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Enhanced Antigen Presentation in the Absence of the Invariant Chain Endosomal Localization Signal

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Summary

The cytosolic tail of the major histocompatibility complex class II–associated invariant chain (Ii) molecule is thought to contain the endosomal localization signal that directs and/or retains newly synthesized class II within the endosomal antigen processing compartment. To determine the role of this signal in class II transport and antigen presentation we have generated class II–positive L cell transfectants that coexpress wild type or truncated forms of Ii. Deletion of the endosomal localization signal from Ii results in rapid transport of class II–Ii complexes to the cell surface. Once at the cell surface, the complex is efficiently internalized, Ii is degraded, and class II free of Ii is recycled back to the plasma membrane. Interestingly, the truncated form of Ii is still able to increase the efficiency of antigen presentation to T cells. These data suggest that the ability of Ii to enhance antigen presentation is not limited to Golgi apparatus–endosomal sorting and raise the possibility that endocytosed class II can form immunogenic complexes with newly processed antigen.

During biosynthesis, MHC class II molecules are associated intracellularly with a glycoprotein called invariant chain (Ii). This association occurs soon after translation of the α and β chains of the class II heterodimer in the rough endoplasmic reticulum (ER)[1] and the complex remains intact throughout Golgi apparatus transport. In a post-Golgi apparatus compartment, possibly in the same endosomal compartment where antigen is degraded, Ii dissociates from class II in a protease-dependent fashion, and the majority of class II appears at the cell surface free of Ii (1, 2). Recently, several functions have been ascribed to Ii that might affect the generation of class II–peptide complexes. Ii has been shown to interfere with peptide–class II association (3–5) and to facilitate class II folding and egress from the ER (6–10), suggesting that Ii may act as a surrogate peptide in the ER to allow proper class II folding and to prevent class II from loading with peptide early in biosynthesis. In addition, Ii has been implicated in localizing class II into endosomal compartments that are thought to be the sites for exogenous antigen processing (11–14). This endosomal localization signal resides within the cytosolic domain of Ii (11, 12). When wild type Ii is expressed in the absence of class II, it is retained intracellularly, apparently localized in the ER and in peripheral vesicles. Truncation of the cytosolic NH_2 terminus eliminates the

steady state endosomal localization of Ii and results in the transport of Ii to the cell surface.

These results demonstrate that Ii can have profound effects on class II folding and transport and predict that association with Ii could increase the opportunity for class II to associate with peptides generated in endosomal compartments. Ii has been shown to increase the efficiency of antigen presentation, but for only a subset of antigens (15, 16). Although the characteristics of protein antigens that determine Ii dependence are not known, the ability of class II to present some antigens in the absence of Ii suggests that class II can gain access to endosomal compartments independently of Ii. Class II could enter endosomes either by direct transport from the trans-Golgi network (Chervonsky, A., and A. J. Sant, personal communication) or after internalization of class II from the plasma membrane (17–19). In most cases the ability of Ii to enhance antigen presentation is restricted to the alternatively spliced Ii gene product, p41 (16). The p41 form of Ii differs from p31 by the inclusion of an additional exon that encodes a 64–amino acid segment near the COOH terminus of Ii (20). The p41 form of Ii is able to impart the same folding (16) and peptide blocking functions as does p31 (Anderson, M. S., and J. Miller, manuscript in preparation). Because p31 and p41 only differ in the lumenal domain, both proteins contain the same endosomal localization signal located in the cytosolic tail. Nevertheless, efficient antigen presentation often requires association of class II with p41 (16).

As a first step in dissecting the mechanism by which p41

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[1] Abbreviation used in this paper: ER, endoplasmic reticulum.

can facilitate antigen presentation, we have tested whether the endosomal localization signal in the cytosolic tail of Ii is required. We find that deletion of the cytosolic tail of Ii does not eliminate the ability of p41 to enhance antigen presentation. Biochemical analyses of class II associated with truncated Ii demonstrate that this complex is rapidly transported to the cell surface and then slowly internalized, allowing for Ii dissociation and class II–peptide loading. These data establish a pathway for class II recycling and raise the possibility that class II may access intracellular peptide loading compartments after internalization from the cell surface.

