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Form and Function of CD8 in T cell Interactions

Anne Margaret Norment

Submitted in partial fulfillment of the requirements for the Ph.D. degree in the Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco.

Dedication

To Adam Wheeler, for patience, friendship and love.

Preface

I am indepted to many individuals for their personal support and intellectual contributions to this work. My thesis advisor Dan R. Littman aided me in both of these capacities, enthusiastically giving guidance, while encouraging me to think critically and independently. Second I would like to thank my parents Miriam B. Norment and Paul J. Norment for their constant love and pride in my endeavors. I am fortunate to have worked with many fun and generous people, including Ned Landau and Julia Turner. In particular I thank Anne Moriarity for organizing the lab and for friendship, despite my constant abuse of her reagents. I owe special thanks to Art Weiss for advice and perspective.

The main body of this thesis contains publications from **The Journal of Immunology** (chapter 2), **Nature** (chapters 3 and 4), and **The European Molecular Biology Organization** (chapter 5). I respectively thank IRL press Ltd., Macmillan Magazines Ltd., and the American Association of Immunologists for permission to reproduce these papers. I have enjoyed a very productive collaboration with Russel Salter and Peter Parham at Stanford University, which includes the experiments described in chapters 3 and 4.

Finally I would like to thank Paul Dazin for FACS analysis and Chris Turk for providing CD8 peptides.

Form and Function of CD8 in T Cell Interactions

Anne Margaret Norment

CD4 positive ($CD4^+$) T cells have T cell antigen receptors (TCRs) restricted for MHC class II molecules, while $CD8^+$ T cells have TCRs specific for MHC class I molecules. To determine whether human CD8 directly binds to MHC class I molecules, *in vitro* assay systems have been developed using human CD8α expressed in transfected cells. Initially, we characterized a soluble form of human CD8α (sCD8α) which is encoded by an alternatively spliced transcript lacking nucleotide sequences from the transmembrane exon. sCD8α homodimers can be detected in culture supernatants of activated peripheral blood lymphocytes and could potentially be used in purified form to analyze CD8-MHC class I binding.

However, CD8-MHC class I binding might be of low avidity and require multivalent interactions for detection. Therefore as an initial approach to studying CD8-MHC class I interactions, an assay has been developed to measure the adhesion of MHC class I^+ B cell lines to transfected cells over-expressing the human CD8 α-chain. MHC class I^+ B cells specifically bind to $CD8\alpha^+$ CHO cells. To determine the binding site of CD8 on MHC class I molecules, the products of individual MHC class I alleles and point mutants have been analyzed. An $HLA\text{-}A^-B^-$ B cell line was transfected with a panel of MHC class I genomic clones, and the subclones tested for adhesion to $CD8\alpha^+$ CHO cells. Of 19 MHC class I molecules analyzed only HLA-Aw68 did not bind CD8α. HLA-Aw68 differs from HLA-Aw69 which binds CD8α, at 6 polymorphic residues of the α_2-domain, and one conserved residue at position 245 of the α_3-domain. In site-directed mutants of HLA-Aw68 a valine to alanine substitution restores CD8 binding. This suggests that CD8 binds conserved residues of the α_3-domain, supporting a model in which CD8 and the TCR can form a high avidity complex with the same MHC class I molecule.

In humans CD8 had been thought to be composed of homodimers of a single 32 kD α-chain, while rodent CD8α was known to form homodimers and heterodimers with a distinct β-chain polypeptide. To determine

whether CD8β is expressed in human T cells, functional human CD8β cDNAs have been isolated and sequenced. Expression of CD8β is conserved, and structural variants of CD8β encoded by alternatively spliced transcripts have been characterized. These differ in their cytoplasmic domains and may transmit regulatory signals to T cells.

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Chapter 1

Introduction

I. Overview

T cell-target cell interaction is central to the vertebrate immune response. T cell education in the thymus, activation by specific antigen in the periphery, and ultimately T cell effector functions involve tightly regulated intercellular communication. Thymocytes are positively selected for growth on thymic stromal cells by interaction of the T cell antigen receptor (TCR) with major histocompatibility (MHC) molecules. Specificity for self peptides on the surface of antigen presenting cells leads to deletion of immature thymocytes and insures against the generation of autoreactive T cells. Mature helper T cells are stimulated by contact with B cells, macrophages and other antigen presenting cells to proliferate and secrete immunoregulatory lymphokines. Finally, cytotoxic T cells (CTL) directly bind to and lyse neoplastic or virus-infected cells that are perceived as foreign.

Each of these parts of the immune response represents a well studied problem of intercellular communication. The cells involved (largely) have been identified and their phenotypes characterized. Many of the cell surface proteins responsible for intercellular adhesion, specificity of interaction, and transmission of regulatory signals are understood at the structural and molecular level. The polymorphic TCR specifically recognizes foreign peptide antigen complexed to MHC molecules on target cells, and transmits regulatory signals to the T cell through the CD3 complex. While it is widely believed that the T cell-target interaction depends upon cooperative activity between the TCR and several other cell surface proteins, little is known about how this activity is coordinated at a mechanistic level.

The goal of these studies is to understand how the T cell surface glycoprotein CD8 coordinates with the TCR during T cell activation. The MHC specificity of mature T cells correlates with the expression of CD4 or CD8. CD4 positive ($CD4^+$) T cells have TCRs specific for MHC class II molecules and $CD8^+$ T cells recognize MHC class I molecules. The studies to follow address whether this correlation arises by direct binding of CD8 to MHC class I molecules, and by determination of the

CD8 binding site on MHC class I polypeptides, whether CD8 and the TCR are capable of binding to the same MHC class I molecule. Characterization of different structural forms of CD8 has also been an important aspect of this work.

II. Major components of the T cell-target cell interaction.

A. T cell surface molecules.

The TCR is specific for peptide antigen presented on target or antigen presenting cells by MHC molecules (reviewed in 1,2). It is composed of two disulfide-bonded 40-50 kD polypeptide chains which form a polymorphic amino-terminal (NH_2-terminal) binding site. Allelic exclusion insures that individual T cells express a single TCR. Most T cells express the Ti-$\alpha\beta$ complex which was initially identified using clonotypic monoclonal antibodies (1,2). Recently, T cells expressing a distinct $\gamma\delta$ TCR complex have been identified, and their function is poorly understood (3). Like variability of immunoglobulins (Igs) in B cells, $\alpha\beta$ and $\gamma\delta$ TCR polymorphism is generated by somatic rearrangement of variable region (V-region) gene segments to exons encoding the carboxy-terminal (C-terminal) constant region. Noncovalently associated with the TCR is the invariant CD3 complex, which is composed of as many as five different transmembrane polypeptides: γ,δ and ϵ are members of the Ig supergene family (4,5); ζ forms homodimers or heterodimers with the η-chain (6) and is phosphorylated on tyrosine residues (7). Association between the TCR and CD3 is required for either complex to be expressed at the cell surface (8). It is generally believed that CD3 transmits activation signals from an externally engaged TCR into the cell. The following observations are consistent with this view: first, in contrast with the three amino acid cytoplasmic domains of the TCR, the CD3 polypeptides have relatively long intracellular domains; second, anti-CD3 monoclonal antibodies (mAbs) can mimic anti-TCR mAbs in stimulating or antagonizing T cell activation; third, stimulation of CD3 or the TCR results in phosphotidylinositol hydrolysis (9); and finally, the CD3

ζ-chain is required for (10) and its phosphorylation correlates with the activation state of T cells (6,7,10).

Several other T cell surface glycoproteins that promote intercellular adhesion and transmit regulatory signals also coordinate with the TCR. LFA-1 is a member of the integrin gene family and promotes conjugate formation by binding ICAM-I (11,12) and ICAM-II (13) on target cells. Recent binding studies with purified ICAM-I suggest that LFA-1 does not act independently of the TCR, but upon TCR ligation is transiently converted from a low avidity to a high avidity state (14). CD2 is expressed on all mature T cells and binds to its ligand LFA-3 with comparable avidity (Kd of 0.4uM) to other cellular adhesion receptors (15-17). Both CD2 and LFA-3 are members of the Ig supergene family. In addition to T cell-target adhesion, CD2 appears to play a direct role in T cell activation (reviewed in 18). This idea was first suggested because pairs of anti-CD2 mAbs stimulate T cells (19,20). It is strongly supported by experiments with cytoplasmic deletion mutants of CD2 that bind to LFA-3, but do not function in transfected cells (21). T cell stimulation by anti-CD2 mAbs requires surface expression of the TCR-CD3 complex (22,23), and there is a single report of co-immunoprecipitation of CD3 glycoproteins with anti-CD2 mAbs, indicating that CD2 physically interacts with the TCR (24).

CD4 and CD8 were initially thought to increase the avidity of T cells for their targets by binding MHC class II and MHC class I molecules, respectively (25,26). While the studies to follow show that CD8 is capable of binding to MHC class I molecules in the absence of the TCR, this binding is likely to be of low avidity. This is consistent with the idea that CD8 does not primarily function in conjugate formation, but plays a role in T cell activation (see below). Like CD2, CD4 and CD8 may also be associated with the TCR, possibly forming a multi-molecular complex with the TCR as it binds to MHC antigens (18,27). The TCR is thought to bind to the polymorphic α-helices of the membrane distal α_1- and α_2-domains of MHC molecules (28). Studies included in this thesis indicate that CD8α binds to nonpolymorphic residues of the MHC class I α_3-domain, supporting the notion that CD8 and the TCR can simultaneously bind to the same MHC class I molecule.

B. Antigen presentation by the target cell.

Peptides are expressed at the surface of vertebrate cells bound to MHC class I and MHC class II molecules (reviewed in 29,30). While highly polymorphic within a species, the ability of MHC molecules to bind a large repertoire of peptides arises primarily from binding of many peptides by a single MHC class I (31-33) or II (34-36) molecule. In addition, a single cell may express several different MHC class I and MHC class II molecules. MHC class II molecules are thought to present peptides of exogenous antigens. Studies with MHC class II restricted T cells have shown that this process is sensitive to chloroquine and utilizes an acidified intracellular compartment (37). Subsequently it was demonstrated that fixed cells effectively present exogenously added peptide fragments on MHC class II molecules (38). In contrast, MHC class I molecules were later shown by Townsend *et al.* to present endogenously generated peptide fragments to CTL specific for influenza nucleoprotein (39). Direct binding of peptide fragments to purified MHC class II molecules has been extensively studied using equilibrium dialysis (34-36,40-42), while binding to purified MHC class I molecules has only recently been demonstrated (43). Binding of individual peptides is restricted to specific MHC molecules and competition studies indicate a single binding site (34-43). Whether all MHC-bound peptides adopt a similar structural motif is still controversial. However, panels of peptides with single amino acid substitutions have been analyzed for TCR recognition and MHC binding, and these studies indicate that T cells recognize some peptides in an α-helical conformation (44,45).

Crystallographic analyis of HLA-A2 has demonstrated that antigenic peptides are presented by a single binding site on MHC class I molecules (32, 33), as is likely to be the case for MHC class II molecules. MHC class I molecules are composed of a polymorphic integral membrane 45 Kd α-chain which requires noncovalent association with the 12 kD nonpolymorphic β_2-microglobulin (β_2-m) protein for surface expression. The extracellular portion of the α-chain is roughly composed of three 90 amino acid domains: α_1, α_2 and α_3. The crystal structure strongly supports epitope mapping studies with

alloreactive and antigen specific CTL which suggested that symmetrical α_1- and α_2- domains bind antigen and contact the TCR (32,33). Polymorphic regions of the α_1-domain (60-80) and α_2-domain (140-170) form two parallel α-helices which stretch across a table of β-pleated sheet. A groove 25 Å long and 10 Å wide lies between the α-helices, is of the appropriate size to bind antigenic peptides, and contains unresolved electron dense material most likely to be endogenous peptides co-purified with HLA-A2 (32). Polymorphic residues along the base of this groove are likely to directly bind antigen fragments and influence the repertoire of peptides presented to T cells (33). The α_3-domain and β_2-m are membrane proximal and each forms Ig-like folds of two anti-parallel β-pleated sheets. Unlike MHC class I, MHC class II molecules are composed of two polypeptide chains of about 34 and 29 kD (α and β, respectively), each of which has a 90 amino acid polymorphic NH_2-terminal domain and a C-terminal Ig-like nonpolymorphic region (30). Because MHC class I and II molecules are conserved in amino acid sequence (46), can be recognized by the same pool of TCRs (28), bind peptides (39-43), and have similar stretches of amino acid polymorphism (at the α_1-domains of MHC class I and II molecules, and α_2-domain of MHC class I and β_1-domain of MHC class II) it is believed that they are structurally similar. Based on the MHC class I crystal structure, Brown *et al.* have been able to generate a hypothetical model for the T cell recognition site (α_1- and β_1-domains) of MHC class II molecules (47). In addition, Davis and Bjorkman have performed modelling studies which indicate that the TCR V-region resembles that of the Ig antigen binding site. They have suggested that the TCR complementarity determining region 1 (CDR1) and CDR2 regions bind the polymorphic MHC α-helices, while the CDR3 region binds peptide antigen in the groove between them (28).

III. Role of CD4 and CD8 in T cell-target cell interactions.

A. Structure of the CD4 and CD8 glycoproteins.

The T cell surface glycoproteins CD4 (human T4, murine L3T4, rat W3/25) and CD8 (human T8, murine Lyt-2,3, rat OX8) are members of the

Ig supergene family (5). Although they appear to perform complementary functions in MHC class II and MHC class I restricted T cells, biochemical and primary sequence analyses suggest little structural similarity (48). In humans and rodents, CD4 is a 55 kd monomer with variable N-linked glycosylation. It is composed of four external domains, a putative hydrophobic transmembrane domain, and a highly charged cytoplasmic domain of about 40 amino acids (49-52). The NH_2-terminal domain is homologous to V-regions of Ig light chains and is followed by three domains of weaker Ig-homology. Unlike the others, the third domain lacks an intrachain disulfide bond. The cytoplasmic domains are highly conserved between humans and rodents (80% of amino acid residues) in comparison to the total protein (55%). This is consistent with the notion that the extracellular portion of CD4 binds to polymorphic MHC class II molecules, while the cytoplasmic domain transmits regulatory signals by interacting with a more conserved intracellular protein.

Unlike CD4, CD8 is known to assume several structural forms. While initial studies had shown that rodent T cells express both CD8 α–α (Lyt-2) homodimers and α–β (Lyt-2,3) heterodimers (53), the human CD8 β-chain polypeptide has only recently been identified (chapter 5). Human CD8α is a 32 kD glycoprotein most homologous to Ig light chains, with an external Ig V-region-like domain, a membrane proximal hinge region with extensive O-linked glycosylation, a nonpolar transmembrane domain, and a highly charged cytoplasmic tail of 28 amino acids (54-56). While this primary structure is conserved in rodent homologs, CD8 adopts additional structural forms that appear to be specific to humans or mice. On the surface of human thymocytes, CD8 forms disulfide-bonded heterodimers with the MHC class I-like CD1 (T6) antigen (57). More recently, intracellular interaction of human CD8 with classical class I molecules has been demonstrated in peripheral T cells (58,59). The functional significance of these complexes is still unclear. In addition, an alternatively spliced murine CD8α transcript lacks sequences encoding the first of two cytoplasmic exons. A consequent shift in the translational reading frame results in a protein (Lyt-2α') with a three amino acid cytoplasmic tail (60). Although Lyt-2α' mRNA

is produced and translated in both thymocytes and mature T cells, only thymocytes express the truncated form at the cell surface (61).

It has been suggested that the NH_2-terminal region of the CD8α–β heterodimer and the TCR may be structurally similar to each other (28,62). Like CD8α, CD8β is composed of V- and joining-like (J-like) sequences, a serine and threonine rich membrane proximal region, and a charged cytoplasmic domain (chapter 4, 62-64). In rodents, the two chains are probably joined by disulfide bonds between cyteine residues in the membrane proximal region, and CD8β requires association with CD8α for surface expression (64). The rodent CD8 α-chain also forms cell-surface homodimers. Like CD4, both the α– and β-chains of CD8 are conserved in sequence between humans and rodents, with greatest homology in the cytoplasmic domains. CD4, CD8α and CD8β are no more homologous to each other than they are to Ig light chains (48). However, it is notable that the CD4 monomer is somewhat similar in size and domain structure to a complex of two CD8 polypeptide chains.

B. CD4 and CD8 increase the avidity of T cells for their targets.

For several years the predominant view was that CD4 and CD8 functioned as accessory molecules; by binding to MHC class II and class I proteins, respectively, they were thought to increase the avidity of T cells for their targets (48). The cytotoxic and helper T cell subsets were initially defined with alloantisera specific for Lyt-1 and Lyt-2 in mice (66,67), and mAbs OKT4/Leu3 and OKT8/Leu2 in humans (68,69). Although Lyt-1 (CD5) was first thought to be the murine homolog of human T4/Leu3, it was not reciprocally expressed with the Lyt-2 antigen, and murine CD4 (L3T4) was later identified with mAb GK1.5 (70). It became clear that anti-CD4 Abs inhibited helper T cells restricted for MHC class II molecules, while anti-CD8 Abs blocked target cell lysis by MHC class I restricted CTL (71-74). Because of a dual correlation with T cell function and MHC restriction, it was uncertain whether the primary role of CD4 and CD8 was to determine helper/cytotoxic activity or to regulate T cell-target interactions. This was resolved by the discovery of $CD8^+$ helper T cells and $CD4^+$ CTL specific for MHC class I and II molecules, respectively (26,75-79).

Abs to CD4 and CD8 blocked proliferation and CTL killing suggesting that CD4 and CD8 were important to the function of these T cells. Hence the stronger correlation existed between the CD4 or CD8 phenotype and the MHC restriction of a particular T cell. The role of these glycoproteins in cell-cell interaction was further supported by results of CTL conjugation assays, in which anti-CD4 and anti-CD8 mAbs blocked T cell-target binding (81-84).

Several findings have suggested that CD4 and CD8 are not absolutely required for T cell function *in vitro* (85-88), but as conditions for TCR triggering become suboptimal, CD4- or CD8-MHC interactions enhance the T cell response (25,89-92). In particular, among panels of T cell hybridomas or clones, an inverse correlation has been demonstrated between the affinity of a TCR for its target and the ability of anti-CD4 or anti-CD8 mAbs to inhibit the response. In these studies, the affinity of the TCR has been defined in terms of the inverse of the minimum antigen concentration required for T cell activation (25,89,90), or of the strength of conjugate formation in cold-target inhibition assays (91,92). In addition, expression of CD4 (93,94) and CD8 (95,96) in T cell hybridomas augments but is not essential for a response to target cells bearing the appropriate antigen and MHC molecule. When the TCR α- and β-chain genes from a $CD8^+$ CTL clone specific for fluorescein and H-$2D^d$ were expressed in a CD8 negative ($CD8^-$) recipient cell, supertransfection of CD8 was only necessary for a response to target cells expressing low amounts of H-$2D^d$ (95,96). However, in another experiment co-transfection of CD8 with the TCR α- and β-chain genes from a CD8 dependent CTL clone was required for transfer of MHC class I specific alloreactivity (97).

The notion that CD4 and CD8 interact with MHC molecules has been substantiated by experiments with T cells in which the putative MHC specificity of CD4 and CD8 is distinct from that of the TCR. $CD8^+$ T cells have been identified which have TCRs that recognize MHC class II molecules, and these cells are generally not inhibited by anti-CD8 mAbs (98-100). MHC class I specific $CD4^+$ T cells are inhibited by anti-CD4, but only if the target cells express MHC class II polypeptides (101-103). The latter results suggest that CD4 is capable of binding MHC class II molecules independently of the TCR. This has been supported

by gene transfer of CD4 or CD8 into T cell hybridomas. For example, T cell reactivity was enhanced by transfer of the human CD4 cDNA into a murine hybridoma reactive against human MHC class II (94). In addition, transfection with a human CD4 cDNA enhanced activity of a murine $CD4^-$ hybridoma specific for MHC class I, but only if the target cells also expressed human MHC class II molecules (93). Conversely, CD8 expression augmented IL-2 production by a murine HLA class II specific T cell only if stimulator cells expressed MHC class I antigens, and this activity was inhibited by anti-CD8 and anti-MHC class I mAbs (104).

Finally, CD4 and CD8 do not appear to require target cell ligands other that MHC molecules to enhance the T cell response. Helper and cytotoxic T cells specifically respond to liposomes and artificial membranes containing purified MHC class II and class I molecules, respectively (reviewed in 105). These responses are inhibited by anti-CD4 and anti-CD8 mAbs under suboptimal conditions such as low avidity TCRs (106) or decreased antigen density (107). Addition of MHC class I molecules not recognized by alloreactive TCRs augments the CTL response to cell size artificial membranes bearing the alloantigen (108). In this system anti-CD8 mAbs are inhibitory, indicating that CD8 binds MHC molecules distinct from those recognized by the TCR. Direct evidence for CD4-MHC class II and CD8-MHC class I binding in the absence of the TCR has recently been obtained using transfected cells (109, chapter 2), and purified proteins in artificial cell membranes (110).

C. CD4 and CD8 are co-receptors with the TCR for MHC molecules.

Despite the demonstration of TCR independent binding between CD4 or CD8 and MHC molecules, current evidence suggests that the functionally important role of CD4 or CD8 is as a TCR co-receptor. By forming a quaternary complex with the TCR, antigen and MHC proteins, CD4 and CD8 could either stabilize TCR-MHC binding or regulate the transmission of T cell activation signals delivered by TCR triggering (see below). Although not directly demonstrated, several lines of evidence suggest physical interaction between CD4 or CD8 and the TCR (reviewed in 27). First, anti-CD8 and anti-CD4 mAbs inhibit T cell

activation in the absence of MHC antigens (111-115). Although it was initially believed that this occured by transmission of negative regulatory signals to T cells, subsequent studies have indicated that these Abs sterically hinder the crosslinking of TCR complexes required for activation (90,116-120). Second, the response of $CD4^+CD8^+$ T cell clones reactive against antigen presented by MHC class II is not inhibited by anti-CD8 mAbs (116), and MHC class I specific double positive cells are not inhibited by anti-CD4 (121). Third, anti-CD4 and anti-CD8 mAbs not only inhibit T cell-target conjugation, but TCR triggering as well (83,84,122,123). Fourth, the TCR co-localizes with CD4 at the T cell-target interface upon contact with target cells bearing specific antigen and MHC (124). Fifth, under certain conditions CD4 is co-internalized with the TCR (125-128), and this does not appear to be a simple consequence of activation or capping (27). Sixth, Janeway *et al.* have obtained Abs directed against a certain TCR V-region epitope which fail to bind specific T cells pre-treated with anti-CD4 Fab fragments (27). Co-immunoprecipitation of CD4 and CD8 with the TCR has not yet been demonstrated, but Janeway *et al.* argue that this is due to the transient nature of the complexes which are induced by MHC molecules on target cells (27).

IV. CD4 and CD8 transmit regulatory signals to T cells.

In contrast to initial studies demonstrating antigen-independent inhibition (negative signalling) of T cells by soluble anti-CD4 and anti-CD8 mAbs, recent evidence indicates that assembly of multimeric CD4- or CD8-TCR complexes is important for T cell activation. A marked synergy in T cell proliferation (129-132), IL-2 receptor expression (133), IL-3 production (134), and release of intracellular Ca^{2+} (135) is observed when anti-CD4 or anti-CD8 mAbs are crosslinked to anti-TCR mAbs. Although several anti-TCR mAbs alone are able to stimulate T cell activation, they may do so by inducing the formation of CD4- or CD8-TCR complexes (127). This is consistent with the idea that T cell inhibition by soluble anti-CD4 and anti-CD8 mAbs may involve the dissociation of such complexes (127,136). An anti-CD4 mAb which

directly activates T cells has recently been identified, although preliminary evidence suggests that it does not induce CD4-TCR association (137).

Both serine and tyrosine phosphorylation have been implicated in the transmission of T cell regulatory signals via CD4 and CD8. Upon TCR stimulation, CD4 or CD8 molecules are rapidly phosphorylated on serine residues, and internalized in the case of CD4 (138-140). This is likely to occur by activation of protein kinase C, since it is also induced by phorbol ester treatment (138-141). These events may be important for T cell activation. For example, stimulation of resting T cells by anti-CD3 alone differs from stimulation mediated by cross-linked anti-CD3 and anti-CD8, in that the latter provides a second signal required for IL-2 production and complete IL-2 responsiveness (142). Notably, this CD8 dependent second signal is sensitive to an inhibitor of protein kinase C and cGMP/cAMP-dependent kinases (142).

The protein tyrosine kinase $p56^{lck}$ is likely to couple formation of CD4- or CD8-TCR complexes to T cell activation. $p56^{lck}$ is a lymphocyte specific *src*-like cytoplasmic protein, and its association with CD4 or CD8 recently has been demonstrated by co-immunoprecipitation (143-145). This interaction is mediated by cysteine residues in the cytoplasmic domains of CD4 and CD8 and the NH_2-terminal domain of $p56^{lck}$ (146). CD4 and CD8 may direct $p56^{lck}$ to the TCR complex, and in this way alter phosphorylation and activity of the CD3 glycoproteins. In T cells, cross-linking of CD4 induces an increase in the tyrosine kinase activity of $p56^{lck}$ and concomitent phosphorylation of the ζ-chain (147). In addition, immune complexes of CD4- or CD8-*lck* phosphorylate the CD3 γ, δ, ϵ and ζ-chains *in vitro* (148). Preliminary evidence suggests that the activity of CD4- or CD8-*lck* complexes is tightly regulated. Protein kinase C mediated serine phosphorylation of $p56^{lck}$ occurs as an early event of T cell activation (144), and this induces dissociation of CD4 but not CD8 from $p56^{lck}$ (150). In addition the phosphotyrosine phosphatase CD45, which is required for TCR signalling (151), enhances T cell activation when cross-linked to CD4 (152), and modestly stimulates $p56^{lck}$ autophosphorylation *in vitro* (153).

