV infection, kept under control by the immune system in lymph node reservoirs, can last as long as 10 years or more. However, the ultimate destruction of T cells by the virus leads inevitably to patient death (54). For adenovirus, a cause of the common cold, there is more latency in the human population than is appreciated (53). Many individuals recover from acute infection and are subsequently immune to reinfection, but the virus can persist for a long time in an apparently recovered individual and still be shed in feces and pulmonary exudate.

While only one HSV gene product has been identified which can prevent class I antigen presentation, both human and murine CMV have multiple gene products, each acting at a different step in the class I antigen presentation pathway. Adenovirus E19 is the first single viral gene product found to affect more than one step in the class I assembly pathway (55). We hypothesize that the redundancy in attack on the class I presentation pathway by both CMV and adenovirus could be a mechanism of viral compensation for allelic variation in class I molecules. If particular class I alleles are weakly affected by viral proteins that directly bind class I molecules, their expression can still be inhibited or delayed by the action of viral proteins at other steps in the class I assembly pathway. Interestingly, HIV Nef takes advantage of class I locus diversity to evade a CTL response but retain inhibition of NK-cell activity (see below).

Viral interference with class I molecule assembly and export

To date, myriad viral strategies for inhibition of class I antigen presentation have been identified (reviewed in (56)). Two human CMV gene products (US2 and US11) interact with class I molecules at the time of translocation and appear to cause reverse translocation, following glycosylation, resulting in proteasome-dependent cytoplasmic degradation of class I molecules (57, 58). This type of degradation has recently been identified as a more general pathway for degradation of misfolded proteins, formerly thought to be degraded in the ER (59). Analysis of the activity of US2 and US11 was crucial to the characterization of this degradation pathway. The HIV protein Vpu can also affect the early stage of class I molecule assembly, leading to degradation (60). Viral strategies for obstruction of proteasome cleavage to generate antigenic peptides have been identified for EBV and CMV. The EBNA1 antigenic protein from EBV is resistant to proteasome degradation due to a Gly-Ala repeat domain, which can be transferred onto another antigenic protein from EBV and render it proteasome resistant (61). CMV encodes a kinase, pp65, which modifies another CMV antigenic protein by phosphorylation and blocks its proteolytic processing (62). Two viral gene products have been identified which interfere with peptide translocation by TAP. HSV ICP47 competes with peptide for binding to TAP on the cytoplasmic side of the ER (63, 64), while human CMV US6 interferes with peptide translocation (65, 66). Interestingly, ICP47 does not bind mouse TAP (63, 64, 67), suggesting co-evolution of the human virus with its host.

The adenovirus E3 19 kDa protein (E19) also has TAP binding activity (Fig. 2). Discovery of this function of E19 was an unexpected outcome of a study revisiting the effects of E19 on class I assembly. It has been known for a number of years that E19 binds class I molecules and prevents their exit from the ER by virtue of a well-characterized ER retention motif in its cytoplasmic domain (68–71). However, E19 can delay the cell surface expression of alleles for which it has poor binding affinity (72, 73). We observed that a truncated form of E19, lacking the ER retention sequence, was capable of delaying the maturation of class I MHC molecules, and we investigated whether this delay occurred prior to class I association with the TAP peptide-loading complex (55). In asking whether the class I/E19 complex could co-immunoprecipitate with TAP, we found that E19 associates with TAP in 721.221 cells lacking classical class I molecules HLA-A, B and C, suggesting an association independent from E19's binding to class I molecules (Fig. 2A, lanes 1–3). We then investigated whether E19 binding to TAP was dependent on the presence of tapasin and found that in a tapasin-negative cell line (721.220/B8) E19 still associates with TAP (Fig. 2A, lanes 4–6). Non-classical class I molecules are thought to associate with TAP via tapasin, as no association is observed between HLA-E and TAP in tapasin-negative cells (44). Thus our analysis of .220/B8 cells confirms that E19 binding to TAP is independent of classical and non-classical class I molecules. E19 binding to TAP was also found to be independent of the ER retention signal in E19's cytoplasmic

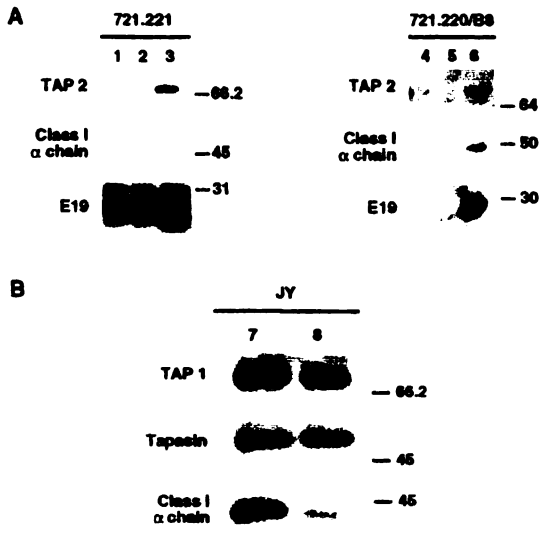


Fig. 2. Adenovirus E19 binds TAP and inhibits tapasin function. A. Adenovirus E19 binds TAP in the absence of expression of classical class I MHC molecules or tapasin. Human B-lymphoblastoid cell lines (LCL) were uninfected or infected with a vaccinia virus construct encoding adenovirus E19 (E19-Vac) or a control virus encoding the nucleoprotein of influenza virus (NP-Vac). E19 was immunoprecipitated from cell lysates. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E19, class I MHC α-chain and TAP2. Proteins were visualized by enhanced chemiluminescence (BCL). Lanes 1 and 4 are uninfected; lanes 2 and 5 are infected with NP-Vac; lanes 3 and 6 are infected with E19-Vac. LCL 721.221 is HLA-A, -B, -C-negative; LCL 721.220/B8 is tapasin-negative and has been transfected with HLA-B8. Data are preliminary results for Bennett et al. (55). **B. Adenovirus E19 acts as a tapasin inhibitor.** The wild-type LCL JY was uninfected (lane 7) or infected with E19-Vac (lane 8). TAP was immunoprecipitated from cell lysates. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for TAP1 and class I MHC α-chain. Proteins were visualized by ECL. The nitrocellulose was then stripped and reprobed for tapasin. Data are preliminary results for Bennett et al. (55).

domain (55). Interestingly, in cells expressing E19, the level of class I α-chain found in the TAP–tapasin complex was reduced compared to levels detected in such complexes isolated from E19-negative cells (Fig. 2B). This result indicates that the association of E19 with TAP and with class I molecules results in inhibition of tapasin function, most likely due to steric constraints. Thus the E19 protein of adenovirus has a dual mechanism for causing immune evasion. E19 causes ER retention of a subset of class I alleles to which it binds avidly (73). It can also delay expression of all class I alleles by interaction with TAP and reduction of tapasin function.

Other viral gene products target the export of class I molecules from the ER to the cell surface. The US3 gene product of human CMV binds class I molecules and delays their exit from the ER, but the mechanism of US3 ER retention is unknown (30). Two gene products from murine CMV affect post-ER transport (48). The first, gp37/40, traps class I molecules in the ER to Golgi intermediate compartment. The other, gp48, binds class I molecules and diverts them for degradation in the endocytic pathway. The general strategy used by the murine and human CMV is similar, but the mechanistic details are completely different, suggesting convergent evolution.