Materials and Methods

Oligonucleotide-directed Mutagenesis. A DNA fragment encoding the amino terminus of Ii with amino acids 2–17 deleted was generated by PCR using a mutagenic 24-base oligonucleotide containing the 5' untranslated region and the translation initiation codon spliced directly to codons for residues 18–21 of murine Ii and a 18-base oligonucleotide corresponding to residues 81–86. The PCR product was subcloned into murine p31 and p41 cDNA clones to generate pcEXV-Δ2-17p31 and pcEXV-Δ2-17p41 and the constructs were confirmed by dideoxynucleotide sequencing.

Reagents. The mAb 10.2.16 (21) is specific for I-A^k, MKD6 (22) for I-A^d, M5/114 (23) for I-A^d, P4H5 (24) for murine Ii, 16-1-11N (25) for class I K^k, and the rabbit antisera α-IiC for the COOH terminus of murine Ii (provided by Drs. J. Lipp and B. Doberstein, European Molecular Biology Laboratory, Heidelberg, Germany). The rabbit antisera 889, specific for a nonpolymorphic peptide sequence of the cytosolic tail of the I-Aβ chains, was generated by immunization with the peptide sequence CQKGPRGPP-PAGLLQ (I-Aβ COOH-terminal sequence with a NH$_2$-terminal cysteine) coupled to KLH as described (26).

Cell Culture and Transfection. The I-A^k–restricted T cell hybridomas 3A9, specific for hen egg lysozyme (27); TS12, specific for bovine ribonuclease (28); and SKK45.10, specific for KLH (29); and the I-A^d–restricted T cell hybridomas 3DO-54.8 and 3DO-18.3, specific for different peptides of OVA (30), were maintained in RPMI supplemented with 10% FCS, 10 mM Hepes, 2 mM glutamine, 0.1 mM nonessential amino acids, and 50 μM 2-ME. Class II–positive Ltk⁻ cells expressing no Ii, wild type Ii (16), or truncated Ii were generated as described (16, 31) and maintained in DMEM supplemented with 10% FCS, 10 mM Hepes, 2 mM glutamine, 0.1 mM nonessential amino acids, 200 μg/ml G418, 250 μg/ml xanthine, 15 μg/ml hypoxanthine, and 6 μg/ml mycophenolic acid. Because cell surface forms of Ii are sensitive to trypsin concentrations required to remove the Ltk⁻ cells from tissue culture dishes, the L cell transfectants used in these studies were maintained as suspension cultures by plating on bacterial petri dishes. To assay their ability to present antigen, L cell transfectants were plated at 2.5 × 10⁴ cells per well with T cell hybridomas at 5 × 10⁴ cells per well with antigen for 24 h at 37°C. Lymphokine production was assayed by [³H]thymidine incorporation into the IL-2-dependent T cell line, CTLL, incubated in the presence of 25% culture supernatant.

Radiolabeling and Immunoprecipitation. Cells were preincubated in leucine-free MEM for 1 h at 37°C, pulse labeled with 300 μCi/ml of ³H-leucine, and then chased in complete media for various times at 37°C. For the trypsin experiments, after the chase time the cells were chilled on ice, washed with PBS, and incubated with or without ice cold 0.05% trypsin/0.53 mM EDTA (GIBCO BRL, Gaithersburg, MD) for 30 min on ice. Ice-cold media containing

10% FCS was added to inhibit the trypsin reaction and the cells were washed in PBS. For cell surface labeling, viable cells (5 × 10⁶) were isolated on Ficoll gradients, washed in PBS, and ¹²⁵I-labeled using the lactoperoxidase method. Labeled cells were solubilized, lysates were precleared, and immunoprecipitation carried out as described (8). For the preclear experiment, lysates were precleared six times with the 889 sera, with the α-IiC sera, or with a control of normal rabbit sera mixed with 16-1-11N (anti–class I mAb) and then precipitated with either 889 or α-IiC. Immunoprecipitates were washed, eluted in SDS with 2-ME, and analyzed on 10% polyacrylamide-SDS gels as described (8). Gels were processed with EN³HANCE (NEN, Boston, MA), dried, and autoradiographed.