V. Role of CD4 and CD8 in T cell development.

The expression of CD4 and CD8 correlates with the maturation state of T cells. Immature cortical thymocytes lacking expression of CD4, CD8 or the TCR differentiate into a recently described $CD4^-CD8^+$ $TCR^{-/lo}$ blast cell population and subsequently into $CD4^+CD8^+$ TCR^{lo} cells (154). Both deletion of autoreactive T cells (155-159), and positive selection of T cells with TCRs reactive with host MHC molecules (159-161) occur during this stage of development. Apparently, most $CD4^+CD8^+$ thymocytes fail either or both selection processes, as only 1% emmigrate from the thymus and the rest are nonfunctional and die *in situ* (162). While initially difficult to establish, experiments with lethally irradiated bone marrow chimeras (163), TCR transgenic mice (158-159), *in vivo* anti-CD4 mAb treatment (156-157), and $CD4^+CD8^+$ thymocyte clones (164) have proven that double positive thymocytes are generally the precursors of mature medullary $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes. It is unclear whether $CD4^-CD8^+$ $TCR^{-/lo}$ blasts also directly differentiate into mature $CD8^+$ cells (154), or if there is any lineage relationship between conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells and the smaller populations of double negative $\alpha\beta$(165) and $\gamma\delta$ (3) T cells, or $\gamma\delta$ T cells expressing CD8 (166).

Both positive and negative selection of thymocytes involve interplay between CD4 or CD8 and the TCR as it binds to MHC molecules. Interaction of the TCR with MHC molecules in the thymus determines the CD4 or CD8 phenotype of mature T cells. This was fist suggested by the observation that treatment of mice with anti-MHC class II (167-168) mAbs abrogates the development of mature $CD4^+$ T cells. Later it was also shown that mice treated with anti-MHC class I mAbs (169) lacked $CD8^+$ T cells. Studies with endogenous (155,170) and transgenic (171-173) TCRs indicate that the MHC specificity of the TCR determines whether a double positive thymocyte retains expression of CD4 or CD8. In support of CD4 and CD8 function in positive selection are experiments demonstrating increased TCR expression following treatment of double positive thymocytes with anti-CD4 (174), and expansion of specific thymocyte populations by crosslinking CD8 to particular TCRs (175). Evidence for participation of CD4 and CD8 in negative selection

is more direct. Intrathymic deletion of autoreactive T cells can be blocked by treatment of neonatal mice with anti-CD4 (156-157). In addition, autoreactive T cells in mice transgenic for a CD8 dependent $\alpha\beta$ TCR specific for the male H-Y antigen and H-$2D^b$ are not deleted if they lose or have decreased expression of CD8 (158). The nature of biochemical signals transmitted to thymocytes via CD4 and CD8 is poorly understood. However, recent evidence suggests that mAbs to CD4, CD8 or the TCR can stimulate tyrosine kinase activity in double positive thymocytes, and this activity correlates with phosphorylation of the CD3 ζ-chain (176).

VI. Structural analysis of CD8-MHC class I binding.

Extensive studies of T cell function indicate that CD4 and CD8 coordinate with the TCR during T cell maturation and activation in the periphery, but little is known about how this process occurs at a mechanistic level. In particular because the TCR, CD4 and CD8 all bind MHC molecules, it has been difficult to independently study their activities using functional assays. The studies described in this thesis utilize a structural approach to determine the binding site of CD8 on MHC class I molecules in the absence of the TCR. Human CD8 was initially chosen because it was thought to be composed of a single 32 kD α-chain, as opposed to murine CD8. In addition a cDNA thought to potentially encode a soluble form of human CD8α was available in the laboratory. A soluble form of CD8α was characterized, but binding of sCD8α in cultured cell supernatants to MHC class I molecules could not be detected, presumeably because of the low avidity of sCD8α for MHC class I molecules. Instead, an assay system to quantitate the binding between MHC class I^+ B cells and transfected cells expressing high levels of human CD8α was developed. In this system multi-valent CD8-MHC class I interactions mediate stable intracellular adhesion, allowing a quantitative comparison of CD8 binding to the products of individual MHC class I alleles and point mutants. Later, a partial genomic clone corresponding to human CD8β was obtained in another laboratory, suggesting that human CD8 also formed α-β heterodimers. In

order to determine the structure of human CD8 and to study MHC class I binding with the complete CD8 glycoprotein, cDNAs corresponding to human CD8β were isolated and characterized.

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Chapter 2

Alternatively Spliced mRNA Encodes a Secreted Form of Human CD8α; Characterization of the Human CD8α gene

Introduction

Initially a soluble form of human CD8α was characterized with the aim of using purified material to study the binding between CD8 and MHC class I molecules. Human CD8 was chosen because it was thought to be composed of a single 32 kD α-chain polypeptide, in contrast to rodent CD8, known to consist of α–β heterodimers. In addition, in the process of obtaining human CD8α cDNA clones, a variant was identified corresponding to an alternatively spliced transcript which lacked sequences from the transmembrane exon (exon IV). This suggested that a secreted form of CD8 might be produced by human T cells. To determine whether transcripts from the human CD8α gene were alternatively spliced, mRNA from a murine L cell fibroblast line transfected with a human CD8α genomic clone was probed with end-labeled oligonucleotides specific for the junction between exon III encoding the hinge region, and exon V, encoding the first part of the cytoplasmic domain. The junctional probe specifically hybridized with mRNA from the CD8α genomic transfectant, indicating that human CD8α transcripts were produced which lacked the transmembrane exon. The studies to follow describe exon mapping of the human CD8α gene, identification of transcripts encoding soluble CD8α, and production of the glycoprotein by human T cells. Possible physiologic functions of secreted CD8α are discussed.

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ALTERNATIVELY SPLICED mRNA ENCODES A SECRETED FORM OF HUMAN CD8α

Characterization of the Human CD8α Gene[1]

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We have determined the organization and nucleotide sequence of the gene encoding the human T cell surface glycoprotein CD8α. This gene spans approximately 8 kb and is organized into six exons which encode separate functional domains of the protein. Exon I encodes the 5′ untranslated region and leader peptide, exon II the Ig V-like region, exon III the hinge-like region, exon IV the transmembrane domain, and exons V and VI the cytoplasmic tail. Alternative splicing that excludes nucleotide sequences from exon IV results in a transcript which encodes a secreted form of the protein. This transcript accounts for approximately 15% of the total CD8α mRNA in human T cell leukemia lines and in normal human tissues. Secreted CD8α protein can be detected in culture supernatants of T cell leukemia lines and PHA-stimulated PBMC by immunoprecipitation with the anti-CD8α mAb OKT8 or with a polyclonal rabbit antiserum specific for the 28 amino acid cytoplasmic domain of CD8α. The secreted CD8α protein forms homodimers; when analyzed by SDS-PAGE, the protein migrates with an apparent molecular mass of 27 or 54 kDa under reducing or non-reducing conditions, respectively. Human secreted CD8α may serve an immunoregulatory role for the interactions of T cells with their targets in vivo.

The CD8 glycoprotein is expressed on the surface of CTL that are specific for Ag presented by MHC class I molecules (1, 2). It is thought to play a major role in thymocyte development and in interactions between T cells and their targets. Low affinity binding between CD8 and MHC class I molecules in the absence of the TCR has recently been demonstrated (3), supporting the suggestion (2, 4) that interaction between CD8 and MHC class I molecules increases the avidity of T cells for their targets. It has also been suggested that CD8 may become physically associated with the TCR and may thus directly participate in signal transduction. This is supported by the demonstration that crosslinking of anti-CD8 mAb to anti-TCR mAb leads to a marked synergy in T cell activation (5–7) and that CD8 and the TCR can be comodulated from the surface of T cells (8).

In humans and rodents CD8 is composed of heterodimers of two distinct chains, α(Lyt-2) and β(Lyt-3), which are highly conserved in their respective transmembrane and cytoplasmic domains (1, 9, 10). In the process of isolating cDNA clones encoding human CD8α (11), we obtained a structural variant which lacked nucleotide sequences encoding the putative hydrophobic transmembrane domain. Partial genomic mapping indicated that this cDNA clone corresponded to a transcript which arose by in-frame alternative splicing that excluded the transmembrane exon. This finding suggested that a secreted form of CD8 may arise by alternative splicing, as has also been shown for transcripts encoding secreted MHC class I molecules (12, 13). sCD8α[2] protein has been detected in human sera and culture supernatants of the human T cell leukemia line HPB-ALL by Fujimoto et al. (14, 15). However, instead of detecting the secretion of CD8α, the authors determined that CD8α is released in vitro by proteolytic cleavage of membrane bound protein from the T cell surface (15). A similar mechanism has been described for the release of a soluble form of the IL-2R α-chain from human T cells (16, 17). It was unclear from the work of Fujimoto et al. (14, 15) whether human CD8 was produced in vivo by secretion or proteolysis. Therefore, we were prompted to determine whether a secreted form of CD8α is produced by normal human T lymphocytes by means of alternative splicing of the transmembrane exon. We have analyzed the structure of the human CD8α gene and its products and have determined that approximately 15% of normal human CD8α transcripts lack nucleotide sequences encoded by the transmembrane exon. A sCD8α homodimer is secreted by cells transfected with a cDNA corresponding to the alternatively spliced transcript. Moreover, sCD8α protein can be detected in the culture supernatants of human T cells, suggesting that it is produced under physiologic conditions.

MATERIALS AND METHODS

Cells. Human T cell leukemia lines Fro2.2, HPB-ALL, and 8402 (11) were grown in RPMI 1640 supplemented with 12.5% FCS (HyClone Laboratories, Logan, UT). Murine L cells (Tk⁻) and their

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[2] Abbreviations used in this paper: sCD8α, secreted CD8α; mCD8α, membrane bound CD8α; NP40, Nonidet P-40; cpm, counts/min.

derivatives, and COS7 cells were maintained in DMEM supplemented with 10% FCS (GIBCO, Grand Island, NY). The L cell derivative F1.2 (9) was a transfected cell line expressing mCD8α and was maintained in 400 μg/ml G418 (Geneticin, GIBCO). Tk7 (11) was transfected with the human CD8α λTM1 genomic clone and was maintained in HAT. B2, A5d, A1b, and C5a were L cell transfectants expressing sCD8α and were maintained in adenine, aminopterin and thymidine. Human PBMC were isolated by Ficoll-Hypaque (Pharmacia, Fine Chemicals, Piscataway, NJ) density gradient centrifugation and stimulated with 15 μg/ml PHA (PHA-E + L, Vector Laboratories Inc., Burlingame, CA) in RPMI 1640 supplemented with 12.5% FCS (HyClone).

Antibodies. The anti-CD8α mAb OKT8 was affinity purified or in the form of ascites. The anti-CD8α mAb 66.2 (18; from Dr. P. Martin, F. Hutchinson Cancer Center, Seattle, WA) was affinity purified. Normal mouse serum was from Zymed (South San Francisco, CA). Rabbit antiserum (prepared at Cattag, South San Francisco, CA) specific for the carboxyl-terminal 28 amino acid residues of human CD8α (CYT) was obtained by s.c. injection of 0.15 mg of unconjugated peptide (synthesized by C. Turk, University of California-San Francisco and Howard Hughes Medical Institute) in 1 ml of FCA (Sigma Chemical Co., St. Louis, MO), followed by weekly booster injections in IFA (Sigma) starting 4 wk later.

cDNA clones, genomic clones, and DNA sequencing. Isolation of the mCD8α F1.1 cDNA (pT8F1) and sCD8α F1.4 cDNA clones from a λgt10 human peripheral T cell cDNA library was as described (11). The nucleotide sequence of the F4.1 cDNA was identical to that of the F1.1 cDNA, except that clone F4.1 lacked nucleotides encoding the amino-terminal 50 amino acids, as well as nucleotides encoding a putative transmembrane domain. The cDNA were subcloned into the plasmid vector SP65 (Promega BioTec, Madison, WI) at the *Eco*RI site, and a hybrid F1/F4 cDNA was constructed by substitution of the *Nae*I-*Bam*HI 3′ fragment of SP65-F4.1 for that of SP65-F1.1. Isolation of CD8α genomic clones from a human genomic library was as described (11). Restriction fragments of CD8α genomic clones were subcloned into the vector M13mp18/19 (New England Biolabs, Inc., Beverly, MA), and sequenced on both strands of single-stranded DNA by dideoxy-chain termination (19). For the sequence ladder of the 680-bp S1 nuclease probe, chemical cleavage reactions were performed according to Benčini et al. (20), using the permanganate reaction described by Rubin and Schmid (21).

Transfection of L-Tk⁻ and COS7 cells. Murine L cells deficient in thymidine kinase (L-Tk⁻) were co-transfected by calcium phosphate precipitation with the plasmid λAT3 (22), and the sCD8α F1/F4 cDNA inserted into the vector pMV7 (23). After selection for expression of adenine phosphoribosyl transferase (aprt) and Tk (22), colonies were expanded and analyzed for expression of sCD8α protein by RIA of culture supernatants. Transfection of L cells to obtain the cell lines F1.2 (9) and Tk7 (11) has been described.

COS7 cells were transfected with the F1.4, F1.1, or F1/F4 cDNA inserted into the vector pSV7d (from Dr. P. Luciw, University of California-Davis), a derivative of pHS210 (24). Cells at 30 to 50% confluency were incubated with 12.5 μg/ml DNA, 50 mM Tris-Cl, pH 7.4, and 400 μg/ml DEAE Dextran in serum free DMEM for 4 h at 37°C. Next, cells were incubated in DMEM supplemented with 2% FCS and 1 μM chloroquine for 3 h at 37°C, followed by incubation in DMEM supplemented with 10% FCS.

S1 nuclease analysis. To identify the transcription initiation site, the 5′ 730-bp *Hin*dIII-*Pst*I fragment of the human CD8α genomic clone λTM1 was subcloned into the plasmid vector pUC18, and a 680-bp *Nar*I fragment (including 0.57 kb of upstream sequences extending from the *Hin*dIII to *Nar*I site) from this construct was isolated as a probe for S1 nuclease mapping. To analyze the alternative splicing of CD8α transcripts, a 211 bp *Sma*I-*Pvu*II fragment of SP64 was inserted into the *Nae*I site of SP65-F1.1, and a 445-bp *Sac*I-*Sau*3A fragment from this construct was isolated. The 680-bp *Nar*I and 445-bp *Sac*I-*Sau*3A fragments were dephosphorylated with calf intestinal phosphatase, and 5′ end labelled with γ[^{32}P]ATP (7000 Ci/mmol, ICN, Irvine, CA). Individual strands of each probe were separated on a polyacrylamide gel, hybridized to RNA samples, and digested with S1 nuclease as described by Favaloro et al. (25).

RIA to detect sCD8α. Round bottomed polyvinyl chloride 96-well microtiter plates were coated overnight with 50 μl of mAb OKT8 ascites diluted 1/100 in PBS, or PBS alone as a control. After blocking with 100 μl of 1% BSA in PBS for 1 h, 50 μl of cell free culture supernatant was added per well and incubated for 2 h. Wells were washed extensively with 0.05% NP40 in PBS, then incubated with 50 μl ^{125}I labeled (Iodogen, Pierce Chemical Co., Rockford, IL) mAb 66.2 (18) diluted to 1 μg/ml in 0.1% NP40 and 2% BSA in PBS. After extensive washing with 0.05% NP40 in PBS, individual wells were counted in a gamma counter. The assay was performed at room temperature. mAb OKT8 and 66.2 recognize distinct epitopes of human CD8α (18).

Immunoprecipitation of sCD8α and mCD8α. L cell transfectants, human T cell leukemia lines and human PBMC were metabolically labeled overnight (14 h) with 100 μCi/ml [^{35}S]cysteine (>600 Ci/mmol, Amersham Corp., Arlington Heights, IL) included in the culture medium. Cultures were harvested and centrifuged (400 × *g*) for 10 min to separate cells and supernatants. Cells were lysed for 1 h at 4°C in PBS containing 1% NP40, 150 mM NaCl, 5 mM ATP, and the following protease inhibitors: aprotinin diluted 1/100, 10 μg/ml pepstatin A, and 1 mM PMSF. Protease inhibitors (all from Sigma) were also added to culture supernatants. After pre-absorption with normal mouse serum and protein A-Sepharose (Pharmacia), 2 × 10^7 acid insoluble cpm (assessed by TCA precipitation) of each L cell, 4 × 10^7 cpm of each T cell leukemia, or 10^8 cpm of each PHA-stimulated PBMC supernatant or lysate was incubated with protein A-Sepharose and saturating amounts of Ab on a turntable at 4°C. Cell lysates and supernatants were immunoprecipitated in a total volume of 200 μl for 2 h, or 2 to 3 ml for 20 h, respectively. The beads were washed twice in 1 ml of 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% NP40, and 1% sodium deoxycholate, and resuspended in 200 μl of 0.5% sodium deoxycholate. After centrifugation (12,000 × *g*) through a sucrose gradient (bottom layer: 800 μl 20% sucrose in 10 mM Tris-Cl pH 7.4, 150 mM NaCl, and 1% NP40; top layer: 400 μl 10% sucrose in wash buffer), beads were washed two more times in wash buffer and resuspended in electrophoresis sample buffer. Samples were analyzed by SDS-PAGE on 12% Laemmli gels.

RESULTS

Organization and nucleotide sequence of human CD8α gene. The isolation of cDNA and genomic clones encoding human CD8α has been described (11). The genomic clone λTM1 is capable of transforming murine L cell fibroblasts to the CD8α$^+$ phenotype (11). We have mapped λTM1 by restriction enzyme analysis, and identified the position of the coding sequences by hybridization to the CD8α cDNA clone F1.1 (pT8F1). The restriction map, sequencing strategy, and nucleotide sequence of the six CD8α exons and their flanking DNA are shown in Figure 1. The intron-exon structure is similar to that of other membranes of the Ig gene superfamily: introns (with the exception of intron V) split codons between the first and second nucleotide, and individual exons roughly correspond to separate functional domains of the protein (28). Exon I encodes the 5′ untranslated region and most of the putative hydrophobic leader peptide, exon II the Ig V-like region, exon III the hinge region, exon IV the putative hydrophobic transmembrane domain, and exons V and VI the cytoplasmic domain. Exon VI also encodes about 1.2 kb of 3′ untranslated sequence as determined by comparing the nucleotide sequence of the genomic clone with that of the CD8α cDNA clone pT8B (11). Within this exon there are two potential polyadenylation consensus signals (AATAAA), and transcripts with a shorter 3′ UT sequence may be produced by the utilization of a site about 0.7 kb 3′ to the translation stop codon. The genomic organization of human CD8α is similar to that of its murine homolog Lyt-2, except that the Lyt-2 gene is composed of five exons, with the 5′ untranslated region, leader peptide and Ig V-like region encoded by the first exon (29).

Transcription initiation site and upstream nucleotide sequences. The transcription initiation site for the human CD8α gene was determined by S1 nuclease mapping (Fig. 2). In human spleen, the major and minor CD8α transcription initiation sites were mapped to 84 and 121 bp, respectively, upstream of the translation initiation codon. The converse was true for the CD4$^+$/CD8$^+$ T cell leukemia line Fro2.2; the major Fro2.2 transcription initiation site was 121 bp and the minor site was 84 bp 5′

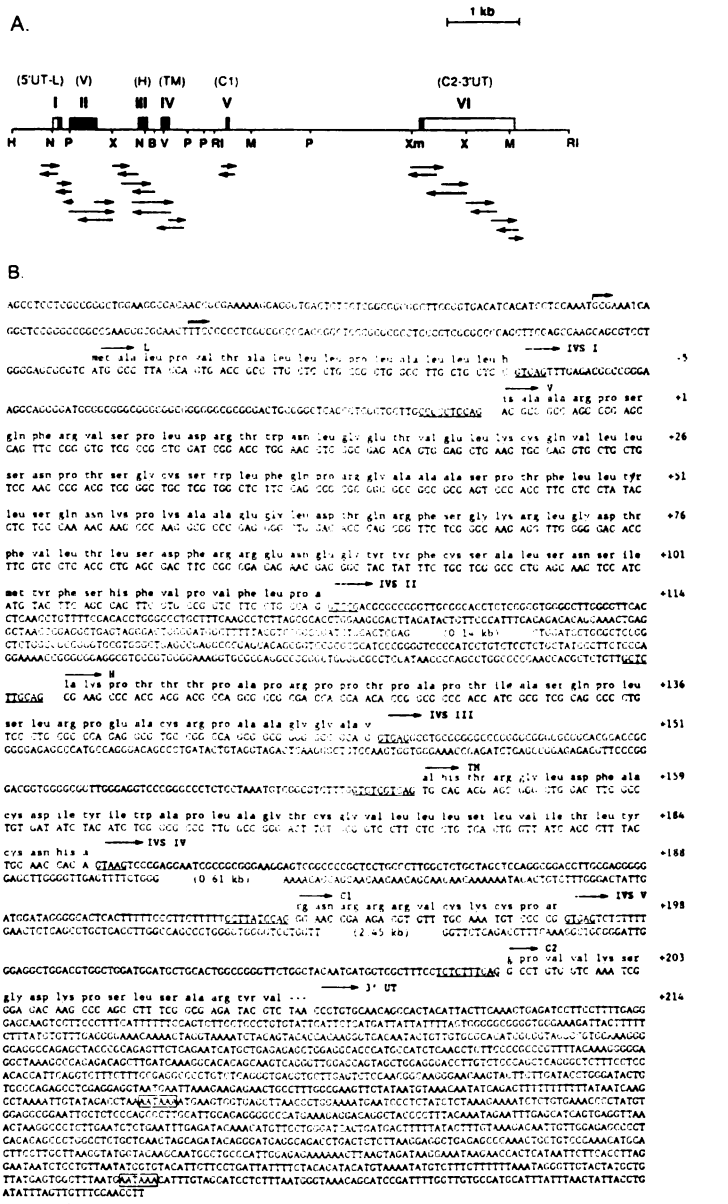

Figure 1. Genomic organization and nucleotide sequence of human CD8α gene. Sequences encoding the 5′ untranslated region (*5′ UT*), leader (*L*), Ig V-like (*V*), hinge (*H*), transmembrane (*TM*), cytoplasmic (*C*), 3′ untranslated (*3′ UT*), and intervening sequence (*IVS*) regions are indicated. *A.* Schematic representation of the human CD8α gene. A 1-kb scale is indicated at the top. *Shaded* and *open boxes* indicate translated and untranslated regions, respectively. Exons are numbered in roman numerals. *Arrows* represent fragments sequenced as described in *Materials and Methods*. Restriction endonuclease sites are as follows: *Hin*dIII (*H*), *Eco*RI (*RI*), *Nae*I (*N*), *Pst*I (*P*), *Xho*I (*X*), *Bg*lII (*B*) *Eco*RV (*V*), *Mst*II (*M*), *Xmn*I (*Xm*). *B.* Sequence of the CD8α gene and the predicted protein. The numbers to the right designate residues in the mature protein, as predicted by N-terminal amino acid sequencing (26). *Bent arrows* indicate transcription initiation sites identified by S1 nuclease analysis (Fig. 2). *Straight arrows* designate domains of the protein corresponding to exons listed above, and intervening sequences numbered in roman numerals. The donor and acceptor consensus sequences at the mRNA splice junctions (27) are underlined. Two potential polyadenylation signals are boxed.

to the start of translation. The lack of consensus mRNA splice acceptor sequences (27) at these sites rules out the possibility of an untranslated 5′ exon. mRNA from Fro2.2 also gave full-length protection of CD8α genomic sequences present in the 5′ end labeled probe from digestion with S1 nuclease (Fig. 2*B*). This suggests that an additional transcription initiation site may lie at least 0.5 kb upstream of the translation start site in this cell line.

The alignment of nucleotide sequences upstream of the translation initiation codon of the human and murine CD8α gene is shown in Figure 2*A*. Both genes lack TATA and CAAT sequences characteristic of most promoter regions. Sixty nucleotide bp upstream of the major spleen transcription initiation site, a decanucleotide sequence found in the promoter region of TCR β-chain genes (30) is conserved. Just upstream of the decanucleotide sequence in the CD8α gene, a GC-rich region is present and contains a consensus binding sequence for the transcription initiation factor Sp1 (31).

Alternative splicing of human CD8α mRNA. The isolation and nucleotide sequences of CD8α cDNA clones from a human peripheral T cell cDNA library have been described (11). The F4.1 cDNA clone (Fig. 3*A*) lacks nucleotides from exon IV, the transmembrane exon, therefore potentially encoding a secreted form of CD8α. Based on a comparison of the sequence of F4.1 to genomic DNA (Fig. 1), it is clear that the corresponding transcript arose by an in-frame alternative splicing event.