Viral interference with CTL and NK-cell recognition at the cell surface

At the cell surface, downregulation of class I molecules by HIV Nef abrogates CTL recognition. The Nef-dependent loss of immunological recognition is described in the review by Collins & Baltimore (51), in this volume. The mechanism for this downregulation is believed to be related to Nef's ability to associate with the adaptor molecules present in clathrin-coated vesicles. Evidence for such association is discussed in the article by Piguet et al. (52), in this volume. Clathrin-coated vesicles are responsible for receptor-mediated endocytosis at the plasma membrane and mediate protein sorting to lysosomes from the TGN (74). Indeed, increased formation of clathrin-coated pits at the plasma membrane is observed when Nef interacts with CD4, correlating with downregulation of CD4 expression (75). However, Nef has never clearly been shown to associate directly with the adaptor molecule, AP2, that functions at the plasma membrane. Rather, it appears to bind AP1, which functions in the TGN (76). Therefore Nef's mechanism of influencing class I and CD4 downregulation may be more complex than direct targeting to clathrin-coated vesicles (77). The recently characterized interaction of Nef with a component of the vac-

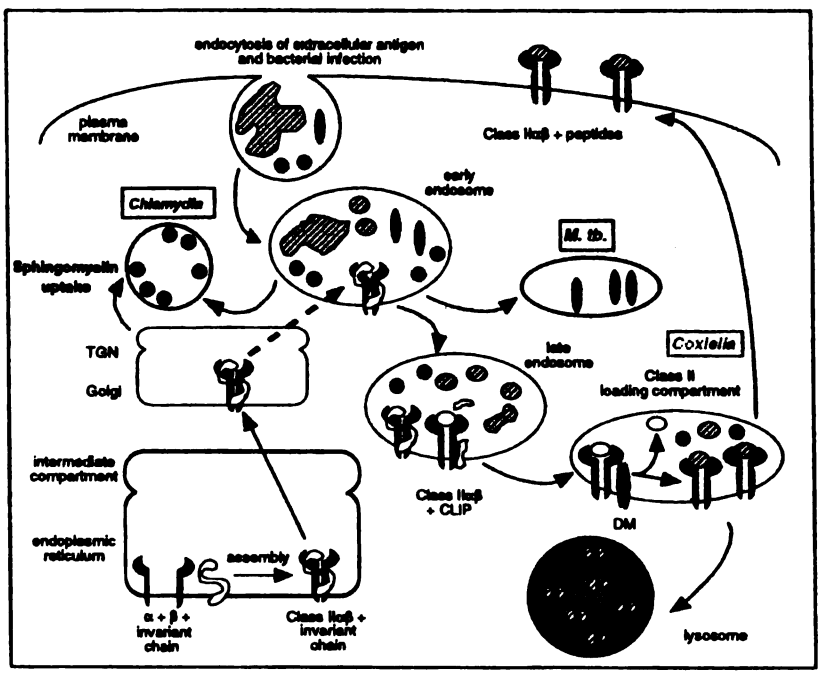


Fig. 3. Pathway for antigen presentation by class II MHC molecules and relationship to parasitophorous vacuoles. The assembly pathway of class II MHC molecules and their acquisition of antigenic peptides is illustrated as described in the text. The residence sites and specialized vacuoles inhabited following infection by *Chlamydia trachomatis* (Chlamydia, red), *Mycobacterium tuberculosis* (M. tb., blue) and *Coxiella burnetii* (Coxiella, green) are shown.

uolar ATPase responsible for endosome acidification could also play a role in Nef's function at the plasma membrane (78).

The binding of peptides by class I molecules is important for activation of CTL. It is also important for inhibition of an NK-cell response. NK cells bear inhibitory receptors that block NK activity only when they are engaged by class I MHC molecules. The complexities of the NK-recognition system are reviewed in the articles by Cosman et al. (49) and Farrell et al. (50), in this volume. NK cells are active in the immune response against viruses which interfere with class I expression because they are cytotoxic against cells that do not express class I molecules. However, several of the viruses described here have mechanisms for avoiding an NK response. The downregulation of human class I molecules by HIV is locus specific (51, 79). Nef causes internalization of HLA-A and B class I molecules but not HLA-C. HLA-C is generally expressed at a lower level than A or B so is less likely to stimulate a vigorous CTL response, while still able to inhibit NK-cell activity by engaging inhibitory receptors. Human CMV expresses a protein, UL18, which resembles a class I α-chain and binds host cell β_2m (80). This protein interacts with an inhibitory receptor on NK cells from the leukocyte immunoglobulin-like receptor family (49) and may thereby protect CMV-infected cells from NK activity. Murine CMV, but not human CMV, expresses a molecule that interacts with class I on the cell surface, possibly blocking its T-cell or NK-cell recognition (48, 50). Murine CMV also expresses a class I homolog which inhibits NK activity, with little sequence homology to UL18 (50), again suggesting convergent evolution towards similar immune evasion strategies for different species.

Antigen presentation by class II MHC molecules

Class II MHC molecules are composed of an α-chain and β-chain, each of which is encoded in the MHC. In humans, there are three functional class II loci, *HLA-DR*, *DQ* and *DP*, each locus encoding at least one α-chain and one β-chain, with minimal cross-locus pairing. These gene products are co-dominantly expressed, but cell surface levels of HLA-DR exceed DQ and DP levels by at least tenfold (81). The intracellular transport pathway of class II molecules, following their assembly in the ER, diverts them to the endocytic pathway, where they bind peptides derived from extracellular proteins for presentation to helper T cells (Fig. 3) (see review by Pieters (82) for original references).

In the ER, three class II α/β heterodimers bind a trimer of invariant chain, a type II glycoprotein, encoded outside the

MHC. The chaperone calnexin aids in the assembly of this non-americ complex. Due to interaction between ER retention and endosomal sorting signals in the cytoplasmic domain of the invariant chain, the class II-invariant chain complex is retained in the ER, until fully assembled, and then exported to the TGN, where it is sorted to the endocytic pathway. This sorting, although dependent on a dileucine sorting motif, is clathrin independent, and may be mediated by the AP3 adaptor, which recognizes such sorting sequences and can function independently of clathrin (83). Detection of the invariant chain–class II complex in early endosomes suggests that this compartment may be the target for sorting from the TGN. However, many invariant chain–class II complexes visit the cell surface, either by evading initial sorting to early endosomes or by being released to the cell surface along the recycling pathway from early endosomes. These surface invariant chain–class II complexes are recaptured into the endocytic pathway by clathrin-coated vesicles at the plasma membrane (83).

As the invariant chain–class II complex is transported further along the endocytic pathway, the invariant chain is gradually degraded by endosomal proteases (84). In most cell types, cleavage by cathepsin S is required to generate an invariant chain-derived peptide called CLIP, which occupies the class II peptide binding site and maintains the class II molecule in a peptide-receptive state. CLIP–class II complexes accumulate in a prelysosomal compartment, the class II-loading compartment. Here, CLIP is removed by interaction of the complex with the HLA-DM molecule (H2-M in mouse). DM stabilizes the empty form of class II molecules and promotes the exchange of an exogenous peptide for CLIP. Class II molecule–DM interaction is regulated in some cell types by the HLA-DO molecule (function reviewed in (85)), which also localizes to the class II loading compartment (86). Class II peptide loading may occur in an assembly complex which contains DM, DO and the tetraspan molecules CD82 and CD63, analogous to the class I molecule assembly/loading complex (85). Once an exogenous peptide is bound tightly by class II molecules, the occupied class II molecule acquires a stable conformation and, because of dissociation from the invariant chain, the peptide-class II complex is released to the cell surface. Molecular stability is detectable biochemically by resistance to dissociation of the α and β-chains by SDS at room temperature. CLIP-loaded or empty class II molecules dissociate under these conditions.

HLA-DM is a non-polymorphic molecule encoded in the MHC, composed of an α and β-chain with primary sequence resemblance to both class I and class II molecules (reviewed in (87)). DM is concentrated in late endosomal compartments, in which peptide loading occurs. Its localization is dependent on a tyrosine-containing sorting motif in its cytoplasmic domain, which is recognized and sorted by clathrin-coated vesicles from the TGN directly to the endocytic pathway, in a process that is probably dependent on the AP1 adaptor molecule (83). It is likely that this sorting targets DM to a compartment that is deeper in the endocytic pathway than the compartment initially accessed by the invariant chain–class II complex. Some HLA-DM molecules do reach the cell surface after their initial targeting to the endocytic pathway. Such molecules are recaptured in clathrin-coated vesicles at the plasma membrane and shuttled into the endocytic pathway once more. It is possible that co-transport of DM and class II molecules to the cell surface is a cellular mechanism to promote their dissociation at neutral pH, though there is no direct evidence for such a mechanism. Association of DM with class II molecules is indeed favored only at the low pH of the class II-loading compartment (87). Thus the DM recapture pathway could serve to target DM back to the loading compartment, leaving the loaded class II molecule on the cell surface.