Endocytosis Assay. The endocytosis assay was adapted from Bretscher and Lutter (32) and modified as described (33, 34). Briefly, ∼8 × 10⁵ cells, plated in 60-cm dishes the day before the assay, were washed in cold PBS supplemented with 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$ PBS-CM. Surface proteins were biotinylated with 1.5 mg/ml NHS-SS-Biotin (Pierce, Rockford, IL) in PBS-CM for 30 min on ice and the reaction was quenched by washing the cells with ice cold 50-mM glycine in PBS-CM. Parallel plates of cells were either kept at 4°C or incubated in prewarmed media for various times at 37°C to allow for internalization of labeled surface molecules. Internalization was stopped by addition of ice cold media. Cells were then treated with or without 50-mM glutathione to remove biotin molecules remaining on the cell surface. Cells were lysed and class I and class II MHC molecules were successively immunoprecipitated with the mAb 16-1-11N and 10.2-16, respectively. Immunoprecipitates were electrophoresed on 10 or 12.5% polyacrylamide-SDS gels under nonreducing conditions and transferred to nitrocellulose. The blots were incubated 1–4 h at room temperature with blocking buffer (3% BSA, 0.5% Tween-20, 1 M D-glucose, 10% glycerol in PBS-CM) followed by 1–4 h with 0.5 × 10⁶ cpm/ml ¹²⁵I-streptavidin (Amersham Corp., Arlington, IL) in blocking buffer to detect biotinylated proteins. The blots were then washed repeatedly with 0.5% Tween-20, dried, and autoradiographed. For quantitation, optical densitometry was performed on films using an AMBIS image acquisition and analysis system (San Diego, CA).

Results

Deletion of the Endosomal Localization Signal from the Cytosolic Tail of Ii Results in Expression of Class II–Ii Complexes at the Cell Surface. Recent studies have indicated that localization and retention of class II within a post-Golgi apparatus, endosomal compartment is enhanced by association with Ii (13, 14, 35). This endosomal localization signal within Ii is thought to reside within the cytosolic tail of Ii, between amino acids 11 and 15 (11, 12). To investigate the importance of this region of Ii on class II transport and antigen presentation, I-A^k- and I-A^d-positive Ltk⁻ transfectants expressing Ii deleted of amino acids 2–17 (Δ2-17; see Fig. 1) were generated. Cells transfected with the truncated form of Ii express significant levels of Ii at the cell surface while cells expressing wild type Ii do not (Table 1).

To determine whether Δ2-17 p41 is expressed at the cell surface in association with class II, transfectants expressing wild type and truncated Ii were cell surface radiolabeled, lysed, and class II and Ii were immunoprecipitated (Fig. 2). In cells transfected with the truncated form of Ii, class II was coprecipitated with the Ii-specific mAb and Δ2-17 p41 was coprecipi-
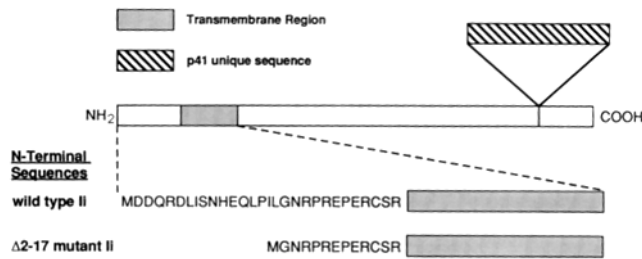
Figure 1. The NH₂-terminal deletion construct of Ii. Murine Ii exists as two distinct forms, p31 and p41, that differ by the inclusion in p41 of a 64–amino acid segment (hatched bar) encoded by an alternately spliced exon (20). Both p31 and p41 are transmembrane glycoproteins oriented such that the NH₂ terminus resides in the cytosol. The sequence of the wild type and NH₂-terminal deletion construct (Δ2-17) of p31 and p41 Ii is shown at the bottom. The Δ2-17 construct is missing the critical amino acid residues between 11 and 15 that are necessary for endosomal localization of Ii (11, 12). Note that the endosomal localization signal and the p41-specific segment required for efficient antigen presentation are located on opposite sides of the membrane.