To determine the relative level of expression of the alternatively spliced sCD8α transcript, we have per-

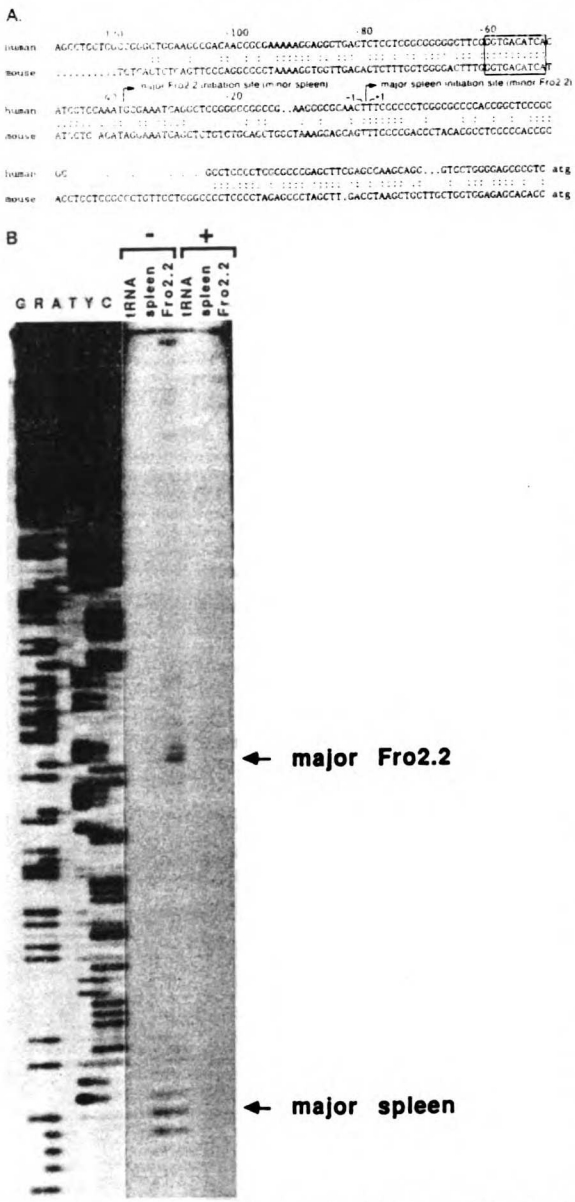

Figure 2. Upstream nucleotide sequence of the human CD8α gene. *A*, Homology between the upstream sequences of the human and mouse CD8α genes. Nucleotide identities are indicated by colons, and gaps are indicated by periods. Numbers shown above the sequence designate nucleotide positions relative to the major spleen transcription initiation site. The major and minor transcription initiation sites for human spleen and the T cell tumor line Fro2.2, as predicted by S1 nuclease protection assay (see below), are indicated by *bent arrows*. The decanucleotide sequence present in the promoter region of human and murine TCR β-chain genes (30) is boxed. The translation initiation codon (atg) is indicated. *B*, S1 nuclease mapping of the transcription start site. The 5′ end labeled 680 nucleotide minus strand probe (−) described in *Materials and Methods* was hybridized with 50 μg of yeast tRNA (tRNA), total human spleen RNA (spleen), or Fro2.2 poly A+ RNA (Fro2.2), and digested with S1 nuclease. Protected fragments were separated on an 8% polyacrylamide/urea gel. As a control, hybridization of the RNA samples to the plus strand probe (+) was performed. As a size marker, the first six lanes show a DNA sequence ladder of the same 680-bp probe. Sequencing reactions were as follows: G, dimethyl sulfate (G only); R, formic acid (A + G); A, NaOH (A > C); T, $KMNO_4$ (T only); Y, hydrazine (T + C); C, hydrazine + NaCl (C only).

formed S1 nuclease analysis on mRNA from transfected L cells, human T cell leukemia lines, and normal human tissues (Fig. 3 *B* and *C*). As expected, hybridization of the 445 nucleotide minus strand DNA probe with mRNA transcribed in vitro from the F1.1 (mCD8α) cDNA resulted in the protection of a 254 nucleotide fragment from S1 nuclease digestion; hybridization with in vitro transcribed F1.4 (sCD8α) mRNA protected a 122 nucleotide fragment (Fig. 3*C*). As shown in Figure 3, L cells transfected with the mCD8α cDNA (F1.2) or the sCD8α cDNA (B2, A5d, A1b, or C5a) expressed the appropriate transcripts. L cells transfected with genomic DNA (Tk7) expressed both transcripts. sCD8α mRNA is expressed by the $CD8^+$ T cell leukemia lines HPB-ALL and Fro2.2, but not by the $CD8^-$ T cell leukemia line 8402. sCD8α mRNA comprises about 15% of total CD8α transcripts in human spleen, thymus, and lymph node. Similar proportions are found in the genomic L cell transfectant Tk7, and in the T cell leukemia lines HPB-ALL and Fro2.2.

Expression of sCD8α protein in transfected cells. To determine whether the sCD8α transcript could encode a secreted protein, COS7 cells were transiently transfected with a functional sCD8α (F1/F4), mCD8α (F1.1), or partial sCD8α (F1.4) cDNA inserted into an SV40 based replicating vector. Aliquots of culture supernatants were assayed for the presence of the CD8α Ag by RIA as described in *Materials and Methods*. By 48 h post-transfection, supernatants of cells transfected with a functional sCD8α cDNA (F1/F4) contained immunoreactive CD8α at levels 35-fold more than supernatants of mock or control transfectants (Fig. 4). Lower levels of soluble CD8α could be detected in culture supernatants of cells transfected with the mCD8α cDNA. This was probably due to proteolytic cleavage of membrane bound CD8α from the cell surface as had been observed by Fujimoto et al. (14, 15). Unlike true sCD8α, this proteolytic product (released CD8α) should not contain amino acid residues from the cytoplasmic domain, and should therefore be of lower M_r.

To assess the relative sizes of the rCD8α and sCD8α proteins, L cells transfected with the mCD8α cDNA (F1.2), genomic CD8α DNA (Tk7), or the sCD8α cDNA (B2, A5d) were metabolically labeled, and their culture supernatants immunoprecipitated with the anti-CD8α mAb OKT8 (Fig. 5). In the supernatants of genomic or sCD8α transfectants we observed a protein doublet migrating with a relative M_r of 25–27 kDa by SDS-PAGE analysis (Fig. 5, *left panel*). S1 nuclease analysis (Fig. 3) excluded the possibility that the heterogeneity in size resulted from alternative splicing of sequences from the hinge or cytoplasmic exons. Given the extensive *O*-linked glycosylation of mCD8α (32), a likely possibility is that sCD8α is heterogeneous in the amount of *O*-linked glycosylation at the serine- and threonine-rich hinge domain. The apparent M_r of sCD8α is consistent with the absence, from the 32 kDa membrane bound form, of 37 amino acid residues encoded by exon IV. As predicted, putative cleavage products of 21 and 24 kDa (visualized on longer exposures of autoradiograms) were immunoprecipitated from supernatants of mCD8α transfectants (data not shown). The 32 kDa CD8α Ag was immunoprecipitated from cell lysates of mCD8α or genomic L cell transfectants, but was not detectable in lysates of sCD8α transfected cells (Fig. 5, *center panel*). As with the membrane bound form, the sCD8α protein appears to form homodimers; a 54-kDa

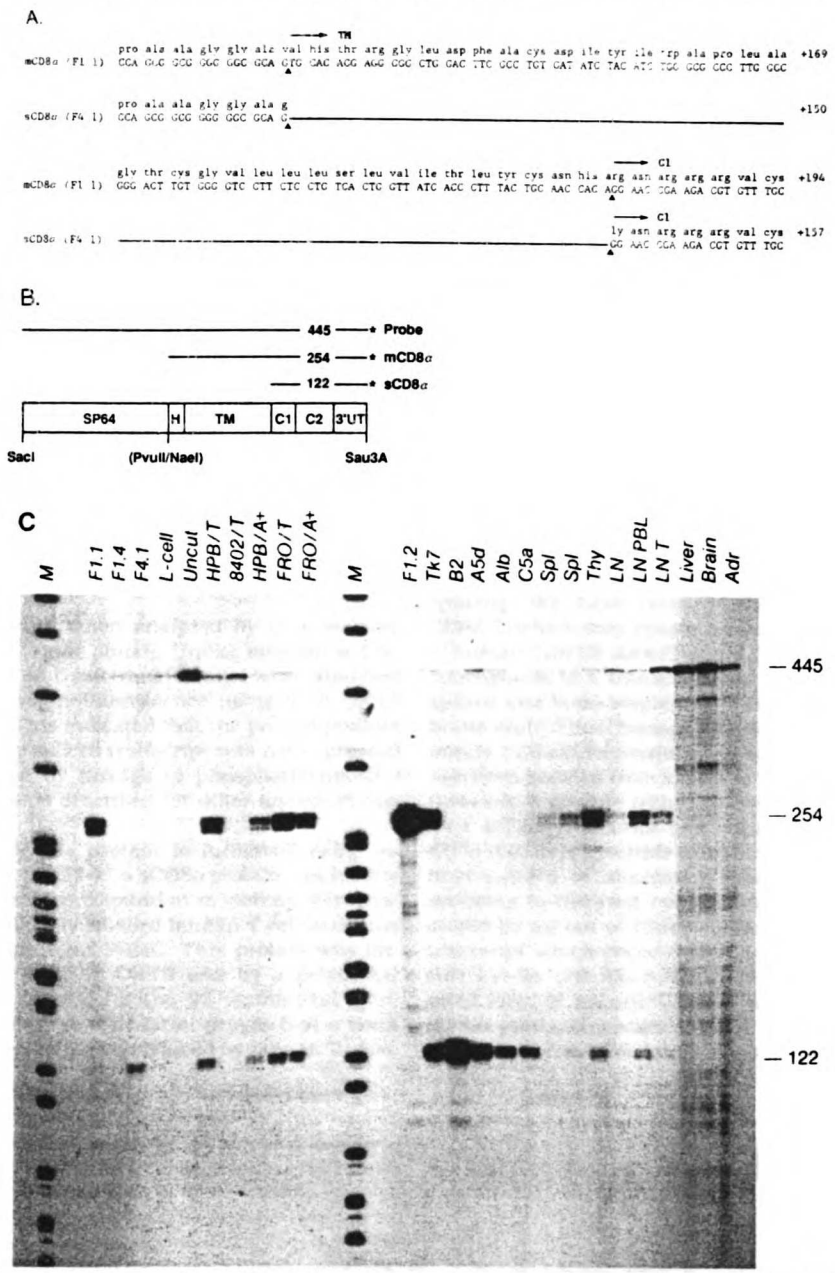

Figure 3. Alternative splicing of human CD8α mRNA. *A*, Nucleotide and amino acid sequence of alternative cDNA forms encoding human CD8α. Sequences encoding the six carboxyl-terminal amino acids of the hinge domain, transmembrane domain (*TM*), and six amino-terminal amino acids (*C1*) of the cytoplasmic domain are shown for the F1.1 and F1.4 cDNA. Numbers to the right designate amino acid residues of the mature protein (Fig. 1*B*). *Solid arrowheads* indicate intron-exon boundaries. As indicated by the *solid lines*, the F4.1 cDNA lacks nucleotide sequences, corresponding to exon IV, which encode the putative transmembrane domain. *B*, Schematic map of the 5′ end labeled S1 probe and protected fragments. *C*, S1 nuclease analysis of alternatively spliced CD8α transcripts. The 5′ end labeled *Sac*I-*Sau*3A probe was hybridized with 2 ng of in vitro transcribed RNA from CD8α cDNA, 10 μg of poly A+ (*A+*) RNA from human T cell leukemias, or 40 μg of total (*T*) RNA from human T cell leukemias, L cells or transfected L cells, or normal human tissues. Sizes of the protected fragments analyzed on a 6% polyacrylamide/urea gel after S1 nuclease digestion are indicated to the right. As a size marker (*M*), a radiolabeled *Msp*I digest of pBR322 is shown. In vitro transcribed RNA were synthesized using the F1.1 (mCD8α), F1.4 (sCD8α anti-sense), or F4.1 (sCD8α) cDNA as a template. Human T cell leukemias included the $CD4^+/CD8^+$ lines HPB-ALL (*HPB*) and Fro2.2 (*FRO*), and the $CD4^-/CD8^-$ line 8402. L cells were transfected with the mCD8α F1.1 cDNA clone (*F1.2*), the human CD8α λTM1 genomic clone (*Tk7*), or the sCD8α F1/F4 construct (*B2 A5d. A1b, C5α*). Normal human tissues included: spleen (*spl*), thymus (*thy*), lymph node (*LN*), lymph node lymphocytes (*LN PBL*), lymph node T cells (*LN T*), liver, brain, and adrenal gland (*adr*).

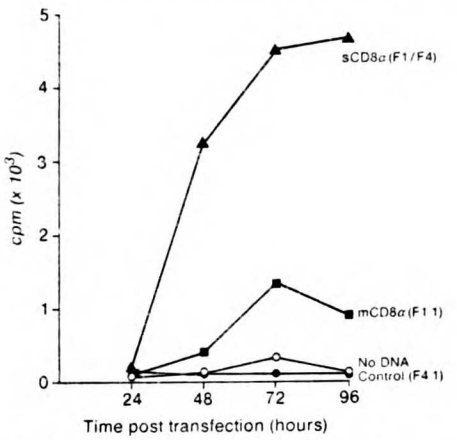

Figure 4. Detection of soluble CD8α protein in the supernatants of transiently transfected COS7 cells as a function of time. COS7 cells were transfected with the sCD8α F1/F4 construct (*triangles*), mCD8α F1.1 cDNA (*squares*), control sCD8α F4.1 cDNA (*circles*), or no DNA (*open circles*). Aliquots of cell supernatants were taken at the indicated times and analyzed for the presence of soluble CD8α by RIA as described in *Materials and Methods*.

species was observed when analyzed by non-reducing SDS-PAGE (Fig. 5, *right panel*). Unlike mCD8α, sCD8α was not detectable on L cell transfectants when analyzed by cell surface immunofluorescence using mAb OKT8 (data not shown). This indicated that the protein product of the alternatively spliced transcript was not expressed at the cell surface by linkage to phosphatidylinositol residues, as has been described for other anchor minus receptors (33).

Expression of sCD8α protein in human T cells. As shown in Figure 6, the 27-kDa sCD8α protein was readily detectable by immunoprecipitation of culture supernatants from metabolically labelled human T cell leukemia lines and PHA-stimulated PBMC. This protein was immunoprecipitated by mAb OKT8 and by a polyclonal rabbit antiserum specific for the 28 amino acid cytoplasmic domain of CD8α. The latter proves that a bona fide secreted CD8α protein is produced by human T cells. Supernatants of Fro2 2 and PBL also contained the 24-kDa proteolytic cleavage product that is reactive with mAb OKT8, but not with the cytoplasmic domain specific antiserum.

DISCUSSION

We have demonstrated that human T cells express an alternatively spliced transcript which lacks sequences encoded by the transmembrane exon and therefore encodes a secreted form of the CD8α protein. The 54-kDa sCD8α homodimer is distinguishable from a proteolytic cleavage product of membrane-bound CD8α by immunoprecipitation with a rabbit antiserum specific for the 28 amino acid cytoplasmic domain. By RIA, Fujimoto et al. (14) have detected low levels of CD8α Ag in normal human sera and 40-fold higher levels in the sera of patients with T cell leukemias. These authors suggested that proteolytic cleavage resulted in the shedding of CD8α Ag from the surface of human T cells (15). Our results indicate that human T cells produce both forms of CD8α in culture.

In humans and in rodents, structural variants of both the α- and β-chains of CD8 appear to arise by alternative splicing. We have recently obtained a cDNA clone, CD8β.3, which may encode a soluble form of the β-chain of human CD8 (9). As with the sCD8α cDNA clone, CD8β.3 corresponds to a transcript that has been alternatively spliced and lacks sequences encoded by the transmembrane exon. Thus, human T cells may secrete an anchor minus CD8α/β heterodimer. A similar CD8β cDNA has also been isolated from a murine T cell cDNA library (34). However, a murine transcript corresponding to the human sCD8α transcript has not been detected. Instead, CD8α (Lyt-2) transcripts in murine T cells display a distinct pattern of alternative splicing: sequences corresponding to the first cytoplasmic domain exon are excluded by an out of frame splicing event, resulting in a transcript which encodes a truncated form of the molecule, Lyt-2α′ (29, 35). Although mRNA encoding the truncated form of murine CD8α is efficiently translated in thymocytes and mature T cells, mature T cells specifically retain the CD8α′ (Lyt-2α′) protein in a late cellular

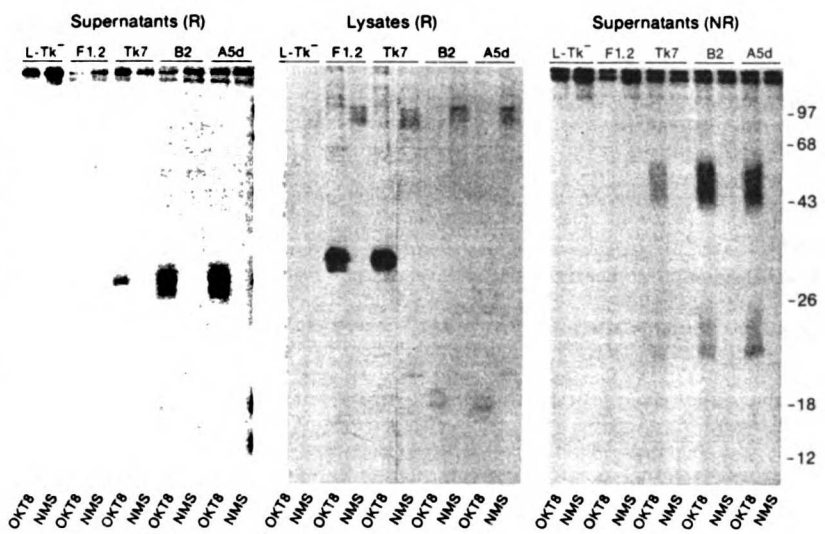

Figure 5. Immunoprecipitation of sCD8α and mCD8α from the supernatants and lysates of transfected cell lines. L cell lines, indicated at the top and described in the legend to Figure 3C, were metabolically labeled overnight with [^{35}S]cysteine. Detergent solubilized cell lysates and cell free supernatants were immunoprecipitated with the anti-CD8α mAb OKT8 or normal mouse serum (*NMS*) as control, and analyzed on a 12% polyacrylamide gel under reducing (*R*) or non-reducing (*NR*) conditions. Relative *M*, are given in kDa to the right of each autoradiogram.

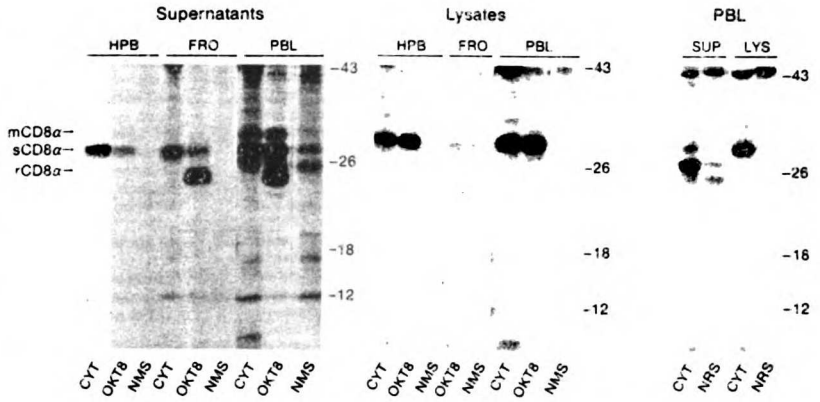

Figure 6. Immunoprecipitation of sCD8α and mCD8α from the supernatants and lysates of human T cell leukemia lines and PHA-activated normal PBMC. HPB-ALL cells (*HPB*), Fro2.2 cells (*FRO*), and PHA-activated normal PBMC (*PBL*) were metabolically labeled overnight with [^{35}S] cysteine. Detergent solubilized cell lysates and cell free supernatants were immunoprecipitated with a rabbit antiserum against the CD8α cytoplasmic tail (*CYT*), OKT8, or NMS, and analyzed on a 12% polyacrylamide gel under reducing conditions. As shown in the panel on the right, immunoprecipitation of supernatants (*SUP*) and lysates (*LYS*) of PBL was also performed using rabbit antiserum against the CD8α cytoplasmic tail (*CYT*), or preimmune rabbit sera (*NRS*) as a specificity control. Gels of immunoprecipitated cell supernatants and lysates were autoradiographed for 3 and 1 days, respectively. *Arrows* indicate the 27-kDa sCD8α, 32-kDa mCD8α, and 24-kDa rCD8α proteins. Relative M_r are given in kDa to the right of each autoradiogram.

compartment and fail to express it at the cell surface (36).

From our experiments, it is unclear whether soluble CD8α/β heterodimers are produced by human T cells. Although human CD8β can be detected on the surface of HPB-ALL and normal human T cells with a recently described mAb 597 (10), the M_r of the mature human CD8β polypeptide is unknown. We have previously isolated three human CD8β cDNA which encode polypeptides that differ in their cytoplasmic domains (9). These variant mRNA probably arise by alternative splicing. CD8β.3 lacks sequences encoded by the transmembrane exon, and with a single N-linked glycosylation site is predicted to encode a protein with a M_r of about 26 kDa. In the absence of any *O*-linked glycosylation, this protein would be expected to co-migrate with sCD8α when analyzed by SDS-PAGE. However, as with sCD8α, sCD8β.3 may be heterogeneous in the extent of *O*-linked glycosylation. In addition, alternative splicing of the transmembrane exon may lead to the production of anchor minus variants of the two other CD8β cDNA, CD8β.1, and CD8β.2.

Binding of CD8α to class I MHC molecules is thought to be a low avidity interaction which stabilizes T cell-target cell binding via multivalent interactions (3). We have recently described a cell-cell adhesion assay in which overexpression of human CD8α is required for the detection of stable binding between transfected cells and MHC class I$^+$ B cell lines (3). Using this assay, 15 different human and 2 different murine MHC class I allelic gene products bound human CD8 with similar avidity (R. Salter, and A. Norment, manuscript in preparation). In light of these results, we expect that sCD8α homodimers or the proteolytic cleavage product rCD8α will have low affinity for the majority of MHC class I molecules. It is therefore unlikely that CD8α Ag in human serum can serve an immunoregulatory role solely by binding to MHC class I molecules. However aggregates of sCD8α, rCD8α, or a soluble CD8α/β heterodimer may have sufficient avidity to stably bind class I MHC molecules in vivo. Alternatively, these forms of CD8α may bind with high affinity to non-classical Qa-like class I molecules (37), the MHC class I-like CD1 Ag (38), or an unidentified cell surface receptor.

The alternatively spliced sCD8α transcript appears to be constituitively expressed by transfected L cells, thymocytes, and mature T cells. However, subpopulations of T cells may regulate the splicing of CD8α transcripts and thereby preferentially express sCD8α mRNA. Tissue specific or developmentally regulated alternative RNA processing has been described for several systems (39). Certain cellular microenvironments may thus contain concentrations of sCD8α protein sufficient to interfere with CD8-MHC class I binding and T cell-target cell interactions.

It is unclear whether CD8 binds to monomorphic determinants of the α_3 domain (40, 41) or to the relatively polymorphic determinants of the α_1 and α_2 domains of MHC class I molecules (42). It is therefore not yet known whether CD8 and the TCR can simultaneously bind to an individual class I molecule (43, 44). The sCD8α homodimer and the putative heterodimer can potentially be used to develop a CD8-class I binding assay allowing quantitation of the affinity of binding of CD8 to different MHC class I molecules. Such an assay will facilitate mapping of amino acid residues of class I molecules involved in binding to CD8. In addition it may be possible to prepare crystals of purified sCD8α dimers suitable for structural analysis.

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Chapter 3

Cell-Cell Adhesion Mediated by CD8 and MHC class I Molecules

Introduction

We wanted to determine whether CD8α could bind MHC class I molecules in the absence of any other lymphoid specific proteins. In initial studies we attempted to detect binding of sCD8α homodimers to MHC class I^+ B cells. Transfected L cells, (described in chapter 2), expressing high levels of sCD8α homodimers were metabolically labeled and their culture supernatants concentrated 10 fold. This material was incubated with human MHC class I^+ Raji cells, or Daudi cells, lacking β_2-m required for surface expression of the MHC class I α-chain. The cells were centrifuged through an oil layer, in order to minimize dissociation of sCD8α, then lysed and analyzed by SDS-PAGE and autoradiography. We failed to detect specific binding of sCD8α to MHC class I^+ Raji cells, suggesting that the affinity of binding between individual CD8 and MHC class I molecules was too low to detect in such an assay.

A cell-cell binding assay involving multi-valent interactions was therefore developed to detect CD8-MHC class I binding. Adherent COS7 cells were transiently transfected with the human CD8α cDNA subcloned in an Simian Virus 40 (SV40) replicating vector. Human B cells were centrifuged onto the COS7 cells, and following a one hour incubation under several conditions, nonspecifically bound B cells were removed by gentle washing. By red blood cell rosetting with an anti-CD8α mAb, it was clear that COS7 cells expressing high levels of surface CD8 specifically bound MHC class I^+ B cells. Specific binding was most efficient at 37 ^oC, and in the presence of 10% fetal bovine serum in phosphate buffered saline. This demonstrated that CD8 and MHC class I molecules bound to each other and could mediate intercellular adhesion. The chapter to follow describes a quantitative assay to measure CD8-MHC class I binding, using stable cell lines expressing up to 100 fold more cell-surface CD8 than human T cells.

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Cell-cell adhesion mediated by CD8 and MHC class I molecules

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CD4 and CD8 are cell-surface glycoproteins expressed on mutually exclusive subsets of peripheral T cells. T cells that express CD4 have T-cell antigen receptors that are specific for antigens presented by major histocompatibility complex class II molecules, whereas T cells that express CD8 have receptors specific for antigens presented by MHC class I molecules (reviewed in ref. 1). Based on this correlation and on the observation that anti-CD4 and anti-CD8 antibodies inhibit T-cell function, it has been suggested that CD4 and CD8 increase the avidity of T cells for their targets by binding to MHC class II or MHC class I molecules respectively[2,3]. Also, CD4 and CD8 may become physically associated with the T-cell antigen receptor, forming a higher-affinity complex for antigen and MHC molecules[4-8], and could be involved in signal transduction[5,6,9,10]. Cell–cell adhesion dependent CD4 and MHC II molecules has recently been demonstrated[11]. To determine whether CD8 can interact with MHC class I molecules in the absence of the T-cell antigen receptor, we have developed a cell–cell binding assay that measures adhesion of human B-cell lines expressing MHC class I molecules to transfected cells expressing high levels of human CD8. In this system, CD8 and class I molecules mediate cell–cell adhesion, showing that CD8 directly binds to MHC class I molecules.