The molecular events involved in peptide loading of class II molecules are fundamentally the same in B cells, macrophages and dendritic cells. There are cell type differences in loading compartment morphology and characteristics (88). There are also cell-specific pathways for regulating the rate of class II synthesis and export and endocytic activity. For example, immature dendritic cells in the periphery have most of their class II molecules concentrated in the endocytic pathway and are endocytically active. In contrast, mature cells which are migrating to lymph nodes from peripheral sites of infection have already had their class II molecules loaded with antigen, and upregulate surface expression of these molecules, while reducing further class II molecule synthesis and their overall endocytic activity. There is some evidence that this regulation of class II traffic during dendritic cell maturation is influenced by regulation of cathepsin S activity by cystatin C, an endogenous protease inhibitor which undergoes intracellular redistribution during dendritic cell maturation (89).

Antigen can reach the class II-loading compartment either by phagocytosis or endocytosis. A discussion of the cell biology of class II-loading compartments in phagocytic cells can be found in the review by Ramachandra et al. (90) in this volume. Dendritic cells and a subset of macrophages are also capable of macropinocytosis, a process that delivers engulfed particles, including some bacteria, to the cytoplasmic compartment of cells. This is believed to be a function of disintegration and/or permeability of the membrane of a macropinocytotic vesicle. This pathway can therefore deliver extracellular antigens to the class I presentation pathway (91).

Antigens generated in the endocytic pathway can be presented to T cells by CD1 molecules as well as class II MHC molecules (reviewed in (92)). CD1 molecules are non-classical class I molecules whose α-chain is encoded outside the MHC. In humans there are five CD1 loci (a–e), of which four are known to be expressed. In mouse only the CD1d equivalent is expressed. CD1 α-chains are non-polymorphic and associate with β_2m. In mouse CD1d, the site where a peptide would bind to classical class I molecules is deeper and more hydrophobic, and could accommodate a lipid or glycolipid. This correlates with the known ability of human CD1b to present glycolipid antigen derived from mycobacteria to responding T cells (93). Within a cell, CD1b localizes to intracellular compartments with the same characteristics as class II loading compartments. This localization is dependent on a tyrosine-containing signal in the CD1b cytoplasmic domain that is homologous to the sorting signal in the DM β-chain (92). Endosomal acidification is required for antigen presentation by CD1b, indicating that this class I-like molecule is indeed acquiring bacterial antigens degraded in the class II presentation pathway (93). Thus both class II molecules and CD1b molecules are dependent upon the endocytic pathway for acquiring antigen. Pathogen disruption of the endocytic pathway can therefore affect the immune response to antigen presented by either of these molecules.

Effects of extracellular bacteria on class II antigen presentation

Bacteria which can multiply extracellularly can mediate effects on class II antigen presentation by secretion of toxic or modulatory molecules. Both inhibitory and stimulatory effects on the class II presentation pathway can be mediated by cholera toxin and heat-labile enterotoxin of *Escherichia coli*, depending on the cell type interacting with the toxin. In addition, bacterial DNA containing unmethylated CpG dinucleotide motifs can have both inhibitory and stimulatory effects on professional antigen-presenting cells. These effects are reviewed by Ramachandra et al. (90) in this issue. The superantigen activity of several bacterial toxins is well characterized and is reviewed by Lavoie et al. in this issue (94). The bacterial superantigens, which bind to both class II molecules and non-cognate TCRs, cause hyperstimulation of T cells, creating havoc in the immune system.

Also in this issue, Lee & Schneewind (95) describe the immunological effects of *Yersinia* and *Salmonella* which use Type III secretion machines to inject bacterial proteins into eukaryotic cells. *Salmonella* use these gene products to alter phagosome function, so that they can multiply in phagosomes. *Yersinia* use these gene products to inactivate macrophages with which they come into contact, disrupting phagocytosis. It is likely that *Salmonella* escape degradation by altering the phagolysosome environment and thus escape antigen presentation by class II molecules. *Yersinia* seem to escape antigen presentation by class II simply by avoiding internalization by the macrophage. Thus, both bacterial species have effects on the class II presentation during infection. Another bacterium, *Helicobacter pylori* secretes a toxin, vac A, which has a direct effect on membrane traffic in the endocytic pathway (96). Vac A disrupts the late endocytic pathway by inducing the formation of late endosome-like vacuoles, a process dependent on the GTPase rab 7. A direct test of Vac A effects on antigen-presenting cells demonstrated that it can block the class II presentation pathway. This immune evasion activity may contribute to the ability of *H. pylori* to establish chronic gut infections leading to ulceration and stomach cancer.

Obligate intracellular pathogens' effects on the class II presentation pathway

Obligate intracellular pathogens multiply in the endocytic pathway of an infected cell and intimately interact with the pathway, using at least three distinct strategies (Fig. 3) (97). One type of organism, represented by *Coxiella burnetii*, resides in lysosome-like compartments and appears to tolerate the acidic and degradative conditions of those compartments (98). A second type of organism, represented by *Mycobacterium tuberculosis*, prevents the endocytic compartment (phagosome) in which it resides from maturing into a degradative compartment. This lack of maturation is characterized by lack of phagosome fusion with existing cellular lysosomes (99) and by lack of phagosome acidification due to vacuolar exclusion of the vesicular proton ATPase (100). Acidification is also believed to be inhibited by mycobacterial products such as ammonia and sulfatides (101). A third type of organism, represented by *Chlamydia trachomatis*, is internalized by cells into a membrane-bound compartment, from which all host membrane markers are removed, rendering it isolated from other host membrane compartments (97). In the case of *C. trachomatis*, the membrane of this so-called privileged vacuole and the bacterial membranes inside the vacuole incorporate sphingomyelin from the host cell TGN but seemingly exclude TGN protein markers (102). The cellular mechanisms by which these three organisms are intially internalized into the host cell are currently under investigation by a number of laboratories, as are the mechanisms by which they interact with the endocytic pathway.

Here we are concerned with how the presence of these pathogens, multiplying in three distinct types of endocytic compartments, can affect the class II MHC antigen presentation pathway. All three of the organisms cited above establish chronic infections, suggesting an ability to evade a vigorous host immune response. Some of this evasion may simply be due to physical sequestration of the bacteria, but part of the mechanism is likely to be attributable to the pathogen's ability to disrupt antigen presentation. C. *burnetii* causes Q fever in infected animals and humans (103). In humans, acute Q fever presents as an atypical pneumonia or a prolonged flu and, in some cases, symptoms relapse years after the initial infection. Despite a vigorous antibody response, the bacteria may not be cleared. Persistence of the bacteria may result in chronic Q fever, which is characterized by inflammatory diseases, such as endocarditis, hepatitis, arthritis and renal failure due to circulating immune complexes. The chronicity of M. *tuberculosis* infection is well characterized, consisting of long periods of latency in which the bacteria survive in alveolar macrophages (104, 105). Latency of infection may be lifelong or lead to active disease in cases where the cellular immunity is compromised. In such conditions M. *tuberculosis* proliferates and produces a pulmonary tuberculosis characterized by granulomatous inflammation, which often results in extensive fibrosis and tissue damage. Tuberculosis remains the most frequent cause of death from an infectious agent. C. *trachomatis* infection is a common sexually transmitted disease and the leading cause of infectious blindness (106, 107). Frequently, C. *trachomatis* infections are asymptomatic, so that the bacterium goes undetected and untreated. In men, untreated chronic infections lead to inflammation in the genital tract and sometimes result in reactive arthritis in peripheral joints and ultimately infertility. Long-term infections in women also result in infertility and perhaps cervical dysplasias. These complications suggest that the immune system responds to the *Chlamydia* but is not effective in eliminating it from the body.

In our laboratory, we have studied the effects of infection by C. *burnetii* and C. *trachomatis* on the class II MHC maturation pathway in γIFN-treated HeLa cells. For C. *trachomatis*, HeLa represents a natural cellular host, since the bacteria infect genital epithelial cells, the tissue from which HeLa cells were derived, in addition to infecting macrophages. γIFN is present during C. *trachomatis* infection, making it feasible that infected epithelial tissue would be expressing class II MHC molecules (11, 108). The natural host cells for C. *burnetii* are macrophages, so our studies of γIFN-treated HeLa cells are only a model system. However, we believe the results are generalizable, since the phenotype of class II MHC disruption caused by swollen lysosomes containing C. *burnetii* is seen also in B cells with swollen lysosomes caused by the genetic disease, Chédiak–Higashi syndrome (CHS) (109), as described below.

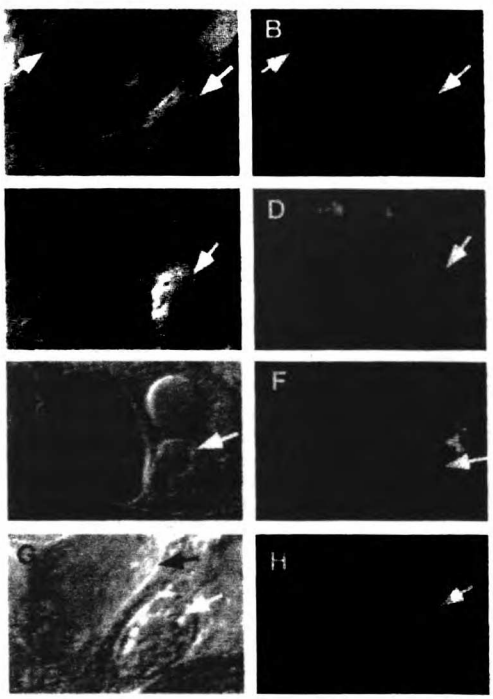


Fig. 4. Localization of HLA-DR and HLA-DM to *Coxiella* but not *Chlamydia* vacuoles. HeLa cells were treated with γIFN for 24 h, then infected with *Coxiella burnetii* (A–D) or *Chlamydia trachomatis* serovar L2 (E-H) for 24 h. Infected cells were fixed and stained for HLA-DR (B, F) or HLA-DM (D, H) with anti-DR mAb L243 or rabbit anti-DM. Nomarski imaging (A, C, E, G) distinguished the bacterial vacuoles (white arrows) from the HeLa nuclei (black arrows).
[Reprinted with permission of Lem L, Riethof DA, Scidmore M, Griffiths G, Hackstadt T, Brodsky FM. Enhanced interaction of HLA-DM with HLA-DR in enlarged vacuoles of hereditary and infectious lysosomal diseases. J Immunol 1999;162:523–532. Copyright 1999. The American Association of Immunologists.]

Our initial studies examined the localization of class II MHC molecules and HLA-DM relative to parasitic vacuoles in infected, γIFN-treated HeLa cells (Fig. 4) (109). The vacuoles established by C. *burnetii* were labeled for both HLA-DR and HLA-DM. This correlates with the fact that all previous characterization has suggested these vacuoles are lysosome like, similar to the properties of the class II-loading compartment. HLA-DR and DM did not localize to chlamydial vacuoles, consistent with previous reports of endocytic marker exclusion.

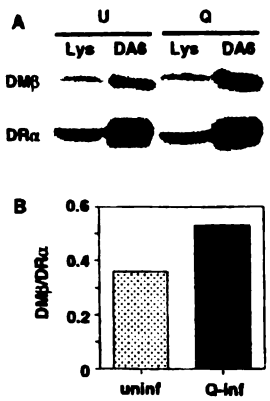


Fig. 5. Increased DM/DR complexes in Coxiella-infected HeLa cells. A. DM/DR complexes were immunoprecipitated with the anti-DRα mAb DA6.147 from lysates of γIFN-treated HeLa cells which were either mock-treated (uninfected, U samples) or infected with Coxiella (Q samples). Lysates were prepared in 1% digitonin at pH 5.0. Immunoprecipitates (DA6) were divided in half and each half was analyzed by SDS-PAGE, adjacent to a sample of whole cell lysate (Lys), for separate immunoblotting with anti-DMβ serum (DMBS1) or anti-DRα mAb (DA6.147), after transfer to nitrocellulose. Data are reproduced from Lem et al. (109). B. DMβ and DRα signals from the DA6 immunoprecipitate were quantitated using NIH image 1.61, and the ratios of the two were graphed. The experiment shown is representative of results from four out of five independent infections of efficiency >65%.
[Reprinted with permission of Lem L, Riethof DA, Scidmore M, Griffiths G, Hackstadt T, Brodsky FM. Enhanced interaction of HLA-DM with HLA-DR in enlarged vacuoles of hereditary and infectious lysosomal diseases. J Immunol 1999;162:523–532. Copyright 1999. The American Association of Immunologists.]

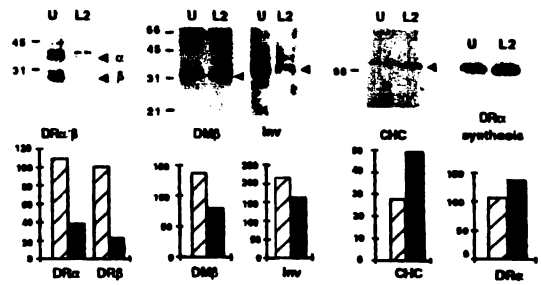


Fig. 6. Reduction of HLA-DR, DM and invariant chain in C. trachomatis-infected γIFN-treated HeLa cells. HeLa 229 cells were treated with γIFN and infected with the L2 strain of C. trachomatis (L2, solid bars) or mock infected (U (uninfected), hatched bars) for 21 h. Cultures were lysed in 1% NP40 and equivalent levels of lysate were analyzed by SDS-PAGE. After transfer to nitrocellulose, samples were immunoblotted to detect DR α and β-chains (mixture of antibodies), DMβ-chain, invariant chain and clathrin heavy chain (CHC). The relevant bands were quantitated by densitometry and are graphed below the blots. A second set of infected and uninfected γIFN-treated HeLa cells were pulse labeled for 1 h with ^{35}S-methionine. These samples were lysed in 1% NP40 and DR α-chain was immunoprecipitated from the lysate, analyzed by SDS-PAGE and detected by autoradiography (DR α synthesis). Densitometry analysis of the autoradiograph is shown below it.

Interestingly, establishment of both types of vacuoles were observed to have effects on class II MHC molecules.

C. burnetii-infected cells were characterized by swollen lysosomes, in which DR and DM accumulated. By immunoprecipitation, we established that the altered morphology of the class II-loading compartment resulted in an enhanced association between DR and DM molecules (Fig. 5) (109). This increased contact suggested a functional phenotype, since the role of DM is to stabilize empty class II molecules and thereby enhance the exchange of the CLIP peptide for an antigenic peptide. Indeed, in a subset of infections, we were able to detect an increase in unstable HLA-DR molecules. In a parallel study we examined the phenotype of B-lymphoblastoid cell lines from patients with CHS (109). This inherited immunodeficiency disease results from a defect in a gene involved in lysosome fission, leading to swollen lysosomes that resemble the intracellular vacuoles formed by *C. burnetii*. In CHS B cells, we also detected an enhanced association betweeen DR and DM. The consequences of this association were more easily analyzed in these cells than in *C. burnetii*-infected cells. We found that the DR expressed on the CHS B cells had a lower level of CLIP associated with it (109). In a complementary study, Faigle et al. (110) reported that CHS B-lymphoblastoid cells displayed a DR-associated peptide repertoire that differed from that displayed by wild-type B cells having the same DR alleles. Furthermore, the maturation rate of DR molecules was slower than normal in CHS B cells (110). These properties of altered DR peptide presentation can be explained by the mechanism of enhanced contact between DR and DM in the swollen lysosomes of both CHS B cells and *C. burnetii*-infected cells (109). These findings imply that *C. burnetii* infection in the endocytic pathway is likely to alter the peptide presentation properties of class II MHC molecules and thereby dampen their immune recognition, favoring chronic infection.