We anticipated that the binding between CD8 and MHC class I molecules might be a low-affinity interaction, similar to that of other cellular adhesion receptors[12,13]. Because detection of such interactions usually requires multivalent binding, stable cell lines with a high degree of CD8 expression were developed (Fig. 1). The relative levels of CD8 expression by the four CHO lines are shown in Fig. 1*a*.

Surface MHC class I expression of several B lymphoblastoid lines used in the cell-cell binding assay was determined using the monoclonal antibody (mAb) W6/32 (Fig. 1*b*) which is specific for monomorphic determinants of class I molecules[14]. The B cells included Raji, RM3 and CIR-A2.1, all of which express high levels of MHC class I (10^6-10^7 molecules per cell, data not shown), and Daudi and CIR, which express significantly reduced levels of surface class I molecules. Daudi cells are deficient in class I because of an absence of β_2-microglobulin expression. CIR cells[15] lack the *HLA-A* and *HLA-B* genes, but nonetheless retain significant binding activity for mAb W6/32, probably because of its reactivity with the *HLA-C* locus product or with the recently described non-classical class I molecules[16]. CIR cells did not react with the HLA-A2, HLA-A28-specific mAb CR11-351 (ref. 17) (Fig. 1*b*). CIR-A2.1, a cell line obtained by transfecting an HLA-A2.1 genomic clone[18] into CIR, expressed levels of class I molecules comparable with Raji.

A cell-cell binding assay was developed for the study of the interaction between CD8 and MHC class I molecules in the absence of the T-cell antigen receptor (TCR). As shown in Fig. 2, MHC class I^+ B cells (Raji, RM3, CIR-A2.1) specifically bound to $CD8^+$ CHO.3 and CHO.4 cells at levels 15-30-fold greater than to $CD8^-$ CHO.1 cells. As many as 90% of the class I^+ B cells remained tightly bound to $CD8^+$ CHO cells and were not removed from the plates with further washing. The extent of cell-cell binding correlated with the relative level of CD8

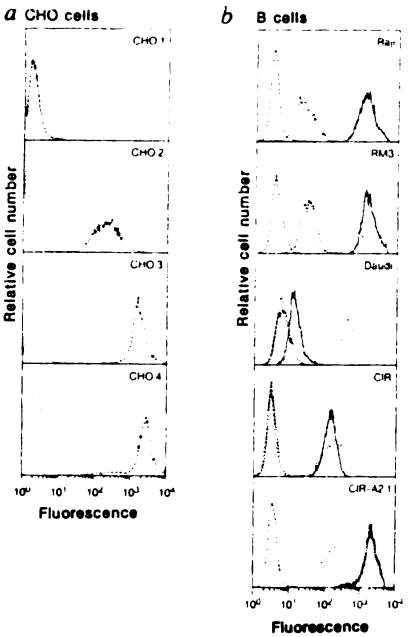

Fig. 1 Cytofluorometric analysis of CHO- and B-cell lines. Cells were incubated with saturating levels of the anti-human transferrin receptor mAb L01.1 (dotted line), the anti-CD8α mAb OKT8 (dotted-dashed line), W6/32 (ref. 14) (solid line), or the HLA-A2, HLA-A28-specific mAb Cr11-351 (ref. 17) (dashed line). Staining used a second step fluorescein-conjugated goat anti-mouse IgG antibody (Becton Dickinson). Fluorescence was measured in arbitrary units using a FACScan cell analyser (Becton Dickinson). *a*, CHO-cell lines. CHO.1 ($CD8^-$) and CHO.2 ($CD8^+$) were derived by selection in media lacking hypoxanthine. CHO.3 and CHO.4 were obtained by methotrexate amplification of CHO.2. *b*, B-cell lines. Raji is a MHC class I^+ Burkitt lymphoma[31]. RM3 (ref. 19) (from M. Peterlin) is a class II^- derivative of Raji. Daudi is Burkitt lymphoma deficient in surface MHC I as a result of a defect in β_2-microglobulin[32]. CIR (Hmy 2 CIR) is an HLA-A, B-negative derivative of Licrlon Hmy 2 (ref. 15). CIR-A2.1 (ref. 18) was obtained by transfection of CIR with an HLA-A2.1 genomic clone under the control of its endogenous promoter.
Methods. The CHO-$DHFR^-$ cell line DXB11 (cell culture facility, UCSF) was co-transfected by calcium phosphate precipitation[33] with the CD8α F1.1 complementary DNA (ref. 24) inserted into the vector pSV7d, a derivative of pHS210 (ref. 34) (from P. Luciw), and the plasmid pMG1P (ref. 35) bearing the hamster DHFR minigene (from D. L. Chasin). Primary selection was in Ham's F12 lacking hypoxanthine as described[36]. Colonies were screened for the expression of CD8 by rosetting[24], then expanded and rescreened by immunofluorescence analysis. A negative (CHO.1) and a positive (CHO.2) clone were chosen. CHO.3 was obtained by selection of CHO.2 in 0.03 μM methotrexate (Sigma). CHO.4 is a pool of colonies obtained by selection of CHO.2 in 0.06 μM methotrexate.

expression on the surface of the CHO cells (Fig. 2). MHC class I^+ B cells failed to bind to the $CD8^-$ line CHO.1 and to CHO.2, which expressed levels of CD8 at least five-fold higher than those on peripheral T cells. Binding was detected only when a threshold level of CD8 expression was achieved. Thus CHO.3 which expressed ~8-fold more surface CD8 than CHO.2 bound class I^+ B cells, and CHO.4, which expressed double the level of CD8 compared with CHO.3, bound even more effectively. Cell-cell binding also correlated with the relative number of class I molecules on the B cells. RM3 (ref. 19) expressed twice as many class I molecules as Raji cells (Fig. 1*b*), and bound the most effectively. CIR did not specifically bind to $CD8^+$ CHO cells. It is possible that the W6/32-reactive molecules expressed by CIR (HLA-C or non-classical class I molecules) may not

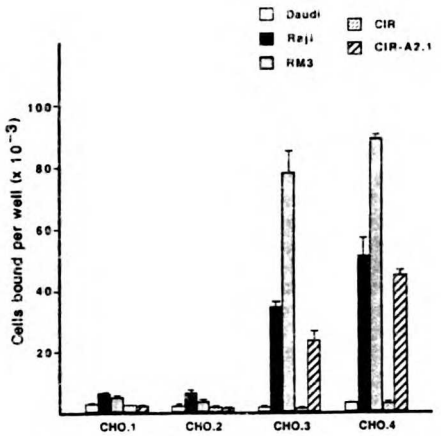

Fig. 2 Binding of B cells to CHO cells expressing different levels of CD8. B-cell lines are indicated by the key at the top of the figure: MHC I^+ lines (Raji, RM3, CIR-A2.1), MHC I^- lines (Daudi, CIR). CHO lines are indicated across the base of the histogram: CHO.1 is $CD8^-$; CHO.2, CHO.3 and CHO.4 express increasing levels of CD8. The number of cells bound per well was determined. Error bars represent standard deviations of triplicate samples.

Methods. Cell-cell binding was assayed in flat-bottom 96-well microtitre dishes (Corning) in phosphate buffered saline (PBS) with 10% fetal bovine serum. B cells were radiolabelled for 2 h with [^{35}S]cysteine (12.5 μCi per 10^6 cells, >600 Ci mmol^{-1}, Amersham). 10^5 B cells were added to wells, each of which contained an adherent confluent monolayer of about 10^5 CHO cells plated two days earlier. The B cells were centrifuged (160*g*) for 5 min on to the CHO cells, and the plates incubated for 1 h at 37 °C on an orbital shaker. Wells were washed gently 10 times with 10% fetal bovine serum/PBS; the cells were solubilized in 1% Nonidet P-40 (Sigma), 400 mM NaCl in PBS, and the amount of radioactivity of the lysate was determined by scintillation counting. The number of cells bound per well was determined on the basis of the known specific activity of the B cells.

bind to CD8, or that their expression level may be insufficient to mediate cell-cell adhesion.

The CD8-MHC class I mediated cell-cell binding was specifically and completely inhibited by anti-MHC I and anti-CD8 mAbs (Fig. 3). W6/32 blocked binding of Raji and CIR-A2.1 to the $CD8^+$CHO.4 cells (Fig. 3*a*). The HLA-A2, HLA-A28-specific mAb CR11-351 did not block binding of Raji cells, which have only slight reactivity with the antibody and express HLA-A1 and A3 (A. Calman, personal communication), but completely blocked binding of CIR cells transfected with HLA-A2.1. The anti-human transferrin receptor mAb L01.1 was reactive with the B-cell lines, but did not block specific binding. Ca206 (ref. 20), a mAb reactive with monomorphic determinants of human HLA-D molecules, also did not block binding (data not shown). Specific cell-cell binding was also completely inhibited by anti-CD8 mAbs that recognize distinct epitopes of human CD8 (refs 21, 22) (Fig. 3*b*).

These experiments demonstrate that the human CD8 glycoprotein directly binds to MHC class I molecules, supporting the idea that CD8 is an adhesion receptor that increases the avidity of T cells for their targets. Although we have detected binding in the absence of the TCR, this binding was of low avidity; over-expression of CD8 was required to mediate stable adhesion between transfected cells and B-cell lymphoblastoid lines. We

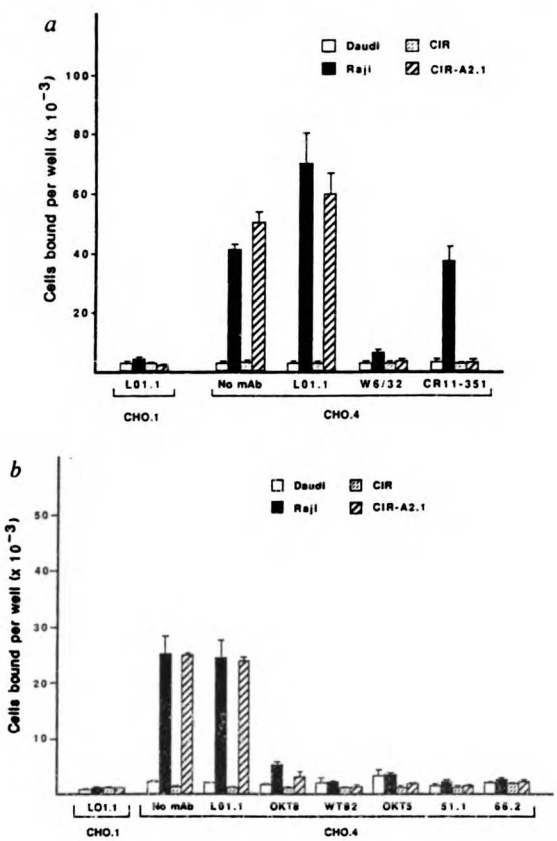

Fig. 3 Inhibition of CD8-MHC I binding by anti-MHC I and anti-CD8 mAbs. B-cell lines are as indicated in Fig. 2. Binding to CHO.1($CD8^-$) or CHO.4($CD8^+$) was determined. *a*, Inhibition by anti-MHC I mAb. B cells were preincubated with saturating levels (determined by fluorescence-activated cell sorting) of the following mAbs: LO1.1 (anti-human transferrin receptor), W6/32 (anti-MHC I monomorphic) or CR11-351 (anti-HLA-A2, HLA-A28-specific). Control cells not incubated with a mAb are also indicated (no mAb). *b*, Inhibition by anti-CD8 mAb. CHO cells were pre-incubated with saturating levels of L01.1 or one of several anti-CD8 mAbs (OKT8, WT82, OKT5, 51.1, 66.2). Control cells (no mAb) are indicated. B cells were pre-incubated with saturating levels of normal mouse serum (Xymed) to prevent binding of anti-CD8 mAb by Fc receptors.

Methods. B cells or CHO cells were incubated with the indicated mAb for 30 min at 4 °C in 0.01% sodium azide, 5% fetal calf serum in PBS, and then washed with 10% fetal bovine serum in PBS. For B cells, incubation used 10 μg antibody per 10^6 cells in 0.1 ml. CHO cells were pre-incubated with 2.5 μg antibody per well in 0.1 ml. Three anti-CD8 mAbs (WT82, 51.1, 66.2) completely blocked binding; two mAbs (OKT8 and OKT5) inhibited 90% of specific binding. The cell-cell binding assay was performed as described in the legend to Fig. 2, except that 0.01% sodium azide was added. Antibodies included in these studies were affinity-purified by protein A Sepharose chromatography, except for OKT5, which was in the form of ascitic fluid and used at a dilution of 1:25. The source of each antibody is as follows: L01.1 (Becton Dickinson); WT82 (Dr W. Tax, University of Nijmegan); OKT5 (Dr E. Reinherz, Dana-Farbar Cancer Institute); 51.1 (ref. 21) and 66.2 (ref. 21) (Dr P. Martin, F. Hutchinson Cancer Center).

cannot exclude the possibility that other molecules on either cell surface contribute to the specific cell-cell adhesion in our assay system. It has been shown, however, that beads coated with lipid bilayers bearing purified class I molecules can effectively stimulate $CD8^+$ T cells in the absence of any other molecules[23].

The binding experiments we describe were performed with only the CD8α chain, which forms homodimers and homomultimers in transfected cells[24]. Experiments with T-cell hybridomas indicate that a CD8-dependent response to antigen can be reconstituted with the TCR and CD8α-chain alone[25]. Rodent CD8, however, is expressed primarily as a heterodimer composed of the Lyt2 (α) and Lyt3 (β) chains[26], and we have recently shown that a CD8β(Lyt3) protein is expressed on human $CD8^+$ T cells[27]. Whether CD8β has a role in class I adhesion and/or signal transduction is not yet known.

Binding has now been observed between transfected cells over expressing CD4 or CD8 and lymphocytes expressing MHC class II (ref. 11) or class I molecules. As these interactions are of low avidity, it is unlikely that CD4 or CD8 alone can initiate cell-cell adhesion, assuming no regulated increase in their binding affinities. It is more likely that CD4 and CD8 stabilize cell-cell binding, probably in cooperation with other T-cell adhesion receptors and with the TCR. CD4 and CD8 could also facilitate TCR-mediated signal transduction by binding to a limited number of MHC molecules after the T cell is already bound to its target. This is supported by the demonstration of synergistic activation of T cells by anti-CD4 or anti-CD8 mAbs and anti-CD3 mAbs[5,6,9,10]. Whether CD4 and CD8 serve to cluster MHC molecules in the vicinity of TCRs and/or effect active signalling remains to be resolved.

It has been proposed that CD8, like the TCR, binds to the α-helices formed by the α_1 and α_2-domains of class I molecules[28-30]. Binding of CD8 to this site would preclude simultaneous interaction of the TCR and CD8 with a single class I molecule. The CD8-class I cell-cell binding assay can be used to map amino-acid residues of class I involved in binding to CD8 and to determine whether CD8 and the TCR bind to the same or different regions of the class I molecule.

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Chapter 4

Polymorphism in the α_3 Domain of HLA-A Molecules Affects Binding to CD8

Introduction

Having developed a quantitative assay to measure CD8α-MHC class I binding, we next wanted to determine the CD8α binding site within the MHC glycoprotein. In this way we hoped to gain insight into how CD8 coordinates with the TCR during T cell interactions. In one model, CD8 could bind to MHC class I molecules distinct from those to which the TCR binds; this would be more consistent with a role for CD8 in intercellular adhesion. Alternatively, CD8 and the TCR could form a high-avidity co-receptor for antigen and MHC class I, more consistent with a direct role for CD8 in T cell signalling. One prediction of the second model is that CD8 and the TCR would bind to different regions of the class I molecule. From epitope mapping studies and crystal structure determination of HLA-A2, it is clear that the TCR recognizes antigen complexed with polymorphic residues of the α_1- and α_2-domains. We therefore wanted to determine whether human CD8α bound to this region, or to conserved residues of the α_3-domain or β_2-m. In collaboration with Russell Salter and Peter Parham at Stanford University, we have analyzed the binding of transfected B cell lines expressing individual MHC class I alleles and point mutants to the CHO cells expressing high levels of CD8α.

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Polymorphism in the α_3 domain of HLA-A molecules affects binding to CD8

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CYTOTOXIC T lymphocytes (CTL) expressing the CD8 glycoprotein recognize peptide antigens presented by class I major histocompatibility complex (MHC) molecules[1,2]. This correlation and the absence of CD8 polymorphism led to the hypothesis that CD8 binds to a conserved site of class I MHC molecules. Using a cell–cell binding assay we previously demonstrated specific interaction between human class I MHC (HLA-A,B,C) molecules and CD8 (ref. 3). Subsequent analysis of the products of 17 HLA-A,B alleles revealed a natural polymorphism for CD8 binding in the human population. Two molecules, HLA-Aw68.1 and HLA-Aw68.2, which do not bind CD8, have a valine residue at position 245 whereas all other HLA-A,B,C molecules have alanine. Site-directed mutagenesis shows that this single substitution in the α_3 domain is responsible for the CD8 binding phenotype and also affects recognition by alloreactive and influenza-specific CTL. Our results indicate that CD8 binds to the α_3 domain of class I MHC molecules.

Various class I MHC alleles were transfected into the HLA-A,B negative B-cell line, CIR. The resulting transfectants were tested for binding to Chinese hamster ovary (CHO) cells expressing the human CD8 gene, using the assay described by Norment *et al.*[3]. Fifteen of 17 HLA-A,B molecules tested gave significant levels of positive binding, as did two murine class I MHC molecules, H-2K^b and H-2D^p (Table 1). These results indicate conservation of the CD8 binding site in class I molecules of humans and mice. It was therefore surprising to find that two human class I molecules; HLA-Aw68.1 and -Aw68.2, showed no specific interaction with CD8 in the cell-cell binding assay (Table I). This effect cannot be attributed to differing levels of HLA-A expression by the various transfectants (Fig. 1) and most probably results from differences in their primary structures. Fortunately, HLA-Aw68.1 and -Aw68.2 are closely related in amino-acid sequence to molecules such as HLA-A2.1 and HLA-Aw69 which do bind CD8. For example, HLA-Aw69 and HLA-Aw68.1 differ at only six positions in the α_2 domain and one position (245) in the α_3 domain[4]. We therefore reasoned that one or more of these substitutions is responsible for the difference in CD8 binding. Furthermore, a comparison of over 50 HLA-A,B,C sequences identified 245 as the only position at which there is a substitution that is specific to HLA-Aw68.1 and -Aw68.2 (ref. 4 and unpublished observations). In these two molecules, Val occurs at position 245 whereas other HLA-A,B,C molecules have Ala. These considerations suggested the involvement of residue 245 and the α_3 domain in the CD8 binding site.

To test this hypothesis we made a pair of mutants: the Ala 245 of HLA-A2.1 was converted to Val, giving the A2.1m245 mutant; and the Val 245 of HLA-Aw68.1 was converted to Ala, giving the Aw68.1m245 mutant. Analysis of transfectants expressing these mutant genes showed that the CD8 binding phenotype correlated with the residue at position 245 (Fig. 1). Thus mutant A2.1m245 showed no binding to CD8 whereas mutant Aw68.1m245 bound CD8 equivalent to wild-type HLA-A2.1. The specificity of this binding was also identical to that obtained with wild-type HLA-A2.1, in that it was dependent on CD8 and could be inhibited by monoclonal antibodies against CD8 and class I HLA molecules. Results of similar experiments with various other mutants argue against the involvement of the α_1 and α_2 domains in binding CD8. In these mutants the six differences (positions 95, 97, 107, 114, 116, 156) between HLA-Aw69, which binds CD8, and HLA-Aw68.1, which does not, were assessed. Conversion of various combinations of these substitutions from one sequence to the other had no effect on the CD8 binding phenotype, which in every case correlated with residue 245 (Table 2).

Previous speculation has suggested an Arg-Phe-Asp-Ser (RFDS) sequence, shared by many class I and class II MHC sequences and related to the fibronectin cell binding tetrapeptide Arg-Gly-Asp-Ser (RGDS), as a possible site for CD8 or CD4 binding[5]. Furthermore, Auffray *et al.* have shown that RFDS-containing peptides derived from class II sequences inhibit T-cell responses[6]. Conversely, the RFDS sequence of HLA-A2.1 (residues 35–38 of the α_1 domain) is, from the crystallographic structure, inaccessible and therefore an unlikely site for CD8

TABLE 1 Binding of CD8 to class I molecules

Experiment	Transfected class I gene	Cells bound per well ($\times 10^{-3}$) CD8$^+$	CD8$^-$
I	HLA-A2.1	32.2±6.4	8.8±0.8
	A2.2Y	38.8±1.0	11.2±1.0
	A2.3	33.1±1.9	9.5±1.2
	Aw69	25.6±3.0	12.5±0.9
	A3.1	49.6±1.2	9.2±1.8
	A3.2	31.4±0.6	5.3±0.4
	A1	27.2±1.4	9.7±2.1
	A24	28.3±1.6	6.5±1.2
	Aw68.1	15.1±1.8	8.2±1.3
	Aw68.2	13.9±1.0	9.7±1.3
	untransfected CIR	18.2±1.1	16.6±1.6
II	HLA-B13	60.4±4.6	25.3±2.3
	B38	41.1±2.9	10.6±0.6
	B44.2	54.4±2.1	21.1±1.2
	B49	41.1±3.8	11.5±1.2
	B51	41.7±0.4	15.5±1.2
	untransfected CIR	26.1±0.3	23.4±0.5
III	HLA-Bw58	39.6±4.7	8.1±2.1
	HLA-B7.1	20.2±0.8	5.7±1.2
	H-2D^p	60.2±9.7	11.7±2.8
	H-2K^b	86.7±6.0	8.8±5.9
	untransfected CIR	17.9±2.2	14.1±1.5

All transfectants were assayed as described in Fig. 1 legend. Results of three experiments are shown. The binding of class I transfected and untransfected CIR cells to CD8 expressing (CD8$^+$) and non-expressing (CD8$^-$) CHO cells is shown. Only HLA-Aw68.1 and HLA-Aw68.2 reproducibly showed no specific binding to CD8. The significance of variation in the positive binding of the other class I molecules is not known; it may result from differences in the properties of the individual transfectants and/or the affinity of the class I–CD8 interaction.

TABLE 2 CD8 binding to mutants of HLA-Aw69 and Aw68.1

HLA molecule	Amino-acid position 95	97	107	114	116	156	245	CD8 binding
Aw69	V	R	W	H	Y	L	A	+
69m95, 97, 245	I	M	W	H	Y	L	V	−
69m107, 245	V	R	G	H	Y	L	V	−
68.1m95, 97	V	R	G	R	D	W	V	−
68.1m107	I	M	W	R	D	W	V	−
Aw68.1	I	M	G	R	D	W	V	−

HLA-Aw69 and Aw68.1 differ only at the seven positions shown. Amino-acid substitutions in the HLA mutants are given by the standard single-letter code. Assays were performed as described in Fig. 1 legend.

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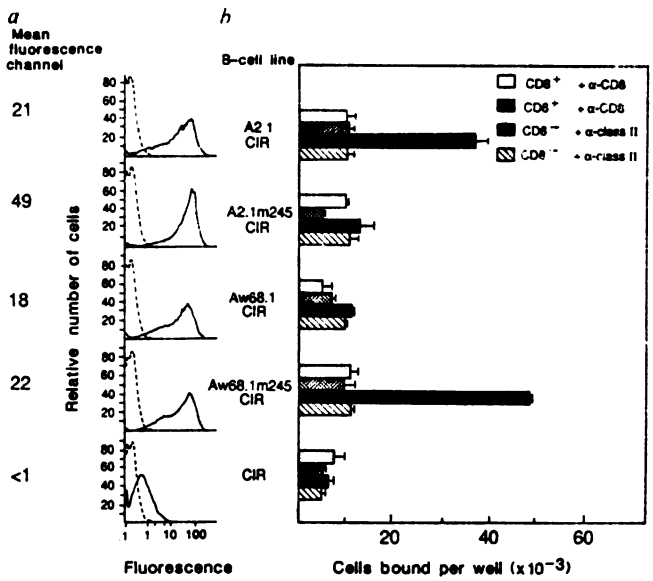

FIG. 1 Substitution at position 245 determines natural polymorphisms in CD8 binding. *a*, Expression of HLA-A2.1, A2.1m245, Aw68.1m245 and Aw68.1 molecules by transfectants of the CIR B-cell line which expresses no endogenous HLA-A,B molecules[18]. Indirect binding of the HLA-A2,A28-specific monoclonal antibody CR11-351 (ref. 19) (solid lines) was compared to an anti-actin control[20] (dotted lines) by flow cytometry. *b*, Binding of class I transfectants to CD8 expressing and non-expressing CHO cells in the presence of either anti-CD8 or anti-HLA-DR. Binding to CHO.1 and CHO.4 cells is labelled $CD8^-$ and $CD8^+$, respectively. In each group of four results the combination of cells and antibodies is, going from top to bottom: $CD8^+$ CHO cells with anti-CD8 (α-CD8); $CD8^-$ CHO cells with anti-CD8; $CD8^+$ CHO cells with anti-HLA-DR (α-class II); $CD8^-$ CHO cells with anti-HLA-DR. The standard errors are indicated by the 'error' bars. The HLA-A2,A28-specific monoclonal antibody CR11 -351 also inhibited cell–cell binding when preincubated with the CIR transfectants (data not shown). A second independently derived set of transfectants had identical CD8 binding characteristics (data not shown).