Although the class II maturation pathway does not directly intersect the *C. trachomatis* vacuole, formation of this vacuole

appears to have a profound effect on expression of class II MHC molecules (111, 112). *C. trachomatis* is internalized into cells in the form of elementary bodies (EBs), the infectious form of the bacteria, and, once inside the cell, they differentiate into reticulate bodies, which divide into progeny. As bacterial maturation takes place, individual vesicles that have internalized the EBs fuse, forming one large perinuclear vacuole where multiplying organisms accumulate (113). Vacuole fusion is detected within 2 h of infection, and large vacuoles are readily visible 18–20 h after infection (Fig. 4). γIFN-treated HeLa cells were infected with *C. trachomatis* and, after establishment of large vacuoles, we analyzed the state of molecules in the class II pathway. Infected cells had significantly reduced steady state levels of HLA-DR, DM and invariant chain, compared to uninfected γIFN-treated HeLa cells (Fig. 6).

Ongoing studies in the laboratory are elucidating the mechanism of reduction of molecules in the class II presentation pathway following *C. trachomatis* infection. Initial analysis has revealed that reduction is not due to inhibition of γIFN induction. The γIFN-signaling pathway is functional and the biosynthetic rate of DR α-chain is similar in both infected and uninfected γIFN-treated HeLa cells (Fig. 6). The reduction in class II and associated molecules possibly reflects an effect restricted to molecules found in endocytic vesicles, since the levels of class I MHC are unaffected (not shown), nor is the level of the cytoplasmic protein clathrin reduced (Fig. 6). Preliminary pulse-chase analysis suggests that the degradation of DR molecules is enhanced, an effect that is detectable within 2 h of biosynthesis, suggesting that the chlamydial vacuole may affect class II molecules at the stage of their sorting in the TGN.

Dramatic changes in lipid flow accompany chlamydial vacuole maturation (102). Uninfected cells loaded with NBD-ceramide produce fluoresent sphingomyelin which accumulates exclusively in the Golgi apparatus and the TGN (114). If labeled cells are infected with *C. trachomatis*, fluorescent sphingomyelin becomes primarily localized to the chlamydial vacuole and chlamydial outer membranes within 2 h (102). Thus the formation of the vacuole has a profound effect on lipid traffic from the TGN. The TGN is a critical site for sorting both class II MHC molecules associated with invariant chain and HLA-DM to their correct destinations in the endocytic pathway. In addition, it is the site for sorting and targeting lysosomal proteases to the endocytic pathway. Chlamydial redirection of lipid traffic out of the TGN may be responsible for missorting molecules to the endocytic pathway, resulting in enhanced degradation of molecules targeted to that pathway.

Infection of macrophages by *M. tuberculosis* has been shown to impair antigen presentation to $CD4^+$ T cells. It is possible that this defect in antigen presentation is a result of downregulation of class II molecule expression on the cell surface. However, while downregulation has been observed in several experimental systems (115–117), other analyses report no effect on the expression of surface class II molecules (118). In a recent study of *M. tuberculosis* infection of the THP-1 monocyte cell line (γIFN-treated), it was demonstrated that class II molecules in infected cells never acquired SDS stability nor localized to late endocytic compartments, suggesting that their transport to a compartment in which DM could remove CLIP was impaired (117). This phenotype could be explained by inhibition of endosomal acidification caused by the intracellular presence of *M. tuberculosis* (100, 119). Downregulation of CD1b molecules on *M. tuberculosis*-infected macrophages has been observed, but it was found to be due to a decrease of CD1b-mRNA, suggesting impaired γIFN induction of CD1b in infected cells (120). This correlates with the observation of Ernst and colleagues (J. Ernst, personal communication) that infection of human macrophages by *M. tuberculosis* blocks macrophage activation due to an inhibitory effect of the bacteria on γIFN signaling. Thus, *M. tuberculosis* may achieve immune evasion by multiple strategies which prevent antigen presentation by class II MHC molecules and CD1b molecules, as well as by inhibition of macrophage activation.

The parasitic protozoan *Leishmania amazonensis*, which multiplies intracellularly, has both a passive and active mechanism for evading presentation of its antigens by class II MHC molecules. These pathogens reside in vacuoles of phagocytic cells, such as macrophages, but the parasitophorous vacuoles never fully acidify into phagolysosomes, suggesting a potential inhibition of antigen degradation and class II molecule maturation (119). In addition, the *Leishmania* inside these parasitophorous vacuoles internalize host class II molecules and degrade them (121).

Viral effects on the class II presentation pathway

A Th1 helper response stimulates the development of CTL and an antiviral inflammatory response, as well as the production of antiviral antibodies. Therefore, it could be in the interest of a virus to reduce antigen presentation by class II MHC molecules to evade an immune response. Several viruses have evolved such strategies. HIV Nef, Vpu and gp120 proteins cause downregulation of CD4 from the infected cell surface, preventing T-cell response to class II antigen presentation (52, 77). Nef also interacts with a subunit of the proton pump responsible for endosome acidification, so has the potential to affect class II MHC processing by disruption of function in the endocytic

pathway (78). The E5 protein of HPV and bovine papillomavirus (BPV) interacts with another proton pump subunit, indicating a common target in the endocytic pathway for viral gene products (122) that could influence antigen presentation by class II molecules. The E6 gene product of BPV binds the AP1 adaptor protein associated with clathrin-coated vesicles in the TGN (123). This interaction with a component of the class II trafficking pathway might also disrupt class II molecule transport or maturation.

Human CMV inhibits γIFN upregulation of class II expression on infected endothelial cells and fibroblasts by disruption of the γIFN-signaling pathway (124). HSV reduces expression of class II molecules on infected neuronal cells, possibly also through an inhibitory effect on induction (125). Measles virus inhibits γIFN upregulation of class II on infected THP-1 cells in culture (126). However, measles virus seems to stimulate class II molecule expression on infected peripheral blood monocytes, while altering the source of antigen presented by these molecules. Measles-infected monocytes exhibit reduced presentation of extracellular exogenous antigens. Lastly, viral superantigen can be considered a mechanism for altering the class II antigen presentation pathway. The viral superantigens, which have been characterized for murine retroviruses, interact with CD4-expressing T cells and induce their deletion by hyperstimulation, a process reviewed by Acha-Orbea et al. in this issue (127).

Concluding remarks: different microbial evasion strategies for different presentation pathways

Consideration of the numerous strategies employed by pathogens to disrupt antigen presentation by MHC molecules reveals a predictable pattern of immune subversion. Viruses are the most dependent on host cells for their persistence and are sensitive to cellular immune attack against class I molecules as well as humoral immune attack. Viruses also co-evolve with the host to maintain their parasitic status and seem to have invested the most in developing host-specific immune evasion strategies. Viral strategies include direct sabotage of class I MHC antigen presentation by inhibition of class I assembly and transport and by enhancing class I downregulation, combined with strategies to avoid NK-cell recognition, as well as epitope mutation to escape recognition. Many viruses have more than one strategy for interfering with antigen presentation and express viral gene products that target additional interactions between immune effector cells. Obligate intracellular bacteria are almost as dependent on host cells for survival as viruses, but those that inhabit intracellular vesicles have the advantage of physical sequestration from class I MHC presentation and thereby reduce stimulation of cell-mediated attack. Therefore, sophisticated mechanisms of interfering with class I MHC presentation are not essential to survival of these organisms. Their disruption of the immune response appears to result from their residence in the endocytic pathway and the consequent effects on class II MHC transport. This latter strategy is certainly advantageous to the pathogen, leading to reduction of Th1 responses and macrophage activation. Lastly, bacteria that can multiply extracellularly must primarily concern themselves with the class II MHC-mediated response. As they have less means to affect cell function directly, these organisms have evolved secreted products that alter class II antigen presentation. The immune evasion strategies of extracellular pathogens which target MHC-dependent pathways are not as sophisticated as those of intracellular organisms, possibly because the extracellular pathogens are less threatened by MHC-induced immune responses. Thus, from the pathogen perspective, the crime fits the punishment.