METHODS. Mutagenesis and transfection of class I genes were as previously described[18]. Flow-cytometric analysis of cells incubated first with monoclonal antibody and then with fluoresceinated goat anti-mouse antibody used a Becton-Dickinson FACS with a 10× neutral density filter. In CD8 binding experiments, CIR transfectants with class I genes were radiolabelled with ^{35}S methionine, washed twice, resuspended in PBS-containing, 10% fetal calf serum, and incubated in 96-well plates with confluent monolayers of transfected CHO cells. The CHO cells were previously incubated (30 min, 20 μg ml^{-1}, 37°) either with monoclonal antibody 51.1 (ref. 21), which is specific for CD8, or CA206, which is specific for HLA-DR (ref. 22), and was used as an irrelevant antibody control. CHO.1 cells express no CD8 whereas CHO.4 cells express ~160-fold more CD8 than peripheral T cells[3]. After incubation at 37° for 1 hour, wells were washed extensively, and bound radioactivity measured.

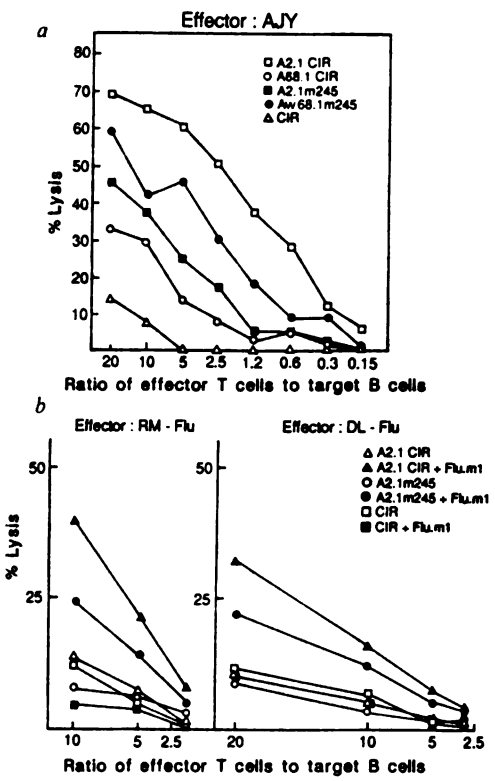

FIG. 2 Cytolysis of class I HLA-expressing CIR transfectants by alloreactive and antigen-specific T cells. *a*, Lysis of CIR transfectants expressing HLA-A2.1, A2.1m245, Aw-68.1m245 and Aw68.1 by an HLA-A2 alloreactive CTL culture AJY. In some experiments, the A2.1m245 transfectants were lysed better than Aw68.1m245-bearing cells. In all experiments, however, the AJY culture recognized HLA-A2.1 more effectively than A2.1m245, and Aw68.1m245 more effectively than HLA-Aw68.1. *b*, Lysis by influenza-specific CTL of HLA-A2.1- and A2.1m245-expressing transfectants after incubation with peptide corresponding to residues 56–68 of the influenza matrix protein. Donor RM (HLA-A1,A2; Bw55, B44), left panel; donor DL (HLA-A2, A11; B27, B44), right panel. All but one of the six donors gave strong influenza-specific, HLA-2.1-restricted CTL. None of the responders lysed HLA-Aw68.1- or Aw68.1m245-bearing transfectants. ▲, △: lysis of A2.1 transfectant with and without the Flu.m1 peptide, respectively. ●, ○; lysis of A2.1m245 transfectant with and without the Flu.m1 peptide, respectively. ■, □; lysis of untransfected CIR cells with and without the Flu.m1 peptide, respectively.

METHODS. ^{51}Cr-release assays were performed as previously described[23]. The AJY culture was generated by *in vitro* stimulation of peripheral blood lymphocytes (PBL) from a single donor AK (HLA-A3; B7, B38) with the HLA-A2.1-expressing cell line JY (ref. 23), and it lyses HLA-A2.1-, Aw69-, and Aw68-bearing targets with varying efficiency. Influenza-specific cultures were generated essentially as previously described[24]. PBL from six HLA-A2 positive donors were stimulated with 10 μg ml^{-1} of a peptide (Flu.m1) derived from residues 56–68 of the influenza matrix protein shown previously to generate HLA-A2-restricted CTL (ref. 25). Three days after the initial stimulation, supernatant containing growth factors from IL-2-stimulated PBL was added. Cultures were restimulated after 7 days to generate secondary cultures. For cytolysis assays, target cells were preincubated overnight with 10 μg ml^{-1} of the Flu.m1 peptide, then used in ^{51}Cr-release experiments.

binding[7,8]. This view is supported by our finding that a mutant of HLA-A2.1 in which Arg 35 is substituted by Val shows no loss of CD8 binding activity (data not shown).

The question arises as to whether the differences in the cell-binding assay have functional consequences for cytotoxic T cells. To address this, we compared recognition of position 245 mutants by both alloreactive and antigen-specific, MHC-restricted CTL. The alloreactive CTL show specificity for HLA-A2.1 and effectively lyse the HLA-A2.1 transfectant. This lysis, however, is reduced by replacement of Ala 245 with Val (Fig.

2). By contrast, these CTL show a poor lysis of the HLA-Aw68.1 transfectant, which is considerably improved by replacement of Val 245 with Ala. Similar effects were observed with HLA-A2.1-restricted CTL lines specific for peptide 56–68 of the influenza matrix protein. Cytolysis was significantly reduced when Ala 245 of the HLA-A2.1 restriction molecule was replaced by Val (Fig. 2). Changes in CTL function can thus be correlated with changes observed in the cell-cell binding assay: greater lysis is observed when the target molecule binds more strongly to CD8.

Both alloreactive and antigen-specific CTL used in these experiments were inhibited by anti-CD8 monoclonal antibodies. Surprisingly, inhibition was not dependent upon whether the class I target could bind CD8 in the cell-cell binding assay. Thus recognition of HLA-Aw68.1 or HLA-A2.1m245 was inhibited by anti-CD8. In representative experiments, cytolysis by alloreactive CTL of HLA-Aw68.1 and HLA-A2.1 was, respectively, reduced from 29 to 8% and from 56 to 39% by anti-CD8 antibody 51.1. Lysis of peptide-sensitized A2.1m245 transfectants by influenza-specific CTL was reduced from 37 to 16% by anti-CD8, whereas no effect was seen on lysis of HLA-A2.1 transfectants. These observations might reflect a residual affinity of these molecules for CD8 that is not detected in the cell binding assay. An alternative is suggested by other studies in which the inhibition of CTL by anti-CD8 antibodies, in a manner that is unrelated to binding of class I molecules, is interpreted as evidence for a regulatory role for CD8 in T-cell activation[9,10].

It is clear that substitution at position 245 alters the interaction of class I HLA molecules with CD8. This could result from a direct involvement of this residue in the interaction or by an indirect conformational effect on a distant site. The crystallographic structures of HLA-A2.1 and -Aw68.1 at high resolution have been determined and they are extremely similar (T. Garrett, M. Saper, P. Bjorkman and D. Wiley, unpublished observations). Thus, it is probable that the distinct functional properties of these two molecules arise from the nature and disposition of the side-chains at the positions of amino-acid substitution and not through gross conformational differences. These observations combined with the complete change in CD8 binding phenotype caused by single substitutions at position 245 in HLA-A2.1 and HLA-Aw68.1 strongly favour the direct involvement of this residue and the α_3 domain in the CD8 binding site.

Further support for this interpretation comes from experiments showing that a mutant of H-2D^d derived *in vitro* with a single substitution at position 227 shows specific loss of interaction with cytotoxic T cells that are dependent on Lyt-2, the murine equivalent of CD8[11,12]. Positions 227 and 245 of the α_3 domain are located proximal to the membrane attachment site in a solvent accessible surface region and are separated by only 10.5 Å (refs 7, 8). Both could therefore contribute to the CD8 binding site.

Davis and Bjorkman have raised the possibility that CD8 interacts with the α_1 and α_2 domains of class I MHC molecules, in a manner analogous to the T-cell receptor[13]. A consequence of this model is that simultaneous interaction of CD8 and the T-cell receptor with the same class I MHC molecule cannot occur. Our results and those of Potter *et al.*[11,12], suggesting interaction of CD8 with the α_3 domain, do not support this view and leave open the possibility that a complex of T-cell receptor and CD8, binding to the same class I MHC molecule (plus its antigenic peptide), is crucial to T-cell activation.

Comparison of the amino-acid sequences of antigen-presenting class I MHC molecules from seven mammalian species (summarized in ref. 14) shows that HLA-Aw68.1 and -Aw68.2 are unique in not having Ala at position 245. This combined with the observation that all 15 molecules with Ala at this position bound CD8 in the cell-cell binding assay suggest that the non-binding CD8 phenotype will be rare among class I MHC products. In most human populations, however, the frequency of HLA-A28, of which HLA-Aw68.1 and -Aw68.2 are subtypes, is 8–9% (ref. 15). The intriguing question is whether reduced CD8 binding confers any selective advantage or disadvantage to individuals expressing these molecules. For example, does it affect the capacity of HLA-Aw68.1 and -Aw68.2 to present antigens, or the repertoire of HLA-Aw68.1- and HLA-Aw68.2-restricted T-cell receptors selected in the thymus? In studies by Rickinson *et al.* CTL specific for Epstein–Barr virus (EBV), generated by *in vitro* priming and restricted to HLA-Aw68, were never obtained, although HLA-Aw69-restricted CTL lysed infected HLA-Aw68 targets[16,17]. This suggests that although HLA-Aw68 can present EBV peptides to specific CTL, it may be deficient in generating such a response. The number of epitopes and restriction patterns described for human class I MHC molecules, however, is still too small to assess the relative use of HLA-Aw68.1 and -Aw68.2. □

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Chapter 5

A Second Subunit of CD8 is Expressed in Human T Cells

Introduction

While we were studying the binding of human CD8α to MHC class I molecules, a human CD8β partial genomic clone was isolated by Pauline Johnson at Oxford University. This clone appeared capabable of encoding a protein homologous to rodent CD8β, and hybridized with transcripts in human T cells. Although experiments with transfected T cell hybridomas indicated that a CD8-dependent response could be reconstituted with the TCR and the CD8α-chain alone, it was still possible that the β-chain of CD8 might also be important for MHC class I binding, or transmit essential regulatory signals *in vivo*. Therefore we wanted to determine whether CD8β was also expressed in human T cells, and if so, to analyze the contribution of CD8β to CD8-MHC class I interactions in the cell-cell binding assay.

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A second subunit of CD8 is expressed in human T cells

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The CD8 glycoprotein plays important functions in T cell development and in T cell activation. In rodents, CD8 is a heterodimer, consisting of an α-chain (Lyt2) and a β-chain (Lyt3). In humans, only the α-chain has been detected, and it has been thought that CD8 consists of homodimers of this protein. We have isolated functional cDNA clones encoding human CD8β, and show that the CD8β protein is expressed on the surface of CD8$^+$ human T cells. cDNA clones encoding multiple forms of the human CD8 β-chain have been isolated and characterized. These structural variants, which are likely to arise by alternative splicing, differ in the sequences encoding the cytoplasmic domain, which can consist of 19, 30 or 52 amino acids. One of the cDNAs lacks nucleotide sequences corresponding to a hydrophobic transmembrane domain, and may encode a secreted CD8β protein. The protein product of the human CD8β gene can be detected by a recently described anti-CD8 monoclonal antibody, 597. Expression of the epitope recognized by this antibody requires co-expression of the CD8α and CD8β gene products. About 90% of human CD8α positive thymocytes and peripheral blood lymphocytes express CD8β at the cell surface. Expression of the CD8β chain is thus conserved between human and rodents, and the variant CD8β polypeptides may have distinct roles in T cell function and development.

Key words: cell–cell interactions/CD8/Lyt3/T cell/alternative splicing

Introduction

The CD4 and CD8 glycoproteins are expressed on separate subpopulations of T lymphocytes and are thought to play major roles in thymocyte development and in interactions between T cells and their targets (reviewed by Littman, 1987). While the T cell antigen receptor (TCR) determines the antigen and MHC specificity of T cells, the expression of either the CD4 or CD8 glycoprotein is usually required for effective T cell activation (Dembic *et al.*, 1987; Gabert *et al.*, 1987). A correlation exists between the expression of CD4 or CD8 and the major histocompatibility complex (MHC) specificity of a particular T cell. T cells that express CD4 respond to class II MHC molecules, while T cells that express CD8 respond to class I MHC molecules (Swain, 1983). Based on this correlation and on the observation that anti-CD4 and anti-CD8 antibodies inhibit T cell function, it was suggested that CD4 and CD8 increase the avidity of T cells for their targets by binding to class II or class I MHC molecules respectively (Marrack *et al.*, 1983; Swain, 1983). Low-affinity binding between CD4 and MHC class II molecules in the absence of the TCR has been recently demonstrated (Doyle and Strominger, 1987), and we have observed similar binding between CD8 and MHC class I molecules (Norment *et al.*, 1988).

In addition to their ability to enhance T cell-target cell binding, CD4 and CD8 appear to alter activation signals in mature T cells. In the absence of cell–cell binding, monoclonal antibodies against CD4 or CD8 will inhibit activation of T cells by anti-TCR antibodies (Moldwin *et al.*, 1987; Blue *et al.*, 1988). It is unclear whether this effect is due to a negative regulatory signal transmitted directly to T cells, or whether there is steric hindrance of TCR cross-linking by the presence of anti-CD4 or anti-CD8 antibodies (Owens and Fazekas de St Groth, 1987). In contrast, when anti-CD4 or anti-CD8 monoclonal antibodies are cross-linked to anti-TCR antibodies, a marked synergy in activation is observed (Emmrich *et al.*, 1987; Walker *et al.*, 1987). It has also been speculated that CD4 or CD8 may become physically associated with the TCR (Saizawa *et al.*, 1987; Takada and Engleman, 1987; Rivas *et al.*, 1988). The transmembrane domains and cytoplasmic tails of CD4 and CD8 are highly conserved between humans and rodents (Littman, 1987), suggesting that these regions may be involved in signal transduction upon contact of T cells with their targets.

Even though CD4 and CD8 are both members of the immunoglobulin (Ig) gene superfamily (Williams and Barclay, 1988), they do not share any sequence or structural similarities to suggest that they perform similar functions. CD4 is a 55-kd glycoprotein that appears to be expressed as a monomer. In humans, CD8 has been shown to be composed of homo-multimers of a 32- to 34-kd glycoprotein. In contrast, rodent CD8 is composed of two distinct chains, α (Lyt2) and β (Lyt3), which are encoded by closely linked genes (Gorman *et al.*, 1988). The α-chain corresponds to the human CD8 polypeptide; no human equivalent of the β-chain polypeptide has been detected, despite extensive biochemical analysis of CD8 (Snow and Terhorst, 1983; Snow *et al.*, 1984). Recently, a human CD8β partial genomic clone has been identified (Johnson, 1987). This clone contains an open reading frame that encodes a protein homologous to rodent CD8β. CD8β transcripts are present in thymocytes and in the human T cell tumor line HPB-ALL at levels comparable to those of CD8α (Johnson, 1987). This result prompted us to search for a CD8β protein in human T cells.

Here we report the isolation and expression of full-length cDNAs encoding human CD8β (Lyt3). The human CD8β cDNAs encode proteins that can be detected by a recently described anti-CD8 monoclonal antibody (mAb). Staining with this mAb demonstrates that the majority of CD8α-positive peripheral blood lymphocytes (PBL) also express

CD8β. In addition, multiple forms of CD8β cDNAs, most likely resulting from alternative splicing, have been identified. These cDNAs encode proteins having different cytoplasmic domains or lacking a transmembrane anchor sequence. These variant CD8 proteins may differ in their functions in cell–cell interactions and T cell activation.

Results

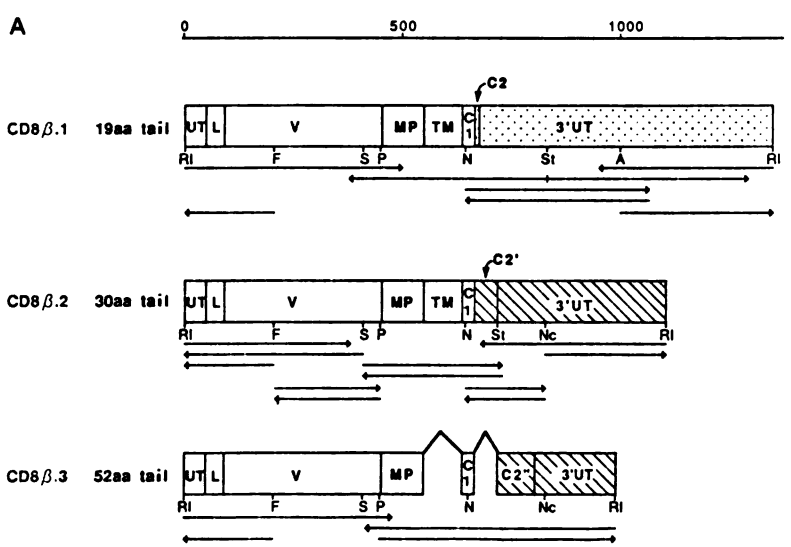
Cloning and sequence of a human CD8β cDNA

A partial human CD8β genomic clone containing exons encoding the Ig-like region, a membrane proximal domain, a putative transmembrane domain and the first part of the cytoplasmic tail has been previously isolated (Johnson, 1987). Sequences encoding the 5′ untranslated region, leader peptide and 3′ untranslated region were not identified, and it was possible that the gene or its product might be defective in humans. In order to establish whether human CD8β transcripts contain an open reading frame capable of encoding a functional protein, we have isolated and characterized human CD8β cDNAs. A λgt10 cDNA library prepared from human peripheral T cells was screened with a fragment from the Ig-like region of the human CD8β (Lyt3) genomic clone (Johnson, 1987). Three cDNA clones were isolated and sequenced. The restriction maps and nucleotide sequences of these clones are shown in Figure 1. The cDNAs are identical from the 5′ untranslated sequence, through an open reading frame that encodes a putative hydrophobic leader peptide, an Ig-like region and a serine- and threonine-rich membrane proximal domain. There is a single site for potential N-linked glycosylation at Asn-81. Beyond the membrane proximal domain, the three cDNAs diverge. A putative hydrophobic transmembrane domain is encoded by β.1 and β.2 but not by β.3 (Figure 1A and B). Based on a comparison with the sequence of genomic DNA (Johnson, 1987), it is clear that the absence of the transmembrane domain from β.3 is the result of an in-frame alternative splicing event that excludes the exon encoding nucleotides 543–633. This finding is of particular interest because similar splicing, which excludes nucleotides encoded by the transmembrane exon from CD8α transcripts, results in the production of a secreted CD8α homodimer (A.Norment and D.Littman, in preparation). The alternatively spliced forms of the two chains may therefore encode products that form a secreted heterodimer.

The differences in the putative cytoplasmic domains encoded by the three CD8β cDNAs also appear to be due to alternative splicing. The lengths of the variant cytoplasmic tails are indicated in Figure 1A. Codons in the transmembrane exon encode the first three amino acids of the cytoplasmic tail. All three cDNA clones have a nucleotide sequence (C1), defined by previous sequencing of genomic DNA, that encodes 13 cytoplasmic amino acid residues. Additional sequences not yet defined in genomic DNA are found 3′ to the C1 region in the three cDNAs. β.1 contains a sequence 3′ to C1 that encodes three additional amino acids. The predicted cytoplasmic domain of β.1 is most like that of rodent CD8β; it is the same size and contains 13/19 identities with the rat homolog (Johnson and Williams, 1986). In β.2, C1 is followed by a distinct sequence that encodes 14 additional amino acids (C2′). In β.3, the C2′ segment is absent, and C1 is followed by C2″, which encodes an additional 36 amino acids. This sequence is identical to the 3′ untranslated sequence of β.2; the transcript corresponding to CD8β.3 therefore probably arises by exclusion of an exon encoding C2′. Another less likely explanation is that this transcript results from the use of an alternate splice acceptor within the 3′ untranslated region of β.2; however, this putative splice acceptor lacks the consensus upstream pyrimidines (Mount, 1982). A search of the NBRF database failed to identify any known proteins with homology to C2′ and C2″. Although each cytoplasmic domain contains unique tyrosine and serine residues, none of these is predicted to be a substrate for tyrosine kinases (Hunter and Cooper, 1985) or protein kinase C (House *et al.*, 1987).

In vitro translation of human CD8β protein

To determine whether the human CD8β cDNA could encode a full-length membrane protein, RNA was synthesized and translated *in vitro* from the CD8β.2 cDNA (Figure 2). A translation product migrating with an apparent relative



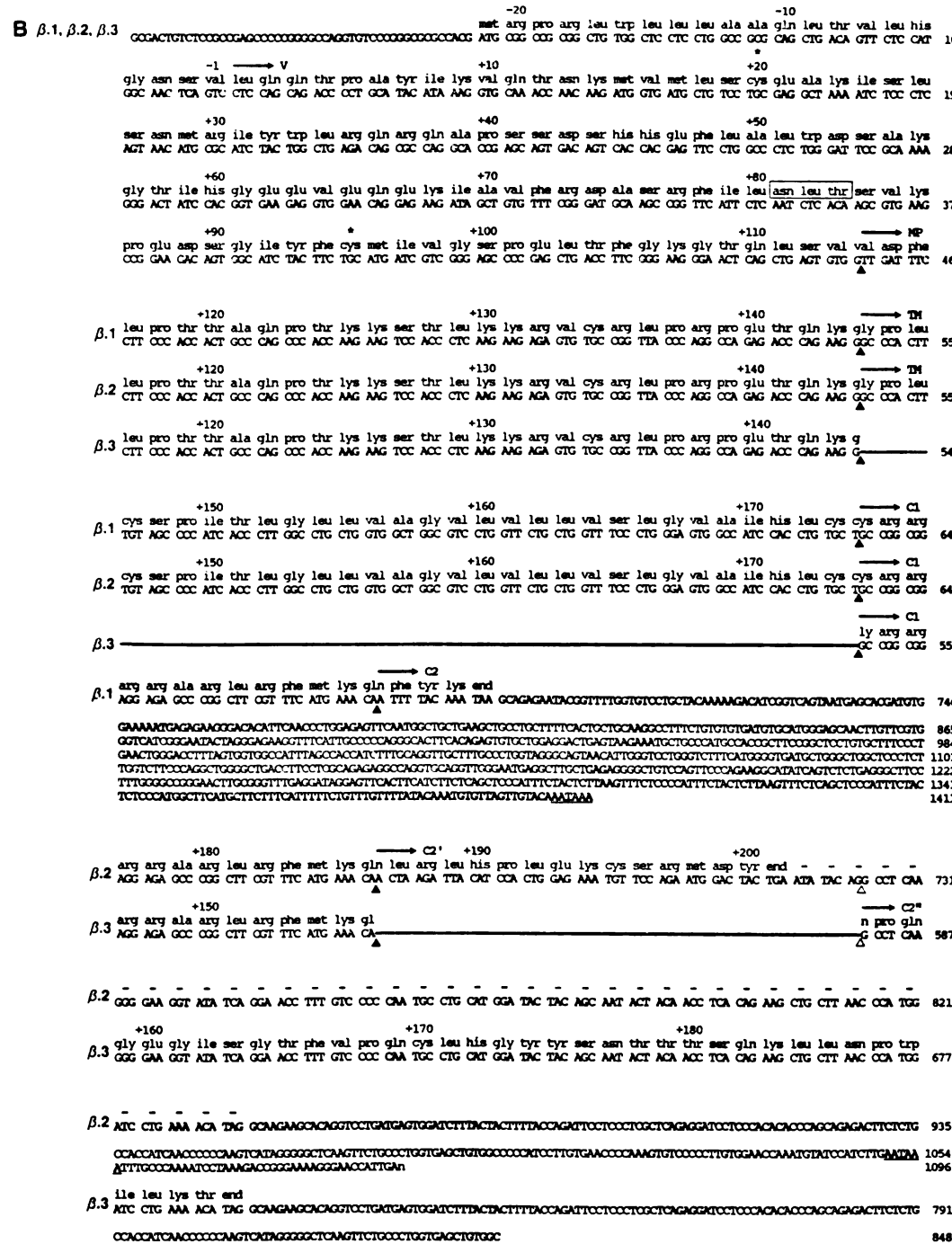


Fig. 1. Nucleotide sequence of human CD8β cDNAs and translated sequence of the corresponding proteins. Sequences encoding the 5′ untranslated (UT), leader (L), immunoglobulin V-like (V), membrane proximal (MP), transmembrane (TM), cytoplasmic (C) and 3′ untranslated (3′UT) regions are indicated. (**A**) Schematic representation of human CD8β cDNAs. A scale in nucleotide base pairs is indicated at the top of the figure. The region of CD8β.1 (stippled) that differs from CD8β.2 and CD8β.3 (stripes) is marked. Arrows represent fragments sequenced as described in Materials and methods. The length of the predicted cytoplasmic tail of each putative CD8β protein is indicated to the left of the figure. Restriction endonuclease sites are as follows: *Eco*RI (RI), *Fsp*I (F), *Sau*3AI (S), *Pvu*II (P), *Nae*I (N), *Stu*I (S), *Ava*II (A), *Nco*I (Nc). (**B**) Sequence of the CD8β cDNAs and the predicted proteins. The numbers to the right show nucleotide positions. Numbers shown above the amino acid sequence designate residues in the mature protein, as predicted by alignment with the rat CD8β (Lyt3) protein sequence (Johnson and Williams, 1986). Arrows designate domains of the proteins listed above. Solid lines indicate sequences absent in CD8β.3. Dashes indicate amino acid residues absent in CD8β.2. Two cysteines believed to form the Ig-like intrachain disulfide bond are marked by an asterisk above the amino acid sequence. A potential N-linked glycosylation site at Asn-81 is boxed. Solid arrowheads indicate known intron−exon boundaries (Johnson, 1987); open arrowheads indicate potential intron−exon boundaries predicted by the sequences of the cDNAs. Polyadenylation signals are underlined.

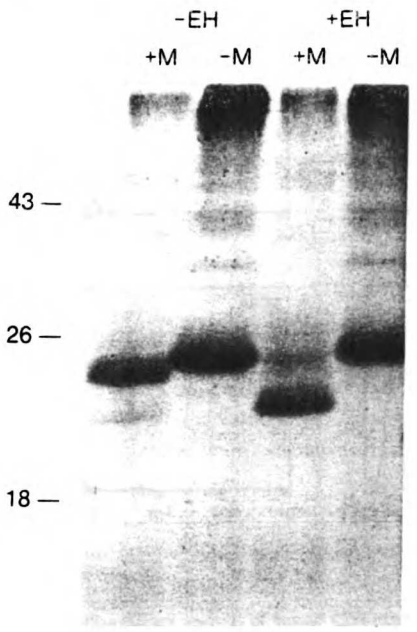


Fig. 2. *In vitro* translation of CD8β.2. The human CD8β.2 cDNA was transcribed *in vitro*, and the RNA was then translated using rabbit reticulocyte lysates in the presence (+M) or in the absence (−M) of microsomal membranes. Samples were then incubated in the presence (+EH) or in the absence (−EH) of endoglycosidase H, and analyzed by SDS−PAGE. Relative molecular masses (M_r) are given in kilodaltons to the left of the figure.

molecular mass of 25 kd was observed by SDS−PAGE. Translation of mRNA derived from the CD8β.2 cDNA in the presence of microsomal membranes resulted in a 24-kd protein that was decreased in size to 22.5 kd following incubation with endoglycosidase H (Figure 2). These results are consistent with the size of the precursor protein (25 236 daltons) and of the cleavage product (22 856) predicted from the primary sequence, assuming that the cleavage of the leader sequence is at a position corresponding to that in rat CD8β (Johnson and Williams, 1986). With a single predicted N-linked glycosylation site, the molecular mass of the mature CD8β.1 and CD8β.2 proteins would be ~25 and ~26 kd respectively. However as in CD8α, there may be additional O-linked glycosylation of the serine- and threonine-rich membrane proximal domain (Snow *et al.*, 1985). The sizes of mature CD8α and CD8β glycoproteins may thus be very similar.

Expression of human CD8β transcripts

To assess the expression of the CD8β gene products, L cells were transfected with expression vectors encoding CD8β.2, CD8α or both (Figure 3). RNA samples prepared from transfected cell lines, from human tissues and from human T cell leukemias were analyzed for the presence of CD8α and CD8β transcripts. As shown in Figure 3, transfected cells expressed a 2.3-kb CD8α transcript and a 1.5-kb CD8β transcript at levels comparable to those of the T cell leukemia line HPB-ALL. A 1.5-kb CD8β mRNA and a minor transcript of 1.0 kb are expressed in human thymocytes (THY), lymph node cells (LN) and spleen cells (SPL)

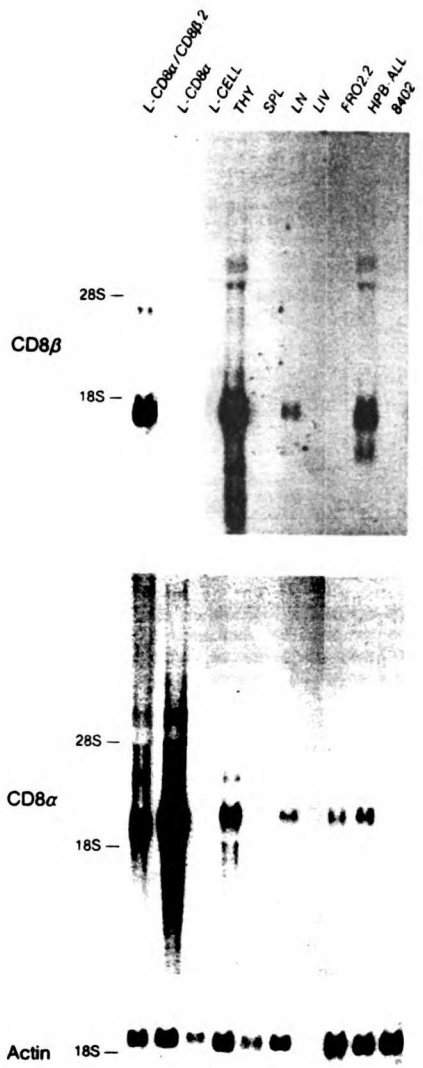


Fig. 3. Northern blot analysis of human CD8 transcripts. Total RNA from L cells, L cells transfected with CD8α or CD8α/CD8β, normal human tissues, and T cell leukemias was analyzed for expression of CD8β, CD8α, and actin transcripts as described in Materials and methods. Normal human tissues include: thymus (THY), spleen (SPL), lymph node (LN) and liver (LIV). T cell leukemia lines include: FRO2.2 ($CD4^+$, $CD8^+$), HPB-ALL ($CD4^+$, $CD8^+$) and 8402 ($CD4^-$, $CD8^-$). The position of 18S and 28S rRNAs is indicated to the left of the figure.

(Figure 3). Although both HPB-ALL and FRO2.2 express CD8α mRNA, only HPB-ALL expresses the CD8β transcript. The T cell leukemia line, 8402, fails to express either CD8 transcript.

Detection of the human CD8β protein

MAbs reactive with human CD8 have been shown to differ in their ability to bind to peripheral T cells and to CD8α-transfected L cells (Martin *et al.*, 1984; Disanto *et al.*, 1987). These observations suggested that some of these antibodies may recognize epitopes of CD8β or combined epitopes of CD8α and CD8β. A panel of anti-CD8 mAb (Disanto *et al.*, 1987) was therefore screened for reactivity with CD8α-

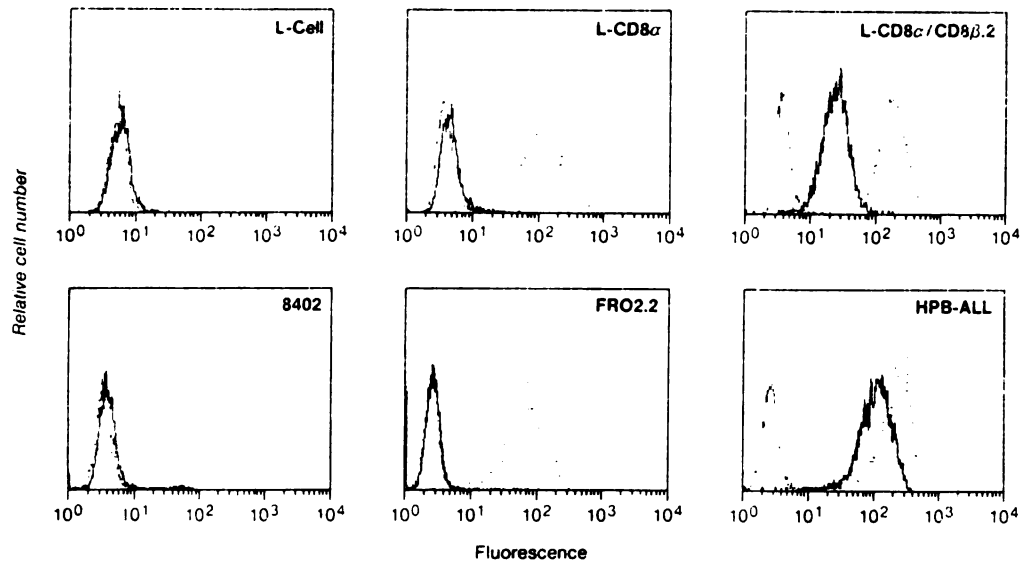


Fig. 4. Surface immunofluorescence analysis of human CD8 expression by transfected cells and T cell lines. Cells are described in Materials and methods, and the legend to Figure 3. Cells were incubated with the anti-CD8α monoclonal antibody OKT8 (fine dotted line), 597 (solid line) or control antibody (heavy dotted line). The control antibodies were W6/32 (anti-MHC class I), for L-tk⁻ cells and their derivatives, and GK1.5 (anti-murine CD4), for the T cell leukemia lines. Staining utilized a second step fluorescein-conjugated goat anti-mouse IgG antibody. Fluorescence is marked in arbitrary units along the *x*-axis.

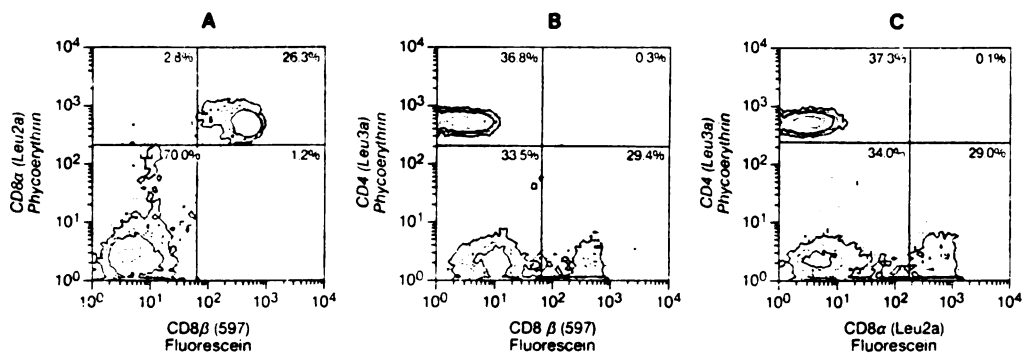


Fig. 5. Dual-color immunofluorescence analysis of human CD8 on PBL. Human PBL were stained as described in Materials and methods using the monoclonal antibodies indicated along the *x*- and *y*-axes of the figure. Fluorescence is marked in arbitrary units along the axes. Statistical markers were set to exclude the dim CD8α⁺ cells; the percentage of gated lymphocytes is indicated in the upper right-hand corner of each quadrant. None of the dim CD8α⁺ cells expressed the 597 determinant.

positive L cells by surface immunofluorescence. Of 40 antibodies tested, 38 bound well to the surface of transfected cells, one (572) bound poorly and another (597) did not bind at all. The mAb 597 has been shown previously not to bind to CD8α-transfected cells (Disanto *et al.*, 1987). When mAbs 572 and 597 were tested for binding to L cells transfected with both of the CD8α and CD8β cDNAs, 597 reacted with the cells (Figure 4). In addition, 597 reacts with HPB-ALL cells, which express mRNA for both CD8 chains, but it does not react with FRO2.2 cells, which express CD8α alone. MAb 597 does not bind to cells transfected with CD8β.2 in the absence of CD8α (data not shown). These results indicate that mAb 597 detects a product of the CD8β gene that is expressed on the surface of transfected cells and on the human T cell leukemia HPB-ALL.

The expression of the 597 epitope on normal human PBL and thymocytes was examined by two-color surface immunofluorescence. About 90% of bright CD8α-positive PBL stain with 597 (Figure 5, panel A); in contrast, none of the CD4 positive cells stain with 597 (panel B). In the same experiment, 23% of dim CD8α⁺ cells specifically bound the natural killer cell specific mAb leu11 (Lanier *et al.*, 1983), but none bound mAb 597 (data not shown). This observation suggests that unlike CD8α, CD8β is not expressed on the human natural killer cell population. Alternatively, a threshold level of CD8α expression may be required for

expression of CD8β at the cell surface. Of human thymocytes, ~90% of CD8α-positive cells express the 597 epitope. Expression of CD8β is similar for $CD4^+/CD8^+$ double-positive thymocytes and mature $CD4^-/CD8^+$ single-positive thymocytes (data not shown). We were unable to detect a population of PBL or thymocytes which express the 597 epitope in the absence of CD8α.

It appears that CD8β-positive T cells may be a subset of CD8α-positive T cells. In mouse, surface expression of the CD8β-chain (Lyt3) is thought to be dependent on expression of the CD8α (Lyt2) subunit (Blanc *et al.*, 1988; Gorman *et al.*, 1988). In our studies, CD8β could be detected by mAb 597 only on cells that also expressed CD8α. The possibility remains that human CD8β can also be expressed at the surface in the absence of CD8α; it has not been determined whether the epitope recognized by mAb 597 resides on the CD8β chain or is specified by both chains.

Discussion

These studies indicate that the human CD8β gene product is expressed on the surface of the $CD8^+$ subset of T lymphocytes. Based on these findings, it is puzzling that the human CD8β protein has not been previously detected. For example, proteolytic analysis of CD8 immunoprecipitated from ^{125}I-surface-labeled HPB-ALL cells and PBL indicates that human CD8 is composed of the single CD8α chain (Snow and Terhorst, 1983). This was confirmed by amino-terminal sequencing of CD8 isolated from HPB-ALL cells (Snow *et al.*, 1984). Several possibilities may account for this paradox: (i) CD8β may co-migrate with CD8α when analyzed by SDS–PAGE; (ii) CD8β may label poorly with ^{125}I; (iii) there may be variations in CD8β expression by leukemic cell lines analyzed in different laboratories; (iv) the amino terminus of the CD8β chain from HPB-ALL cells may be blocked; (v) the anti-CD8 mAbs used for immunoprecipitation or purification may react with the CD8α homodimer, but not with the heterodimer; and (vi) unlike rodent CD8β, the human homolog may not be disulfide linked to CD8α, and association of the two chains may be disrupted upon detergent solubilization. When mAb 597 was used to immunoprecipitate lysates of ^{125}I-surface-labeled PBL, no protein was detected (Disanto *et al.*, 1987). However, we have been able to detect a small amount of protein by immunoprecipitation of [^{35}S]cysteine-labeled lysates of HPB-ALL cells using mAb 597. This co-migrates with CD8α under reducing and non-reducing conditions (data not shown).

The three cDNAs that we have isolated encode CD8β polypeptides that differ in their cytoplasmic domains. This is interesting in light of recent evidence that the CD4 and CD8 glycoproteins may function not only in adhesion, but also in transduction of regulatory signals in the course of T cell activation (Emmrich *et al.*, 1987). The variant CD8β polypeptides may differ in their abilities to interact with components of the cytoskeleton and with other T cell surface proteins, or they may be subject to alternative means of regulation through covalent modification. These forms of CD8β may also have different requirements for association with CD8α and surface expression.

The structure and pattern of expression of CD8β appear to be conserved between rodents and humans, suggesting that this molecule serves an important function. Gene transfer experiments indicate that CD8α and the α- and β-TCR genes are sufficient to reconstitute antigen-specific T cell activation (Gabert *et al.*, 1987). It is unclear whether CD8β would have any function in this system. We have detected binding of CD8α to class I MHC molecules in the absence of the T cell antigen receptor (Norment *et al.*, 1988). It is possible that CD8α homo-multimers may be sufficient for binding to some MHC class I molecules, but that $\alpha\beta$ heterodimers may extend the range of polymorphic class I molecules to which CD8 can bind. Alternatively CD8β may be primarily involved in transmission of regulatory signals to T cells upon binding of CD8α to class I MHC molecules on the target cell.

Materials and methods

Library screening and DNA sequencing

Preparation of a λgt10 human peripheral T cell cDNA library was as described (Littman *et al.*, 1985). By hybridization with a nick-translated (Bethesda Research Laboratory) CD8β genomic *NcoI–PvuII* fragment (a gift of Drs P.Johnson and A.Williams, University of Oxford), 11 positive plaques were identified out of 6×10^5 screened. Hybridization was performed overnight in 30% formamide, 5 × SSCPE and 5 × Denhart's solution. Washes were at 55°C in 0.1% SDS and 2 × SSC. Three plaques were isolated, and the insert DNA subcloned into M13mp18 (New England Biolabs) or Bluescript M13-KS (Stratagene) at the *Eco*RI site. Sequencing was performed on both strands of single-stranded DNA by dideoxy-chain termination (Sanger *et al.*, 1977).

In vitro transcription and translation

The CD8β.2 cDNA was subcloned into the vector pSP72 (Krieg and Melton, 1987), linearized with *Hin*dIII, and transcribed according to standard protocols with T7 polymerase. *In vitro* translation was subsequently performed using rabbit reticulocyte lysates (Promega) according to the manufacturer's instructions, with [^{35}S]methionine (>600 Ci/mmol, Amersham) included in the reaction, in the presence or absence of canine pancreatic microsomal membranes [a gift of Drs V.Lingappa and D.W. Andrews, University of California—San Francisco (UCSF)] for 60 min at 26°C. Endoglycosidase H (Boehringer Mannheim) digestion was performed overnight at 37°C in the presence of 0.02 U/ml of enzyme, 0.1 M sodium citrate, pH 5.5, 0.1% SDS. Control samples received no endoglycosidase H. Samples were analyzed by SDS–PAGE on a 12.5% Laemmli gel.

Transfection of L-tk$^-$ cells

Murine L cells deficient in thymidine kinase (L-tk$^-$) were transfected by calcium phosphate precipitation with the CD8α cDNA pT8F1 (Littman *et al.*, 1985) inserted into the vector pMV6Tkneo (Maddon *et al.*, 1985), and selected in 400 μg/ml of G418 (Geneticin, Gibco). Colonies expressing CD8α were detected by rosetting using the monoclonal antibody OKT8, and human red blood cells conjugated with rabbit anti-mouse IgG (Zymed) as described (Littman *et al.*, 1985). One $CD8\alpha^+$ clone (L-CD8α) and L-tk$^-$ cells were subsequently co-transfected with pTk, and the CD8β.2 cDNA subcloned into the vector pSV7d (from Dr P.Luciw, University of California—Davis), a derivative of pHS210 (Stuvé *et al.*, 1987). After selection in hypoxanthine, aminopterin and thymidine (HAT), colonies were expanded and analyzed for expression of CD8β mRNA on Northern blots and a representative colony was chosen for further analysis.

Cells

Murine L cells (tk$^-$) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS, Gibco). Human T cell leukemia lines FRO2.2, 8402, and HPB-ALL (Littman *et al.*, 1985) were grown in RPMI 1640 supplemented with 12.5% FBS (Hyclone). Human PBL were isolated by Ficoll-Hypaque density gradient centrifugation. Human thymocytes (from Dr L.Bockenstedt, UCSF) were isolated from fresh tissue samples, and frozen with liquid nitrogen in RPMI 1640, 10% FBS and 10% DMSO. Aliquots of human thymocytes were thawed in the presence of 1 μg/ml DNase I.

Northern blots

Eight micrograms of total RNA was fractionated on a 1% agarose gel containing 40 mM morpholinopropanesulfonic acid (MOPS), 10 mM sodium

acetate and 1 mM EDTA plus 7% formaldehyde, and transferred to Genescreen (New England Nuclear) in 20 × SSC. RNA was crosslinked to the filter by UV irradiation. Hybridization was performed according to Church and Gilbert (1984) in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1% bovine serum albumin (BSA), 1 mM EDTA and 25% formamide for 24 h at 65°C with 10 ng/ml of nick-translated (Bethesda Research Laboratory) probe. The filter was washed in 40 mM sodium phosphate, pH 7.2, 1% SDS and 1 mM EDTA. The filter was first probed with the CD8β.2 cDNA (2 × 10^8 c.p.m./μg), and then stripped and reprobed with the CD8α cDNA T8F1 (Littman *et al.*, 1985), followed by a hamster actin cDNA as a control for RNA loading and degradation.

Antibodies and immunofluorescence analysis

Antibodies included in these studies were as follows. The panel of anti-CD8 antibodies was provided by the Third International Leukocyte Typing Workshop, Dr A.McMichael, director (Disanto *et al.*, 1987), and included the antibody 597 (2ST85H7). These antibodies were in the form of ascites and were diluted 1:100 for immunofluorescence studies. Additional 597 (IgG2a) was a gift of Dr E.Reinherz, and was used at a dilution of 1:200. OKT8 (IgG2a) and W6/32 (a gift of Dr P.Parham, Stanford University) were affinity purified by protein A sepharose chromatography and used at 10 μg/ml. GK1.5, Leu2a (unconjugated and phycoerythrin conjugated), Leu3a (phycoerythrin conjugated) and goat anti-mouse IgG (fluorescein conjugated) were from Becton Dickinson and were used according to the manufacturer's instructions. For immunofluorescence studies, 1 × 10^6 cells were incubated with saturating levels of antibody in 0.1 ml of PBS and 5% FBS for 30 min at 4°C. Cells were washed twice with 3 ml of 5% FBS in PBS at 4°C. For dual color immunofluorescence analysis, PBL and thymocytes were stained as follows: cells were incubated with 597 or Leu2a, and then with fluorescein-conjugated goat anti-mouse IgG; this was followed by blocking with saturating levels of normal mouse serum (Zymed) and staining with phycoerythrin-conjugated Leu2a or Leu3a antibody. Fluorescence was measured in arbitrary units using a FACScan cell analyzer (Becton Dickinson).

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Chapter 6

Characterization of Structural Forms of Human CD8β

Introduction

In humans and rodents CD8 is composed of two distinct polypeptide chains, α(Lyt-2) and β(Lyt-3). In rodents the β-chain is disulfide-bonded to the α-chain through cysteine residues in the membrane proximal domain (1), and requires association with CD8α for surface expression (2). While human CD8β can be detected on the surface of T cells by immunofluorescence staining with a recently described monoclonal antibody (mAb) 597 (chapter 5), the nature of the association between the human CD8α- and β-chains has been unclear. For example, co-immunoprecipitation of human CD8β with CD8α has not yet been demonstrated. In order to determine whether the formation of CD8$\alpha\beta$ heterodimers is conserved, and to better understand the role of CD8β in T cell function, we have immunoprecipitated CD8 from human T cells and analyzed the samples by Western Blot analysis with rabbit antiserum against CD8α or CD8β. It appears that about 20% of CD8 on human peripheral blood lymphocytes (PBL) is composed of α–β heterodimers, and the rest forms α–α multimers. In addition, it is clear that human CD8α co-migrates with CD8β.1, accounting for past difficulty in detecting human CD8β.

Materials and Methods

Cells. HPB-ALL, a $CD4^+CD8^+$ human T cell leukemia line (3), and human PBL were grown in RPMI 1640 supplemented with 12.5% Fetal Bovine Serum (FBS, Hyclone Laboratories). Human PBL were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals) density gradient centrifugation and stimulated for 72 hours with 15 ug/ml Phaseolus Vulgaris Haematoagglutinin (PHA-E + L, Vector Laboratories Inc.). COS7 cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FBS (GIBCO Laboratories). Chinese hamster ovary (CHO) cells were maintained in Ham's F12 lacking hypoxanthine and supplemented with 10% dialyzed FBS (Hyclone Laboratories). CHO.4 and CHO$\alpha\beta$.1 were selected and maintained in 0.06 and 0.03 uM methotrexate (Sigma Chemical Co.), respectively.

Antibodies and immunofluorescence analysis. The anti-CD8$\alpha\beta$ mAb 597, a gift of Dr. E. Reinherz, was in the form of ascites and used at a dilution of 1:200. mAb OKT8 was affinity purified by protein A sepharose chromatography and used at 10 ug/ml. mAb GK1.5 and goat anti-mouse IgG (fluorescein conjugated) were from Becton Dickinson and used according to the manufacturer's instructions. Normal mouse serum (NMS) was from Zymed, Inc. Rabbit antiserum (prepared at Caltag, South San Francisco, CA) specific for the carboxy-terminal (C-terminal) 28 amino acid residues of human CD8α, C-terminal 30 amino acid residues of human CD8β.3, and the human CD8β Ig-like domain (residues 36-64) were obtained by subcutaneous injection of 0.15 mg of unconjugated peptide (synthesized by C. Turk, University of California-San Francisco and Howard Hughes Medical Institute) in 1 ml of Freunds Complete Adjuvent (Sigma Chemical Co.), followed by weekly booster injections in incomplete Freunds Adjuvent (Sigma Chemical Co.) starting 4 weeks later. For immunofluorescence studies, 10^6 cells were incubated with saturating levels of antibody in 0.1 ml of phosphate buffered saline (PBS) and 5% FBS for 30 min at 4^oC. Cells were washed twice with 3 ml of 5% FBS in PBS and fluorescence was measured in arbitrary units using a FACscan cell analyzer (Becton Dickinson).

Transfection of CHO and COS7 cells. Transfection of the CHO-DHFR$^-$ cell line DXB11 to obtain CHO.1(CD8$^-$) and CHO.4(CD8α^+) has been described (Chapter 3). In a similar manner, CHO$\alpha\beta$.1 was obtained by co-transfection of DXB11 cells with the plasmid pMG1P bearing the DHFR minigene (4), and the CD8α F1.1 (3) and CD8β.1 (chapter 5) cDNAs separately subcloned into the SV40 based replicating vector pSV7d (5). After primary selection in Ham's F12 lacking hypoxanthine, colonies were expanded and screened for expression of CD8α and CD8β.1 by immunofluoresence analysis using mAb 597. Cells expressing high levels of surface CD8$\alpha\beta$.1 were isolated using a FACS IV from Becton Dickinson (Paul Dazin, University of California-San Francisco and Howard Hughes Medical Institute), then selected in 0.03 uM methotrexate, and rescreened and sorted using mAb 597. By comparing the staining with saturating levels of the anti-CD8α mAb OKT8 to that of mAb 597, those cells which expressed more CD8α than CD8$\alpha\beta$.1 were excluded.

COS7 cells were transfected overnight by calcium phosphate precipitation with 30 ug of sCD8α pSV7d, sCD8β.3 (chapter 5) pSV7d or both plasmids in the presence of 100 uM chloroquine, followed by incubation in DME supplemented with 10% FBS.