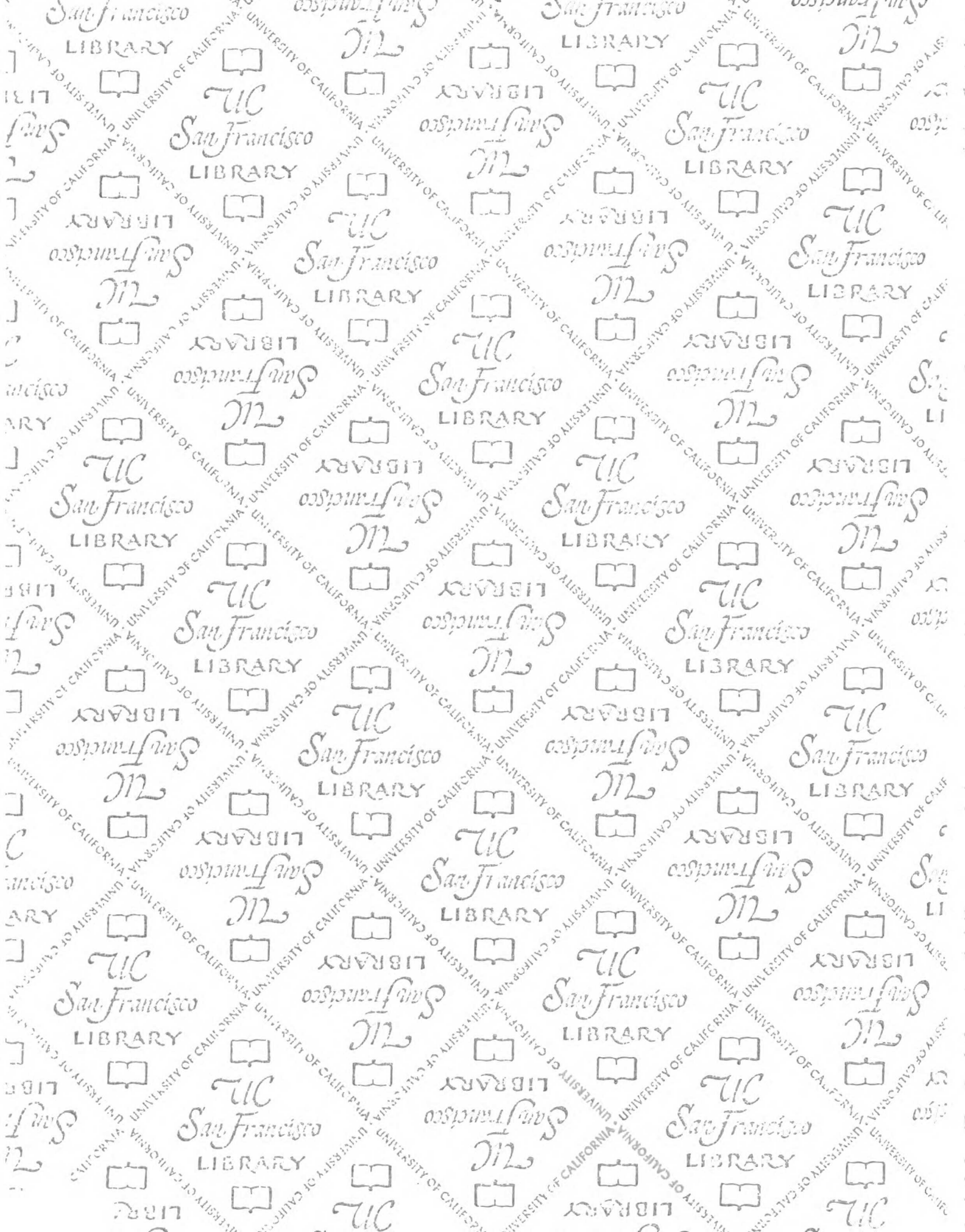
References

1. Madden DR. The three-dimensional structure of peptide-MHC complexes. Annu Rev Immunol 1995;12:181–208.
2. Bodmer JG, et al. Nomenclature for factors of the HLA system, 1996. Tissue Antigens 1996;49:297–321.
3. Van Bleek GM, Nathenson SG. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. Nature 1990;348:213–216.
4. Wodarz D, Nowak MA. Evolutionary dynamics of HIV-induced subversion of the immune response. Immunol Rev 1999;168:75–89.
5. De Campos-Lima PO, et al. HLA-A11 epitope loss isolates of Epstein-Barr virus from a highly A11$^+$ population. Science 1993;260:98–100.
6. Wang H, Eckels DD. Mutations in immunodominant T-cell epitopes derived from the non-structural 3 (NS3) protein of hepatitis C virus have the potential for generating escape variants that may have important consequences for T-cell recognition. J Immunol 1999;162:4177–4183.
7. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 1995;13:251–276.
8. Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. Annu Rev Immunol 1995;13:151–177.
9. Su H, Caldwell HD. CD4$^+$ T cells play a significant role in adoptive immunity to *Chlamydia trachomatis* infection of the mouse genital tract. Infect Immun 1995;63 3302–3308.