Immunoprecipitation of mCD8 and sCD8. CHO cells, HPB-ALL cells, human PBL, and COS7 cells were metabolically labeled for 12-16 hours with 100 uCi/ml [^{35}S] cysteine (>600 Ci/mmol, Amersham Corp.) included in the culture medium. Cultures were harvested and centrifuged (400 x *g*) for 10 min to separate cells and supernatants. The cells were lysed for 1 hour at 4^{o}C in PBS containing 1% NP40, aprotinin (Sigma Chemical Co.) diluted 1:100 and 1mM PMSF (Sigma Chemical Co.). After pre-absorption with NMS and protein A-Sepharose (Pharmacia Fine Chemicals), lysates containing 10^6 equivalents of CHO$\alpha\beta$.1 or HPB-ALL cells, or 2 x 10^6 equivalents of PHA stimulated PBL were immunoprecipitated using protein A-Sepharose and saturating amounts of Ab for 2 hours at 4^{o}C in a total volume of 0.2 ml. Supernatants from 10^6 COS7 cell equivalents (0.6 ml) were immunoprecipitated for 20 hours. The beads were washed 4 times in 1% NP40 in PBS at 4^{o}C, resuspended in sample buffer, and the samples analyzed by SDS-PAGE.

Western Blot Analysis. Cell lysates containing 1 mg protein (determined using a Bio-Rad protein assay, Bio-Rad Chemical Div.) from HPB-ALL and CHO cells were pre-absorbed with NMS and protein A-Sepharose, and immunoprecipitated with mAb OKT8 or NMS in a total volume of 0.2 ml as described above. The beads were washed 3 times with 1% NP40 in PBS and resuspended in sample buffer. The samples were electrophoresed on a 12% polyacrylamide gel, and transferred to a nitrocellulose filter (Schleicher and Schuell) in 10 mM Tris-Cl pH 8.0, 150 mM NaCl, and 0.5% tween 20 (TBST). The filter was blocked with 5% nonfat dry milk in TBST, incubated with rabbit anti-CD8β antiserum diluted 1:200 in TBST containing 5% milk, washed twice in TBST 5% milk and once in TBST, and then probed with 0.25 Ci/ml [^{125}I] protein A (affinity purified, Amersham Corp). Following two washes in TBST and one in water, the filter was autoradiographed. The filter was stripped in 2% SDS, 0.1 M 2-mercaptoethanol and 50 mM sodium phosphate pH 7.2 at 65^{o}C and re-probed with rabbit anti-CD8α antiserum in a similar manner.

Results

In previous experiments, a single band with a relative molecular mass of 32 kD was observed following immunoprecipitation and SDS-PAGE of CD8α from metabolically labeled or surface iodinated human T cells. A faint 32 kD band was also observed if the anti-CD8αβ mAb 597 was used (immunoprecipitated in 1% NP40 in PBS with 150 mM NaCl added). These results could be accounted for if the anti-CD8α mAb OKT8 did not recognize α–β heterodimers and m597 bound poorly under the conditions used, or if human CD8β was not disulfide linked to CD8α on the T cell surface. Another possibility was that human CD8α co-migrated with CD8β when analyzed by SDS-PAGE.

To determine whether human CD8α and CD8β are associated, lysates of the CD8αβ$^+$ T cell leukemia line HPB-ALL, and CHO cell lines transfected with cDNAs encoding human CD8α (CHO.4), CD8α and CD8β.1 (CHOαβ.1), or neither (CHO.1) were subjected to immunoprecipitation with OKT8 or NMS; the immunoprecipitates were then probed with a polyclonal rabbit antiserum specific for the human CD8β Ig-like domain (Fig. 2, left panel). A specific band or triplet of bands corresponding to human CD8β was co-immunoprecipitated from HPB-ALL or CHOαβ.1 cells, respectively, but not from the CD8β$^-$ CHO.4 and CHO.1 cells. These results indicate that CD8β is associated with CD8α on the surface of human T cells and that the resulting heterodimer is recognized by the anti-CD8α mAb OKT8. With a single predicted N-linked glycosylation site the molecular mass of the mature CD8β.1 protein would be about 25 kD (chapter 5). The major 32 kD form of the β-chain polypeptide expressed in human T cells co-migrates with CD8β.1 immunoprecipitated from transfected CHO cells, and therefore CD8β.1 appears to be the form that is predominately expressed in both humans and rodents (6). As in CD8α, additional O-linked glycosylation may account for the difference between the predicted and observed molecular mass of the protein. The heterogeneity of CD8β co-immunoprecipitated with CD8α in CHOαβ.1 cells may be due to O-linked glycosylation differences resulting from over-production of the β-chain. However,

there may also be differential proteolytic processing or partial degradation of the CD8β-chain in the CHO$\alpha\beta$.1 cells.

To assess the relative sizes of human CD8α and CD8β.1, the western blot containing CD8$\alpha\beta$ immunoprecipitates was stripped and reprobed with a polyclonal antiserum specific for CD8α (Fig. 2, right panel). The bands corresponding to CD8α and CD8β.1 from HPB-ALL cells were precisely superimposed, demonstrating that the two CD8 polypeptides co-migrate when analyzed by SDS-PAGE.

CHO$\alpha\beta$.1 cells were selected to express similar levels of the M597 and OKT8 epitopes (Fig. 1) in order to insure that almost all of the cell surface CD8 was in the form of the heterodimer. However western blot analysis of total cell lysates using rabbit anti-CD8β antiserum indicates that CHO$\alpha\beta$.1 cells overexpress CD8β relative to CD8α by about 10 fold (data not shown), suggesting that α–β heterodimers form less efficiently than α-α homodimers. In total cell lysates of CHO$\alpha\beta$.1 most of the β-chain polypeptide migrates with a relative molecular mass of 22 or 37 kD. These species can be immunoprecipitated from metabolically labeled CHO$\alpha\beta$.1 cells using rabbit anti-CD8β antiserum (Fig. 3, lane 3), which apparently does not recognize CD8$\alpha\beta$ heterodimers. Therefore, the 22 and 37 kD β-chain polypeptides are likely to be precursors not yet associated with the α-chain.

As shown in Fig. 3, mAb 597 is as effective as OKT8 in immunoprecipitating CD8α–β heterodimers from CHO$\alpha\beta$.1 cells. This allows for a comparison of the relative levels of α–β heterodimers and α–α homodimers in metabolically labeled human T cells. By visual inspection, about 20% of CD8 appears to be in the form of the heterodimer in HPB-ALL cells and about 10% in PHA stimulated PBL (Fig. 3), consistent with estimates from previous cell-surface immunofluorescence analysis (chapter 5).

In addition to CD8β.1, several cDNAs corresponding to alternatively spliced transcripts that encode different forms of human CD8β have been identified (7,chapter 5). However, it is unclear whether multiple β-chain polypeptides are produced by human T cells. The mCD8β.2 cDNA is capable of encoding a cell surface protein when transfected into CD8α^+ L cells (Chapter V). However, association between CD8β.2 or CD8β.3 and CD8α has not previously been demonstrated.

By substitution of 5' cDNA sequences encoding the Ig-like, hinge and transmembrane sequences of mCD8β.1 for those of sCD8β.3, which lacks sequences from the transmembrane exon, an additional construct capable of encoding a membrane bound form of CD8β.3 has been obtained (data not shown). COS7 cells were transiently transfected with the SV40 replicating vector pSV7d encoding mCD8β.1, mCD8β.2 or mCD8β.3 with or without co-transfection of mCD8α. Immunofluorescence staining with mAb 597 showed that all three forms of CD8β could be expressed at the cell surface in the presence of the α-chain (data not shown). Although the human CD8α- and β-chains are likely to interact via cysteines in their membrane proximal domains, co-transfection of sCD8α with any of the mCD8β constructs did not result in surface expression of the M597 epitope (data not shown). mAb 597 is likely to recognize a co-determinant of CD8α and CD8β; it fails to recognize CD8β precursor forms (Fig. 3) or soluble CD8β.3 monomers produced in transfected cells (Fig. 4). Therefore the possibility remains that one or more of the forms of human CD8β may not require association with the α-chain for surface expression.

To determine whether sCD8β.3 protein could be produced and/or form secreted heterodimers with sCD8α, COS7 cells were transiently transfected with sCD8α, sCD8β.3 or both cDNAs. The cells were metabolically labeled and their culture supernatants were immunoprecipitated with a panel of anti-CD8 Abs (Fig. 4). As expected, the supernatants of cells transfected with sCD8α contained the 27 kD protein doublet specifically reactive with rabbit antisera against the CD8α cytoplasmic domain (Fig. 4, lanes marked 1). sCD8β.3 protein was specifically recognized by rabbit antiserum against the sCD8β.3 C-terminus (sCD8β, lane 2) or Ig-like domain (sCD8β, lane 3). Surprisingly, sCD8β.3 was produced in the absence of sCD8α. Unlike sCD8α it fails to form disulfide-linked homodimers; sCD8β.3 migrates with an apparent M_r of 36-39 kD under reducing (Fig. 4) or nonreducing conditions (data not shown). In addition, sCD8β.3 fails to form sCD8α-linked heterodimers recognized by mAb 597 (sCD8α+β, lane 4). The amount of sCD8α and sCD8β.3 is decreased in culture supernatants of co-transfected cells as compared to cells transfected with a single sCD8α or sCD8β.3 construct. Experiments in which the sCD8α and sCD8β.3

plasmids were co-transfected with control plasmid DNA have excluded the possibility that this result is an artifact of transfection efficiency (data not shown). In addition, attempts to obtain stable cell lines producing sCD8α–β heterodimers have failed. Together these results suggest that sCD8α–β.3 heterodimers may be degraded intracellularly.

Discussion

These studies indicate that human and rodent CD8 are structurally conserved, since α–β heterodimers and α–α homodimers are expressed on the surface of T cells from humans, rats and mice. Human CD8β had previously gone undetected primarilly because it co-migrates with human CD8α when analyzed by SDS-PAGE. In addition HPB-ALL cells are heterogeneous in their expression of CD8β (7), and it is likely that the cells used by Snow *et al.* for purification and NH_2-terminal sequencing of human CD8 lacked expression of the β-chain (8).

Whether CD8α–β heterodimers play a role in T cell-target cell adhesion or signal transduction is unknown. CD8α homomultimers are sufficient for binding to MHC class I molecules (Chapter 3), and for reconstituting antigen specific T cell activation when co-expressed with the α- and β-TCR genes (9,10). CHO$\alpha\beta$.1 cells failed to specifically bind MHC class I^+ Raji cells in the CD8-MHC class I cell-cell binding assay described in chapter 3 (data not shown). However CHOCD8$\alpha\beta$.1 cells may be below the threshold level of CD8 expression that is required to mediate stable intracellular adhesion; they express 20% as much CD8α (in heterodimer form) at the cell surface as do CD8α^+ CHO.3 cells, which bind MHC class I^+ B cells (chapter 3). Therefore it is only possible to conclude that CD8β does not markedly increase the avidity of CD8α for MHC class I molecules. Three human CD8β cDNAs which encode CD8β polypeptides that differ in their cytoplasmic domains have previously been isolated (chapter 5). In this study we have demonstrated that all three forms of CD8β can be expressed at the cell surface and associate with the CD8α-chain. In T cells, alternatively spliced transcripts may encode CD8β polypeptides which differentially regulate signal transduction through the CD8α-chain.

Both humans and rodents express alternatively spliced mRNA transcripts predicted to encode soluble CD8β proteins (2, chapter 2). Although human PBL produce sCD8α homodimers encoded by transcripts lacking transmembrane sequences, soluble CD8β protein has not been detected in T cell culture supernatants. Over-expression of sCD8β.3 monomers may lead to their release from transfected cells, and this may

not occur with PBL *in vivo*. When we attempted to obtain large amounts of sCD8β.3 protein using the DHFR amplification system, CHO cells were about 50 fold less effective in the production of sCD8β.3 monomers relative to sCD8α homodimers. In addition, sCD8$\alpha\beta$.3 heterodimers were not detected in the culture supernatants of transfected cells. Hence it is possible that sCD8β.3 is nonfunctional in human T cells, but that soluble CD8 heterodimers may arise by association of sCD8α with other anchor minus variants of CD8β. An sCD8β.1 cDNA lacking sequences corresponding to the transmembrane exon has been isolated from murine T cells (2), and we have constructed similar human CD8β.1 and CD8β.2 cDNAs which confer production of soluble CD8β proteins in transfected cells.

CD8α binds monomorphic determinants of the MHC class I α_3-domain (chapter 4), suggesting that CD8 and the TCR can simultaneously bind to a single MHC class I molecule. It is unclear whether the β-chain of CD8 also binds MHC class I molecules and plays a role in the formation of a putative complex between CD8, MHC class I, and the TCR. Purified sCD8β protein can potentially be used to determine whether CD8β binds to other regions of MHC class I molecules (perhaps β_2-microglobulin).

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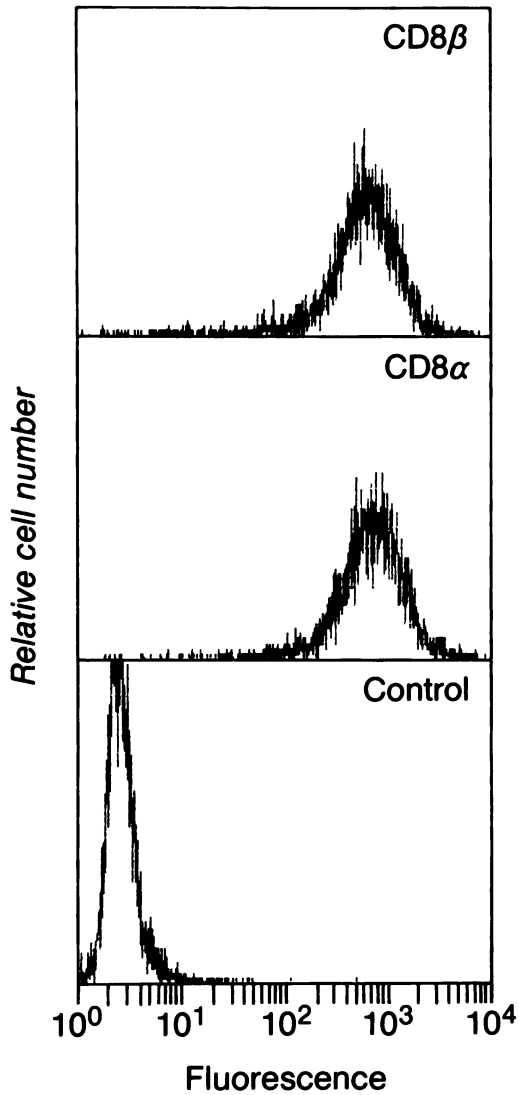

Fig. 1 Surface immunofluorescence analysis of CHO cells transfected with CD8α and CD8β.1. Cells were incubated with saturating levels of mAb 597 (CD8β), the anti-CD8α mAb OKT8 (CD8α), or the anti-CD4 mAb GK1.5 (Control). Staining utilized a second step fluorescein-conjugated goat anti-mouse IgG antibody. Fluorescence was measured in arbitrary units indicated along the *x*-axis.

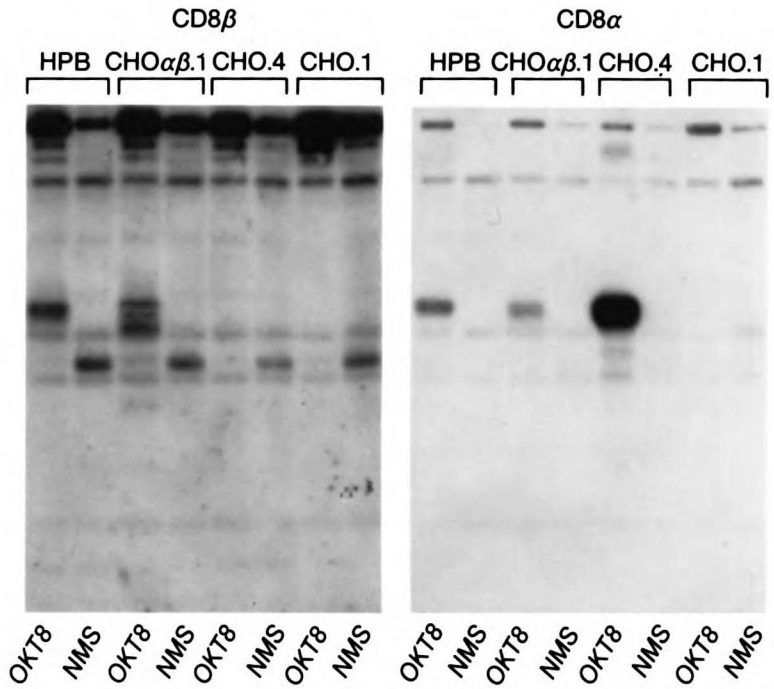

Fig. 2 Human CD8β.1 co-migrates with CD8α. Detergent solubilized cell lysates from HPB-ALL cells, CHO cells transfected with CD8α and CD8β.1 (CHO$\alpha\beta$.1), CD8α (CHO.4), and CD8$^-$ CHO cells (CHO.1) were immunoprecipitated with the anti-CD8α mAb OKT8 or normal mouse serum (NMS) as control. The samples were electrophoresed on a 12% Laemmli gel under reducing conditions, and transferred to a nitrocellulose filter. The filter was first probed with rabbit antiserum against CD8β (left panel), and then stripped and re-probed with rabbit anti-CD8α antiserum (right panel), followed by autoradiography for 3 and 1 days, respectively. Western blotting utilized a second step [^{125}I]-conjugated protein A. The mobility of CD8β.1 and CD8α was identical relative to non-specific Ig heavy chain bands at the top of the figure.

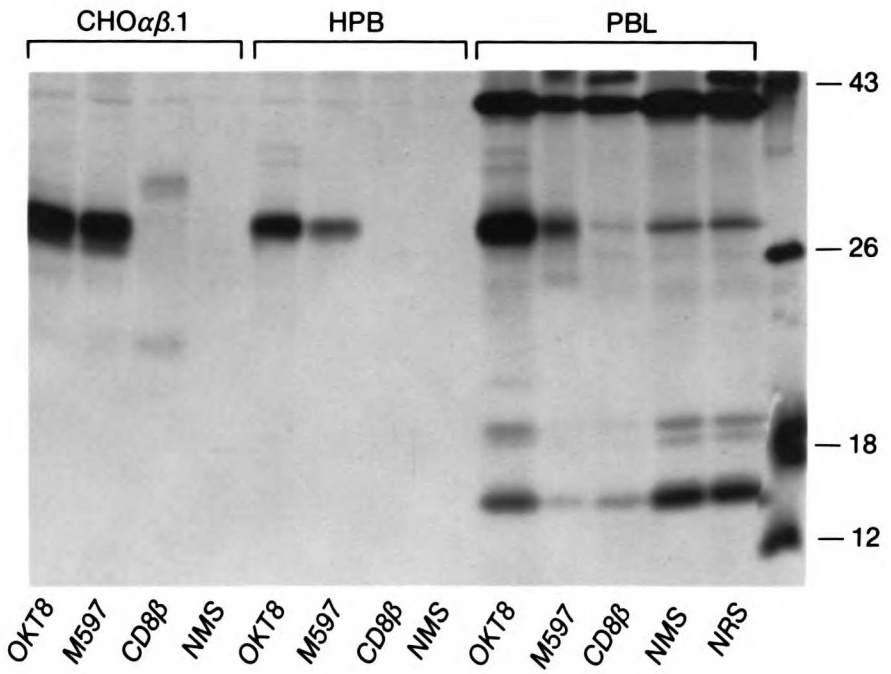

Fig. 3 Immunoprecipitation of CD8 from lysates of transfected cells and human T cells. CHO cells transfected with CD8α and CD8β.1 (CHO$\alpha\beta$.1), HPB-ALL cells, and PHA-activated normal peripheral blood mononuclear cells (PBL) were metabolically labeled with [^{35}S] cysteine. Detergent solubilized cell lysates were immunoprecipitated with mAb OKT8, M597, a rabbit anti-serum against the CD8β external domain (CD8β), or normal mouse serum (NMS). PBL were also immunoprecipitated with pre-immune rabbit serum (NRS) as a specificity control. Samples were analyzed on a 12% polyacrylamide gel under reducing conditions. Relative M_r are given in kD to the right of the autoradiogram.

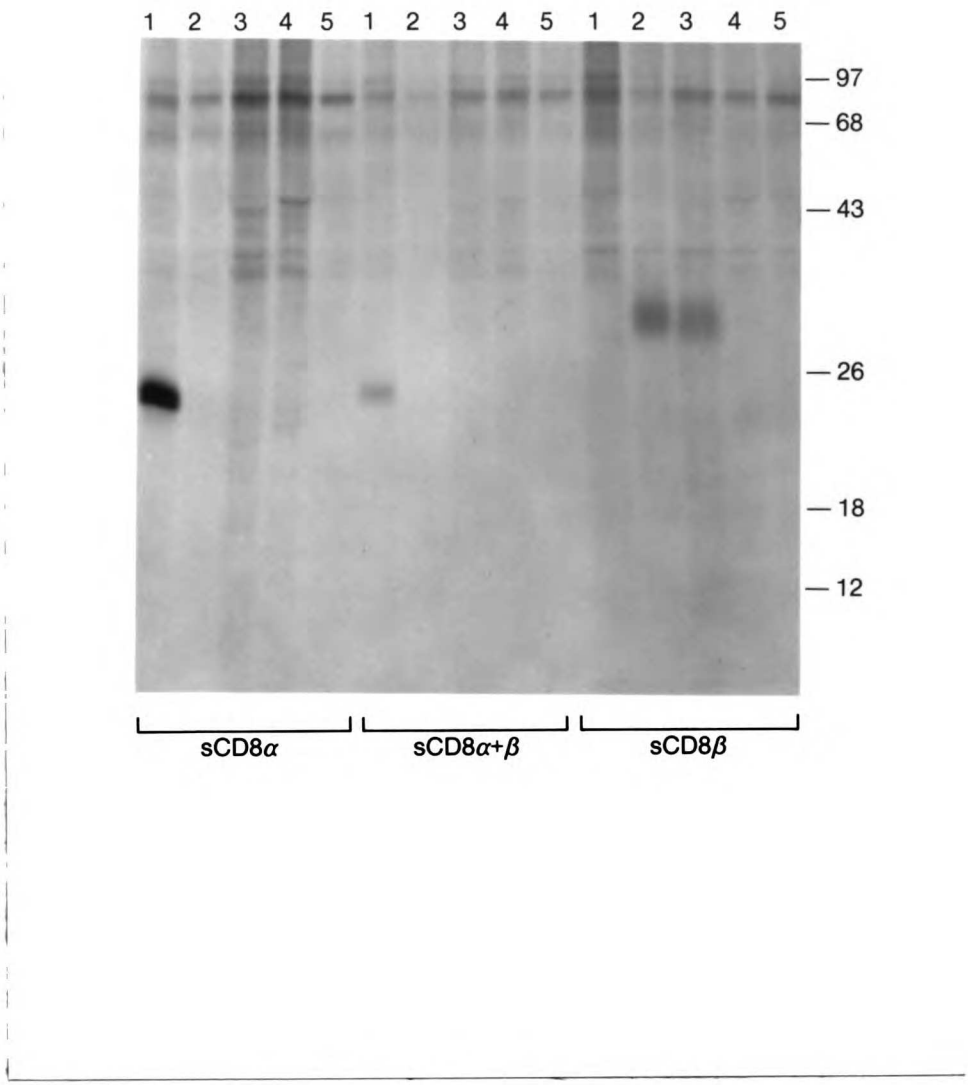

Fig. 4 Immunoprecipitation of sCD8α and sCD8β.3 from the supernatants of transiently transfected COS7 cells. COS7 cells were transiently transfected with the sCD8α, sCD8β.3, or both cDNAs, and metabolically labeled with [^{35}S] cysteine for 12 hours. Cell free supernatants were immunoprecipitated with rabbit antiserum against the CD8α (1) or CD8β.3 (2) cytoplasmic domains, rabbit antiserum against the CD8β external domain (3), M597 (4), or normal rabbit serum (5) and analyzed on a 12% polyacrylamide gel under reducing conditions. Relative M_r are given in kD to the right of the autoradiogram.

Chapter 7

Conclusion

I. Summary.

The studies included in this thesis indicate that the human CD8α-chain directly binds to nonpolymorphic residues in the α_3-domain of MHC class I molecules. Prior to this work, experiments with T cells had shown that CD8 and CD4 were important for T cell-target cell interactions as well as activation by MHC class I and II restricted TCRs, respectively. Although it was suggested early on that CD8 and CD4 directly bound to MHC molecules (1,2), functional T cell assays did not distinguish this activity from the engagement of MHC molecules by the TCR. For example, conclusions from experiments which utilized anti-CD8 and anti-CD4 mAbs were confounded by the possibility that the Abs sterically hindered TCR-MHC binding, due to the proximity of CD8 or CD4 to the TCR (3-5). In addition, it has been difficult to interpret experiments with cultured T cell hybridomas and clones because of variation in TCR avidity for antigen and MHC molecules, thought to determine their dependence on CD8 or CD4 function (6,7). To study CD8 function in the absence of the TCR, we have developed an assay which measures the adhesion of B cell lines expressing MHC class I molecules to transfected CHO cells expressing high levels of human CD8α (chapter 3). In this system, human CD8α binds with low avidity to MHC class I molecules. Of 18 individual MHC class I molecules tested (including HLA-A, HLA-B, and murine MHC class I molecules) only HLA-Aw68.1 and HLA-Aw68.2 did not bind. Using site directed mutagenesis, it is clear that a single valine to alanine substitution at position 245 of the MHC class I α_3-domain is responsible for this difference in binding. Subsequent analysis of CD8 binding to site directed mutants of HLA-A2.1 indicates that CD8α binds to an exposed negatively charged loop (residues 220-229) which extends from the MHC class I α_3-domain (7a).