10. Perry LL, Feilzer K, Caldwell HD. Immunity to Chlamydia trachomatis is mediated by T helper 1 cells through IFN-γ-dependent and -independent pathways. J Immunol 1997;158:3344–3352.
11. Igietseme JU, et al. Route of infection that induces a high intensity of γ interferon-secreting T cells in the genital tract produces optimal protection against Chlamydia trachomatis infection in mice. Infect Immun 1998;66:4030–4035.
12. Ortiz L, et al. Chlamydia trachomatis major outer membrane protein (MOMP) epitopes that activate HLA class II-restricted T cells from infected humans. J Immunol 1996;157:4554–4567.
13. Lewinsohn DM, Alderson MR, Briden AL, Riddell SR, Reed SG, Grabstein KH. Characterization of human CD8+ T cells reactive with Mycobacterium tuberculosis-infected antigen presenting cells. J Exp Med 1998;187:1633–1640.
14. Canaday DH, Ziebold C, Noss EH, Chervenak KA, Harding CV, Boom WH. Activation of human CD8+ αβ TCR+ cells by Mycobacterium tuberculosis via an alternate class I MHC antigen-processing pathway. J Immunol 1999;162:372–379.
15. McCutcheon JA, Gumperz J, Smith KD, Lutz CT, Parham P. Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. J Exp Med 1995;181:2085–2095.
16. Krause K-H, Michalak M. Calreticulin. Cell 1997;88:439–443.
17. Bergeron JJ, Brenner MJ, Thomas DY, Williams DB. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. Trends Biochem Sci 1994;19:124–128.
18. Srivastava SP, Fuchs JA, Holtzman JL. The reported cDNA sequence for phospholipase Cα encodes protein disulfide isomerase, isozyme Q-2 and not phospholipase-C. Biochem Biophys Res Commun 1993;193:971–978.
19. Urade R, Nasu M, Moriyama T, Wada K, Kito M. Protein degradation by phosphoinositide-specific phospholipase C-α family from rat liver endoplasmic reticulum. J Biol Chem 1992;267:15152–15159.
20. Oliver JD, van der Wal FJ, Bulleid NJ, High S. Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. Science 1997;275:86–88.
21. Sadasivan B, Lehner PJ, Ortmann B, Spies T, Cresswell P. Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. Immunity 1996;5:103–114.
22. Hughes EA, Cresswell P. The thiol oxidoreductase ERp57 is a component of the MHC class I peptide-loading complex. Curr Biol 1998;8:709–712.
23. Lehner PJ, Trowsdale J. Antigen presentation: coming out gracefully. Curr Biol 1998;8:R605–R608.
24. Nössner E, Parham P. Species specific difference in chaperone interaction of human and mouse major histocompatibility complex class I molecules. J Exp Med 1995;181:327–337.
25. Suh W-K, Mitchell EK, Yang Y, Peterson PA, Waneck GL, Williams DB. MHC class I molecules form ternary complexes with calnexin and TAP and undergo peptide-regulated interaction with TAP via their extracellular domains. J Exp Med 1996;184:337–348.
26. Carreno BM, Solheim JC, Harris M, Stroynowski I, Connolly JM, Hansen TH. TAP associates with a unique class I conformation, whereas calnexin associates with multiple forms in mouse and man. J Immunol 1995;155:4726–4733.
27. Lindquist JA, Jensen ON, Mann M, Hämmerling GJ. ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. EMBO J 1998;17:2186–2195.
28. Morrice NA, Powis SJ. A role for the thiol-dependent reductase ERp57 in the assembly of MHC class I molecules. Curr Biol 1998;8:713–716.
29. Solheim JC, Harris MR, Kindle CS, Hansen TH. Prominence of β_2-microglobulin, class I heavy chain conformation, and tapasin in the interactions of class I heavy chain with calreticulin and transporter associated with antigen processing. J Immunol 1997;158:2236–2241.
30. Früh K, Gruhler A, Krishna RM, Schoenhals GJ. A comparison of viral immune escape strategies targeting the MHC class I assembly pathway. Immunol Rev 1999;168:157–166.
31. Lewis JW, Elliott T. Evidence for successive peptide binding and quality control stages during MHC class I assembly. Curr Biol 1998;8:717–720.
32. Androlewicz MJ, Anderson KS, Cresswell P. Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. Proc Natl Acad Sci USA 1993;90:9130–9134.
33. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol 1992;8:67–113.
34. Townsend A, Trowsdale J. The transporters associated with antigen presentation. Semin Cell Biol 1993;4:53–61.
35. Powis SJ, et al. Effect of polymorphism of an MHC-linked transporter on the peptides assembled in a class I molecule. Nature 1992;357:211–215.
36. Driscoll J, Brown M, Finley D, Monaco JJ. MHC-linked LMP gene products specifically alter peptidase activities of the proteasome. Nature 1993;365:262–264.
37. Gaczynska M, Rock K, Goldberg A. γ-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature 1993;365:264–267.
38. Van Kaer L, et al. Altered peptidase and viral-specific T cell response in LMP2 mutant mice. Immunity 1994;1:533–541.
39. Fehling HJ, et al. MHC class I expression in mice lacking the proteasome subunit LMP-7. Science 1994;265:1234–1237.
40. Vinitsky A, Anton LC, Snyder HL, Orlowski M, Bennink JR, Yewdell JW. The generation of MHC class I-associated peptides is only partially inhibited by proteasome inhibitors. J Immunol 1997;159:554–564.
41. Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL. A proteolytic system that compensates for loss of proteasome function. Nature 1998;392:618–622.
42. Wei ML, Cresswell P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. Nature 1992;356:443–446.
43. Henderson RA, et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 1992;255:1264–1266.
44. Braud VM, Allan DSJ, Wilson D, McMichael AJ. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. Curr Biol 1997;8:1–10.
45. Elliott T, Willis A, Cerundolo V, Townsend A. Processing of major histocompatibility class I-restricted antigens in the endoplasmic reticulum. J Exp Med 1995;181:1481–1491.
46. Snyder HL, Yewdell JW, Bennink JR. Trimming of antigenic peptides in an early secretory compartment. J Exp Med 1994;180:2389–2394.
47. Roelse J, Gromme M, Momburg F, Hammerling G, Neefjes J. Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. J Exp Med 1994;180:1591–1597.
48. Hengel H, et al. Cytomegaloviral control of MHC class I function in the mouse. Immunol Rev 1999;168:167–176.
49. Cosman D, Fanger N, Borges L. Human cytomegalovirus, MHC class I and inhibitory signaling receptors: more questions than answers. Immunol Rev 1999;168:177–185.
50. Farrell HE, Degli-Esposti MA, Davis-Poynter NJ. Cytomegalovirus evasion of natural killer cell responses. Immunol Rev 1999;168:187–197.

51. Collins KL, Baltimore D. HIV's evasion of the cellular immune response. Immunol Rev 1999;168:65–74.
52. Piguet V, Schwartz O, Le Gall S, Trono D. The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. Immunol Rev 1999;168:51–63.
53. Mahr JA, Gooding LR. Immune evasion by adenoviruses. Immunol Rev 1999;168:121–130.
54. Cohen OJ, Kinter A, Fauci AS. Host factors in the pathogenesis of HIV disease. Immunol Rev 1997;159:31–48.
55. Bennett EM, Yewdell JW, Bennink JR, Brodsky FM. Cutting edge: adenovirus has two mechanisms for affecting class I MHC expression. J Immunol 1999;162:5049–5052.
56. Ploegh HL. Viral strategies of immune evasion. Science 1998;280:248–253.
57. Wiertz EJHJ, et al. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 1996;384:432–438.
58. Wiertz EJHJ, Jones TR, Sun L, Bogyo M, Geuze HJ, Ploegh HL. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell 1996;84:169–119.
59. Hughes EA, Hammond C, Cresswell P. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. Proc Natl Acad Sci USA 1997;94:1896–1901.
60. Kerkau T, et al. The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. J Exp Med 1997;185:1295–1305.
61. Levitskaya J, Sharipo A, Leonchiks A, Ciechanover A, Masucci MG. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proc Natl Acad Sci USA 1997;94:12616–12621.
62. Gilbert MJ, Riddell SR, Plachter B, Greenberg PD. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. Nature 1996;383:720–722.
63. Ahn K, et al. Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus protein ICP47. EMBO J 1996;15:3247–3255.
64. Tomazin R, et al. Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. EMBO J 1996;15:3256–3266.
65. Hengel H, et al. A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter. Immunity 1997;6:623–632.
66. Ahn K, et al. The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. Immunity 1997;6:613–621.
67. Früh K, et al. A viral inhibitor of peptide transporters for antigen presentation. Nature 1995;375:415–418.
68. Burgert H-G, Kvist S. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell 1985;41:987–997.
69. Andersson M, Pääbo S, Nilsson T, Peterson PA. Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. Cell 1985;43:215–222.
70. Jackson M R, Nilsson T, Peterson PA. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J 1990;9:3153–3162.
71. Nilsson T, Jackson M, Peterson PA. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell 1989;58:707–718.
72. Cox JH, Yewdell JW, Eisenlohr LC, Johnson PR, Bennink JR. Antigen presentation requires transport of MHC class I molecules from the endoplasmic reticulum. Science 1990;247:715–718.
73. Beier DC, Cox JH, Vining DR, Cresswell P, Englehard VH. Association of human class I MHC alleles with the adenovirus E3/19K protein. J Immunol 1994;152:3862–3871.
74. Schmid SL. Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem 1997;66:511–548.
75. Foti M, et al. Nef-mediated clathrin-coated pit formation. J Cell Biol 1997;139:37–47.
76. Bresnahan PA, Yonemoto W, Ferrell S, Williams-Herman D, Geleziunas R, Greene WC. A dileucine motif in HIV-1 nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. Curr Biol 1998;8:1235–1238.
77. Oldridge J, Marsh M. Nef - an adaptor adaptor? Trends Cell Biol 1998;8:302–305.
78. Lu X, Yu H, Liu S-H, Brodsky FM, Peterlin BM. Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4. Immunity 1998;8:647–656.
79. Le Gall S, et al. Nef interacts with the μ subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. Immunity 1998;8:483–495.
80. Browne H, Smith G, Beck S, Minson T. A complex between the MHC class I homologue encoded by human cytomegalovirus and β_2-microglobulin. Nature 1990;347:770–772.
81. Robbins PA, Maino VC, Warner NL, Brodsky FM. Activated T cells and monocytes have characteristic patterns of class II antigen expression. J Immunol 1988;141:1281–1287.
82. Pieters J. MHC class II restricted antigen presentation. Curr Opin Immunol 1997;9:89–96.
83. Liu S-H, Marks MS, Brodsky FM. A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. J Cell Biol 1998;140:1023–1037.
84. Chapman HA. Endosomal proteolysis and MHC class II function. Curr Opin Immunol 1998;10:93–102.
85. Hammond C, Denzin LK, Pan M, Griffith JM, Geuze HJ, Cresswell P. The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. J Immunol 1998;161:3282–3291.
86. Liljedahl M, Kuwana T, Pung-Leung WP, Jackson MR, Peterson PA, Karlsson L. HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. EMBO J 1996;15:4817–4824.
87. Kropshofer H, Hämmerling GJ, Vogt AB. How HLA-DM edits the MHC class II peptide repertoire: survival of the fittest? Immunol Today 1997;18:78–82.
88. Mellman I, Turley SJ, Steinman RM. Antigen processing for amateurs and professionals. Trends Cell Biol 1998;8:231–237.
89. Pierre P, Mellman I. Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. Cell 1998;93:1135–1145.
90. Ramachandra L, et al. Pathogen subversion of cellular immunity. Immunol Rev 1999;168:217–239.
91. Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 1997;15:821–850.
92. Porcelli SA, Segelke BW, Sugita M, Wilson IA, Brenner MB. The CD1 family of lipid antigen-presenting molecules. Immunol Today 1998;19:362–368.
93. Ernst WA, et al. Molecular interaction of CD1b with lipoglycan antigens. Immunity 1998;8:331–340.

94. Lavoie PM, Thibodeau J, Erard F, Sékaly R-P. Understanding the mechanism of action of bacterial superantigens from a decade of research. Immunol Rev 1999;168:257–269.
95. Lee VT, Schneewind O. Type III secretion machines and the pathogenesis of enteric infections caused by *Yersinia* and *Salmonella* spp. Immunol Rev 1999;168:241–255.
96. Molinari M, et al. Selective inhibition of Ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. J Exp Med 1998;187:135–140.
97. Hackstadt T, Fischer ER, Scidmore MA, Rockey DD, Heinzen RA. Origins and functions of the chlamydial inclusion. Trends Microbiol 1997;5:288–293.
98. Heinzen RA, Scidmore MA, Rockey DD, Hackstadt T. Differential interactions with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. Infect Immun 1996;64:796–809.
99. Clemens DL, Horwitz MA. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is limited. J Exp Med 1995;181:257–270.
100. Sturgill-Koszycki S, et al. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science 1994;263:678–681.
101. Deretic V, Via LE, Fratti RA, Deretic D. Mycobacterial phagosome maturation, rab proteins, and intracellular trafficking. Electrophoresis 1997;18:2542–2547.
102. Hackstadt T, Scidmore MA, Rockey DD. Lipid metabolism in *Chlamydia trachomatis*-infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. Proc Natl Acad Sci USA 1995;92:4877–4881.
103. Raoult D, Marrie T. Q fever. Clin Infect Dis 1995;20:489–496.
104. Le HQ, Davidson PT. Reactivation and exogenous reinfection: their relative roles in the pathogenesis of tuberculosis. Curr Clin Top Infect Dis 1996;16:260–276.
105. Rook GA, Stanford JL. The Koch phenomenon and the immunopathology of tuberculosis. Curr Top Microbiol Immunol 1996;215:239–262.
106. Terho P. *Chlamydia trachomatis* and clinical genital infections: a general review. Infection 1982;10:S5–S9.
107. Centers for Disease Control. *Chlamydia trachomatis* genital infections–United States 1995. Morbid Mortal Weekly Rep 1997;46:193–198.
108. Ljunggren G, Anderson DJ. Cytokine induced modulation of MHC class I and class II molecules on human cervical epithelial cells. J Reprod Immunol 1998;38:123–138.
109. Lem L, Riethof DA, Scidmore M, Griffiths G, Hackstadt T, Brodsky FM. Enhanced interaction of HLA-DM with HLA-DR in enlarged vacuoles of hereditary and infectious lysosomal diseases. J Immunol 1999;162:523–532.
110. Faigle W, et al. Deficient peptide loading and MHC class II endosomal sorting in a human genetic immunodeficiency disease: the Chédiak-Higashi syndrome. J Cell Biol 1998;141:1121–1134.
111. Rodel J, Groh A, Vogelsang H, Lehmann M, Hartmann M, Straube E. β-Interferon is produced by *Chlamydia trachomatis*-infected fibroblast-like synoviocytes and inhibits γ-interferon-induced HLA-DR expression. Infect Immun 1998;66:4491–4495.
112. Zhong G, Lu H, Hu H. Effect of *Chlamydia trachomatis* infection on MHC class II antigen processing and presentation. In: Stephens RS, ed. Chlamydial Infections. Proceedings of the Ninth International Symposium on Human Chlamydial Infection; 1998 Jun 21–26; Napa (CA). San Francisco (CA): International Chlamydia Symposium; 1998. p. 379–382.
113. Moulder JW. Interaction of chlamydiae and host cells in vitro. Microbiol Rev 1991;55:143–190.
114. Pagano RE. A fluorescent derivative of ceramide: physical properties and use in studying the Golgi apparatus of animal cells. Methods Cell Biol 1989;29:75–85.
115. Gercken J, Pryjma J, Ernst M, Flad HD. Defective antigen presentation by *Mycobacterium tuberculosis*-infected monocytes. Infect Immun 1994;62:3472–3478.
116. Mazzaccaro RJ, et al. Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci USA 1996;93:11786–1191.
117. Hmama Z, Gabathuler R, Jefferies WA, de Jong G, Reiner NE. Attenuation of HLA-DR expression by mononuclear phagocytes infected with *Mycobacterium tuberculosis* is related to intracellular sequestration of immature class II heterodimers. J Immunol 1998;161:4882–4893.
118. Henderson RA, Watkins SC, Flynn JL. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. J Immunol 1997;159:635–643.
119. Russell DG. *Mycobacterium* and *Leishmania*: stowaways in the endosomal network. Trends Cell Biol 1995;5:125–128.
120. Stenger S, Niazi KR, Modlin RL. Down-regulation of CD1 on antigen-presenting cells by infection with *Mycobacterium tuberculosis*. J Immunol 1998;161:3582–3588.
121. De Souza Leao S, Lang T, Prina E, Hellio R, Antoine J-C. Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. J Cell Sci 1995;108:3219–3231.
122. Andresson T, Sparkowski J, Goldstein DJ, Schlegel R. Vacuolar H⁺-ATPase mutants transform cells and define a binding site for the papillomavirus E5 oncoprotein. J Biol Chem 1995;270:6830–6837.
123. Tong X, Boll W, Kirchhausen T, Howley PM. Interaction of the bovine papillomavirus E6 protein with the clathrin adaptor complex AP-1. J Virol 1998;72:476–482.
124. Miller DM, et al. Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. J Exp Med 1998;187:675–683.
125. Lewandowski GA, Lo D, Bloom FE. Interference with major histocompatibility complex class II-restricted antigen presentation in the brain by herpes simplex virus type 1: a possible mechanism of evasion of the immune response. Proc Natl Acad Sci USA 1993;90:2005–2009.
126. Leopardi R, Ilonen J, Mattila L, Salmi AA. Effect of measles virus infection in MHC class II expression and antigen presentation in human monocytes. Cell Immunol 1993;147:388–396.
127. Acha Orbea H, et al. Interplays between mouse mammary tumor virus and the cellular and humoral immune response. Immunol Rev 1999;168:287–303.
128. Abendroth A, Arvin A. Varicella-zoster virus immune evasion. Immunol Rev 1999;168:143–156.
129. Frazer IH, et al. Pathogen subversion of cellular immunity. Immunol Rev 1999;168:131–142.


UC
San Francisco
LIBRARY
UNIVERSITY OF CALIFORNIA