With the aim of better understanding the roles or CD8α and CD8β in MHC class I binding, an anchor-minus variant of human CD8α and the human CD8β-chain polypeptide have been characterized. An alternatively spliced transcript lacking sequences from the transmembrane exon accounts for about 15% of the total CD8α mRNA, and encodes soluble CD8α homodimers produced by human T cells (chapter 2). Human CD8 was

initially thought to be composed of homodimers and higher multimers of the 32 kD α-chain. We have isolated 3 cDNAs encoding structural variants of human CD8β that differ in their cytoplasmic domains, and appear to arise by alternative splicing (chapter 5). Unlike rodent CD8, the human CD8 β-chain polypeptide went undetected in T cells because the major form CD8β.1 co-migrated with the α-chain when analyzed by SDS-PAGE, and many human T cell leukemia lines lacked expression of CD8β (chapter 6).

II. A complex between CD8, the TCR and MHC class I molecules.

Based on the observations that CD8α binds to the MHC class I α_3-domain and that the TCR binds to polymorphic residues of the α_1- and α_2-domains, it is likely that CD8 and the TCR can simultaneously bind to the same MHC class I molecule. According to the model developed by Davis and Bjorkman using sequence data from the Ig and TCR V-regions, the CDR1 and CDR2 regions of the TCR directly bind to the polymorphic MHC α-helices, while the highly variable CDR3 region interacts with peptide antigen in the central groove (8). This would place the TCR directly over top the surface of the MHC-peptide complex, as a T cell contacts its target cell. Because CD8 is composed of two distinct chains, each of which contains Ig V-like and J-like sequences, it has also been suggested that CD8 α–α homodimers and α–β heterodimers may form Ig-like combining sites (8,9). Davis and Bjorkman have suggested that CD8 is the most direct evolutionary precursor of the TCR and binds to MHC class I molecules in a similar manner (8). In the cell-cell binding assay, CD8α directly interacts with the MHC class I α_3-domain. However, the role of β_2-m in CD8α binding is unknown. We cannot rule out the possibility that in the presence of other T cell specific molecules, CD8α may undergo a conformational change and bind to determinants other than the α_3-domain.

The α_3-domain of MHC class I molecules is likely to be accessible to CD8. While β_2-m appears to closely interact with the underside of the MHC class I α_1- and α_2-domains, the α_3-domain is relatively exposed and flexible (7a). Residues 35-38 (RFDS) of the α_1-domain form a conserved salt bridge with β_2-m below. As the HLA-A2 crystal structure

was solved using papain digested material lacking the transmembrane anchor, the angle of orientation of the MHC class I molecule relative to the cell surface is unknown (10). Upon contact with CD8 and the TCR, the MHC class I molecule may tilt and further expose the α_3-domain to CD8. In addition, the CD8α- and β-chains contain membrane proximal hinge regions of 48 and 30 amino acid residues, respectively, which may extend the NH_2-terminal Ig-like domain from the T cell membrane (9,11). The serine- and threonine-rich hinge is the site of extensive O-linked glycosylation (11,12), which may form a supporting scaffold and/or protect the otherwise exposed polypeptide from proteolytic degradation.

The role of the CD8β-polypeptide in T cell-target cell interactions is unknown. CD8β may increase or decrease the avidity with which the α-chain binds to MHC class I molecules, possibly by interacting with non-covalently associated β_2-m. Alternatively, CD8α–β heterodimers may be similar to the TCR and directly bind polymorphic residues of the MHC class I α_1- and α_2-domains, as suggested by Davis and Bjorkman (8). Two naturally occuring human MHC class I molecules, HLA-Aw68.1 and HLA-Aw68.2, do not mediate stable adhesion to CD8α^+ CHO cells. α–β heterodimers may extend CD8 binding to include these MHC molecules, or specifically bind to other types of class I molecules such as Qa (13), or CD1 antigens (14). A distinct possibility is that CD8β does not directly bind to MHC class I molecules, but is responsible for coupling CD8 to the TCR.

III. Different biochemical signals transmitted through CD4 and CD8.

Upon engagement of MHC molecules, CD4 and CD8 may transmit different regulatory signals to T cells. Aside from the cysteine motif (++XCXCP, where + represents a basic residue), which mediates association between CD4 or CD8α and $p56^{lck}$ (15), the cytoplasmic domains of CD4 and CD8 are not homologous to each other. In addition, the 38 amino acid cytoplasmic domain of CD4 is longer than that of CD8α, which is composed of only 28 amino acids residues. The proximal portion of the cytoplasmic domain of CD4 contains the cysteine motif, but the function of the distal portion of CD4 is unknown. The cytoplasmic domain of the CD8 β-chain polypeptide and the distal CD4

cytoplasmic region may each function to diffentially modulate association of CD8α or CD4 with $p56^{lck}$, or couple CD8 and CD4 to distinct intracellular mediators, such as the lymphocyte specific variant of the tyrosine kinase *fyn* (16). Preliminary evidence suggests that $p56^{lck}$ differentially associates with CD4 and CD8. Activated protein kinase C rapidly phophorylates CD4 and CD8 on serine residues, but only CD4 is modulated from the surface of T cells (17-19) and this activity correlates with dissociation of CD4 from $p56^{lck}$ (20). In addition, the cytoplasmic domain of murine CD4 appears to have greater avidity than murine CD8α for $p56^{lck}$ (15).

It is tempting to speculate that differential signal transduction through CD4 or CD8 might influence the helper or cytotoxic phenotype of developing T cells. For example, in $CD4^+CD8^+$ thymocytes simultaneous engagement of a specific MHC class I molecule by CD8 and the TCR could transmit regulatory signals resulting in the transcription of genes specific to cytotoxic T cells and the loss of CD4 expression. This model predicts that treatment of thymocytes with Abs cross-linking CD4 or CD8 to the TCR will lead to differential lymphokine production and phosphorylation of intracellular mediators.

IV. CD4 and CD8 function differs between virgin and activated T cells.

In initial experiments, T cell hybridomas and clones appeared to require CD4 and CD8 only under suboptimal stimulatory conditions (6,7,21). These observations lead to the conclusion that CD4 and CD8 were accessory cell adhesion molecules, and appeared to conflict with later studies implicating an important role for CD4 and CD8 in T cell activation (22-24). In fact, it is difficult to obtain cultured T cells which are completely inhibitable with anti-CD4 and anti-CD8 mAbs (C. Clayberger, personal communication). The results of Connolly, Potter *et al.* suggest that this paradox can be resolved by the notion that unlike activated or cultured T cells, virgin T cells absolutely require CD4 and CD8 function (25). The generation of primary CTL but not secondary CTL is inhibited by mAbs against the MHC class I α_3-domain or CD8, as well as a mutation at position 227 in the MHC class I α_3-domain, likely to interfere with CD8 binding (25,26). The studies

described in chapter 4 appear to support this conclusion. The presence of valine instead of alanine at position 245 interferes with the interaction between HLA-Aw68 molecules and CD8, and decreases but does not abrogate the antigen specific or alloreactive response of cultured CTL. Rickinson *et al* failed to obtain an Epstein-Barr virus (EBV) specific CTL response restricted for HLA-Aw68, unless the cells were first stimulated with HLA-Aw69, a similar MHC class I molecule capable of binding CD8 (27,28). CD4 and CD8 function may also be absolutely required for the development of $CD4^+$ and $CD8^+$ thymocytes. For example, unlike conventional $CD4^+$ T cells, the differentiation of MHC class II-reactive $CD8^+$ T cells is not inhibited by anti-class II mAbs. This suggests that although the TCRs of these $CD8^+$ cells are cross-reactive with MHC class II molecules *in vitro*, they may be thymically selected *in vivo* by interaction with MHC class I (29).

The difference between primary and secondary CTL with regard to CD8 function is unclear. One possibility is that the secondary CTL population is composed of T cells bearing high affinity TCR, which have been selectively expanded, and never required the function of CD4 or CD8. TCRs do vary in their requirement for CD8; in one case, transfer of TCR α- and β-chain genes required co-transfection of CD8 to confer T cell activation (30). Such a requirement was not observed with another TCR (31). Variation of TCR affinity by somatic mutation and subsequent expansion of high affinity T cell clones that are CD4 or CD8 independent probably does not occur; unlike Igs, the genes encoding TCRs do not undergo somatic mutation (32).

Alternatively, high affinity TCRs may be able to compensate for low avidity CD8-MHC class I binding and drive the formation of multimolecular complexes between CD8, the TCR and MHC class I molecules. The cell-cell binding assay is very sensitive to small differences in the levels of CD8 and MHC class I on CHO and B cells, respectively (chapter 3). It is therefore quite possible that CD8 may bind to HLA-Aw68, albeit with lower avidity. The formation of a high affinity complex composed of the TCR, CD8 and MHC class I may effectively compete with anti-CD8 or anti-MHC class I mAbs. CD4- or CD8-MHC interactions do appear to be required for peripheral T cell responses; tolerance to exogenous antigens (33,34) and enhanced

survival of skin grafts (25) is obtained by anti-CD4 and anti-MHC class I α_3 mAbs *in vivo*. However, in these experiments the Abs may also be acting in the thymus.

A final possiblity is that the phenotype of activated T cells may be different from virgin T cells so that signals transmitted through CD4 or CD8 are no longer required for stimulation. With activation, T cells increase production of lymphokines and their receptors, and bear cell-surface LFA-1 with high affinity for ICAM-1 (35). Regulatory signals transmitted through CD8 may effect in part this phenotypic change. For example, cross-linking of CD8 to CD3 appears to provide a second signal required for IL-2 production and IL-2 responsiveness in resting T cells, which then results in autocrine proliferation (36). In addition, multivalent crosslinking of CD8 on resting T cells induces the generation of CTL resistant to blocking with anti-CD8α and anti-CD8β mAbs (37). Eichmann *et al* have observed that splenic T cells expressing low levels of Pgp-1 (a marker for naive T cells), differ from Pgp-1^{hi} T cells (memory T cells) in that cross-linking of CD8 to the TCR results in their synergistic proliferation (38).

CD8 itself appears to be altered upon T cell activation. *In vivo* administration of anti-CD3 causes a transient increase in CD8α surface expression (39), and TCR triggering stimulates protein kinase C mediated serine phosphorylation of CD8 (17,18). Changes in CD8 transcription appear to be mediated by IL-4 which transiently induces the expression of CD8 on $CD4^+$ as well as $CD8^+$ T cell clones (40). Perhaps altered expression of the amount or form of CD8β affects the ratio of heterodimers to homodimers, and the nature of the signals transmitted to the T cell via CD8.

V. Role of CD8 in the function of $\gamma\delta$ T cells and natural killer cells.

The expression of CD8 correlates with the tissue localization of $\gamma\delta$ T cells in humans, mice and chickens, suggesting that CD8 is important for the function of these cells. In all three species, CD8 is expressed on some $\gamma\delta$PBL, and on a large proportion of the $\gamma\delta$ T cell subsets which preferentially home to the splenic sinusoids (as opposed to the white pulp which contains $\alpha\beta$ T cells), and intestinal epithelia

(41-44). A $CD8^-$ $Thy\text{-}1^+$ dendritic epidermal $\gamma\delta$ T cell population that was initially identified in mice (45) has recently been demonstrated in humans (46). The physiologic function of any of these T cell populations has yet to be illucidated. However, $\gamma\delta$ TCRs are structurally homologous to $\alpha\beta$ TCRs (8), and are also believed to recognize antigen in the context of MHC molecules (47-48), so they are likely to function in a similar manner. Other $\gamma\delta$ TCRs appear to be restricted for MHC class I-like molecules, including the MHC-linked T1 antigens in mice (49), and $\alpha\beta$ and $\gamma\delta$ T cell clones specific for CD1 antigens in humans (50). As in $\alpha\beta$ T cells, CD8 may bind to MHC class I or MHC class I-like antigens on the target cell and form a multimolecular complex with the $\gamma\delta$ TCR important for T cell activation. As most $\gamma\delta$ TCR^+ thymocytes are $CD8^-$, CD8 probably does not function in their intrathymic positive and negative selection (41). CD8 expression may be induced as part of the T cell activation process in the periphery, and this would predict that $\gamma\delta$ T cells have different requirements than $\alpha\beta$ T cells for primary stimulation. However, a small population of $CD8^+$ $\gamma\delta$ thymocytes may have gone undetected.

In addition to T cells, natural killer (NK) cells also express CD8 (51). NK cells are large granular lymphocytes which kill tumor and virus infected host cells, and it has been suggested that this lineage represents an evolutionary precursor to CTL (52). Some activation pathways appear to be conserved with T cells; NK cells can be activated by CD2 stimulation and express the ζ-chain component of the TCR-CD3 complex (53). However, neither the receptor responsible for NK specificity nor its ligand on target cells has been identified. It is possible that in these cells CD8 functions independently of the TCR to bind target MHC molecules, or CD8 may interact with a distinct NK receptor specific for non-MHC or minor histocompatibility antigens. NK cells only express the α-chain of CD8 (chapter 5), but this may complex with a distinct polypeptide chain, altering the ligand specificity or possibly coupling CD8 to the NK antigen receptor.

VI. Anchor minus and cytoplasmic variants of CD8.

Both humans and mice express structural variants of the α- and β-chains of CD8; these arise by alternative splicing and vary in their transmembrane or cytoplasmic domains. However, the splicing pattern differs between the two species, and it is uncertain whether these variants are functionally conserved. cDNAs lacking sequences from the transmembrane exon of human CD8α (chapter 2, 54), human CD8β.3 (chapter 5), and murine CD8β (55) have been identified, but expression of the corresponding transcript and polypeptide has been determined only for human CD8α. In contrast, murine T cells express a transcript which encodes a membrane bound form of CD8α with a truncated cytoplasmic domain, Lyt-2α′ (56). This form is retained in a late intracellular compartment in mature T cells, but forms α–β heterodimers in developing thymocytes (57). Lyt-2α′ fails to interact with the protein tyrosine kinase $p56^{lck}$, and may thus alter the transmission of developmentally specific regulatory signals. By surface immunofluorescence Lyt-2α′ cannot be distinguished from full-length Lyt-2, and it is possible that particular populations of developing thymocytes homogeneously express one form or the other.

While human T cells do not express transcripts encoding a Lyt-2α′ homolog (chapter 2), it is possible that soluble human CD8α may interact with proteins on the surface of developing thymocytes and provide a similar function. For example, a soluble form of the IL-6 receptor is capable of associating with cell surface gp130 in the presence of IL-6, and in this way transmits signals to T cells (58). However co-transfection of CD8β with sCD8α does not confer surface expression of the M597 epitope (unpublished results). Alternatively, sCD8α may associate with CD1 antigens or MHC class I molecules on the same cell (59-61), or even interact with cell surface TCRs. Human CD8α is also released from the surface of T cells by proteolytic clevage (chapter 2), and this may also occur in mice.

A more likely possibility is that human sCD8α is functionally divergent from Lyt-2α′, perhaps binding to proteins on the surface of cells outside of the thymus. In contrast to Lyt-2α′, sCD8α is produced by activated mature T cells (54,62). Although human CD8α appears to

bind with low avidity to HLA-A and HLA-B molecules (chapter 2,3), aggregates of sCD8α may bind MHC class I molecules, or MHC class I-like molecules with higher avidity *in vivo*. Soluble forms of the TCR have recently been identified, and appear to form multimolecular complexes greater than 500 kD (63,64). Perhaps sCD8α or putative α–β heterodimers are present in these complexes and increase their avidity for MHC class I molecules. This is interesting in light of recent reports describing signal transduction through MHC class I antigens in B cells and T cells (65,66). sCD8 could also function as a lymphokine or chemotactic agent, binding with high avidity to non-MHC encoded cell surface receptors. For example thymotaxin is a chemotactic agent which attracts T cell precursors into the thymus, and has recently been shown to be equivalent to β_2-m (67). Finally, sCD8 may bind to and inhibit the as yet unknown function of soluble class I molecules which also arise by alternative splicing (68,69) or even thymotaxin/β_2-m.

VII. Future studies.

There is now good evidence that CD8α transmits regulatory signals which are required for the activation of mature resting T cells. This is likely to occur with the formation of a multimolecular complex in which the TCR binds to antigen in the central groove between the α-helices of the MHC class I α_1- and α_2-domains, and CD8α binds to the MHC class I α_3-domain. In this way CD8 may bring the lymphocyte specific tyrosine kinase $p56^{lck}$ into close proximity with TCR, resulting in phosphorylation of intracellular mediators such as the ζ-chain (Fig. 1). In contrast little is understood about the role of CD8 in thymocyte development and the function of the CD8β-chain. It may be possible to address the first point by obtaining mice which lack CD8 expression through homologous recombination of defective CD8 genes into germline DNA. The CD8 signalling function as well as MHC class I binding would be absent in this system. If CD8 is involved in positive selection of MHC class I reactive T cells, then the thymic repertoire will be skewed towards MHC class II reactivity. Alternatively, CD8 may play its major role in negative selection, and autoreactive T cells will escape into the periphery. However, these cells may be quiescent,

because they would lack CD8 required for primary antigen specific responses. It may be possible to expand and identify them with polyclonal T cell activators.

To specifically study the role of CD8-MHC class I interactions in thymocyte development, MHC class I molecules defective in CD8 binding will be introduced into the murine germline by Nigel Killeen in the laboratory. These will include H-2D^b and H-2K^d mutants at positions 245(A to V) and 227 (E to K) of the α_3-domain. The precursor frequency of CTL specific for the mutant MHC class I molecules can then be compared to endogenous or wild-type MHC class I molecules. Although this experiment will probably show that CD8-MHC class I interactions are necessary for the development of T cells, it will be difficult to determine if a defect lies in thymocyte development or peripheral expansion because of a large background of T cells with TCR specific for normal endogenous MHC class I molecules. This problem can be overcome by mating mice carrying mutant H-2D^b molecules with mice developed by von Boehmer *et al.* (70) transgenic for a CD8 dependent TCR specific for the male H-Y antigen and H-2D^b. If CD8 is required for positive selection, it may be possible to identify the specific stage of thymocyte development at which this occurs. It is formally possible that the mutant MHC class I molecules may also be defective in their ability to interact with the TCR or other molecules required for positive selection. This can be addressed by screening for compensatory mutations in CD8 *in vitro* which allow binding to the MHC class I mutants, introducing the corresponding sequences into the mouse germline, and asking whether thymocytes expressing compensatory CD8 mutations can now be selected on MHC class I mutants *in vivo*.

Learning the function of CD8β will also be important for understanding the role of CD8 in T cell development. To determine whether the β-chain modulates CD8-MHC class I binding, purified CD8 α–α homodimers and α–β heterodimers can be isolated using mAb OKT8 and mAb 597 respectively, inserted into planar membranes and compared for their ability to bind MHC class I$^+$ B cells. Both the avidity of binding and the range of MHC class I or class I-like molecules bound should be determined. It is also possible to test whether CD8β alters the association of the cytoplasmic domain of the α–chain polypeptide to

lymphocyte specific tyrosine kinases. The relative amount and activity of $p56^{lck}$ co-precipitated with CD8 α–α homodimers and α–β heterodimers can be determined in normal and activated PBL. Perhaps distinct regulatory molecules will be co-precipitated with the structural variants of CD8β that differ in their cytoplasmic domains.

Finally, soluble CD8α–α homodimers will hopefully be purified and used for structure determination. In collaboration with H. Spits, (DNAX Research Inst.) sCD8 will be added to mixed lymphocyte reactions to determine whether this molecule has immunomodulating activity. sCD8β.3 monomers could be purified and analyzed in a similar manner, and may be potentially used to determine whether the CD8β-chain polypeptide also directly binds MHC class I molecules.

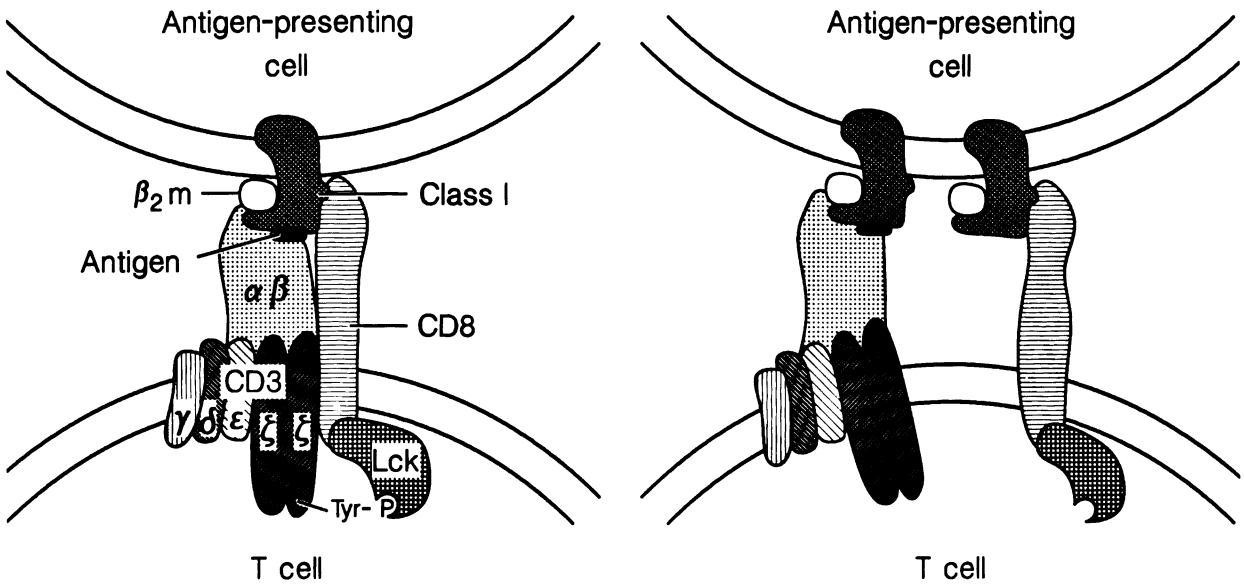

Fig. 1 Models for CD8 function. CD8 may form a multimolecular complex with the TCR, antigen and MHC class I molecules and transmit regulatory signals to T cells (left panel). Under certain conditions, CD8 may complex with distinct MHC molecules from that which the TCR cell binds and increase the avidity of T cells for their targets (right panel).

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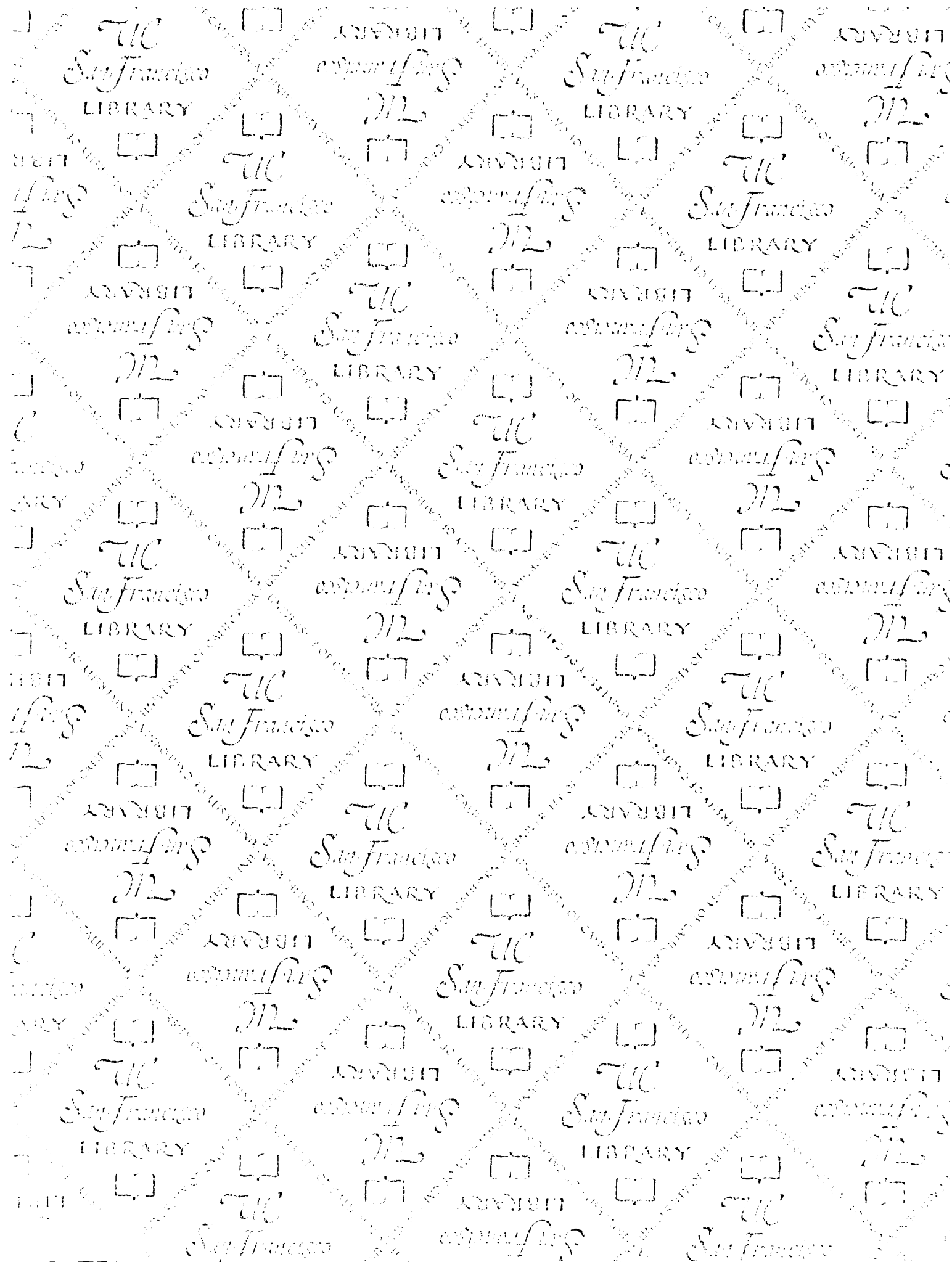
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