Figure 2. Δ2-17 p41 is expressed at the cell surface associated with class II. L cell transfectants expressing I-Aᵏ with Δ2-17 p41 Ii (Δp41), I-Aᵏ with genomic Ii (gIi), or I-Aᵈ with genomic Ii (LADgIi) were surface labeled with ¹²⁵I. I-Aᵏ was immunoprecipitated with 10.2.16, I-Aᵈ with M5/114, and Ii with P4H5, and analyzed by SDS/PAGE. Class II α chains do not generally label well with iodine; however, when associated with Ii, the labeling efficiency of α is increased. The position of molecular mass markers, class II α and β chains, Δ2-17 p41 Ii, and the p55 protein that coprecipitates with I-Aᵏ, but not with I-Aᵈ, are indicated.

tated with the class II–specific mAb, clearly indicating that Δ2-17 p41–class II complexes are present at the cell surface. In wild type Ii-positive transfectants, Ii was not detectable at the cell surface in either anti–Ii or anti–class II immunoprecipitates, confirming that class II dissociates from wild type Ii before it reaches the cell surface. Both Δ2-17 p31 and Δ2-17 p41 remain associated with class II at the plasma membrane in a manner that effectively inhibits presentation of pep-

tide antigens to T cells (reference 5; Anderson, M. S. and J. Miller, manuscript in preparation). These results demonstrate that deletion of the endosomal localization signal in the cytosolic tail of Ii interferes with the post-Golgi apparatus processing events that are necessary for Ii degradation and dissociation from class II before transport of class II to the cell surface.

Only a Subpopulation of Class II Is Associated with Δ2-17 p41 at the Cell Surface. Reciprocal preclear experiments (Fig. 3) indicate that although the vast majority of cell surface Δ2-17 p41 is associated with class II, ~30–45% of the total class II at the cell surface was free of Ii. This population of Ii-free class II was not derived from cells that have turned off Δ2-17 Ii expression because flow cytometry analysis (summarized in Table 1) indicates that these transfectants are quite uniform in their expression of Δ2-17 Ii. Although we cannot exclude the possibility that some class II molecules have not associated with Ii during biosynthesis, pulse chase analyses (see next section) argue against this accounting for such a large percentage of Ii-free class II at the cell surface. These analyses indicate that a sizable population of cell surface class II has dissociated from Ii in cells expressing Δ2-17 p41.

Table 1. *Truncation of Cytosolic Tail of Ii Results in Cell Surface Expression of Ii*

Cell line	GαM	P4H5	10.2.16
LAK	5	5	618
LAK p41	4	6	570
LAK Δ2-17 p41	8	140	437

	GαM	P4H5	MKD6
LAD	4	5	446
LAD gIi	5	6	594
LAD p31	5	6	376
LAD p41	6	6	392
LAD Δ2-17 p31	5	68	437
LAD Δ2-17 p41	5	62	376

Ii-negative, class II–positive Ltk⁻ transfectants expressing I-Aᵏ (LAK) or I-Aᵈ (LAD) were retransfected with a genomic clone of Ii (gIi) that expresses both p31 and p41, or with cDNA clones expressing either wild type (p31 or p41) or truncated (Δ2-17 p31 or Δ2-17 p41) Ii. The cloned transfectants were stained with P4H5 (anti-Ii), MKD6 (anti-I-Aᵈ), or 10.2.16 (anti-I-Aᵏ) followed by FITC-coupled goat anti-mouse IgG (GαM) and analyzed by flow cytometry. Background staining was determined by staining with the GαM reagent alone. Numbers indicate the mean fluorescence intensity for each antibody.
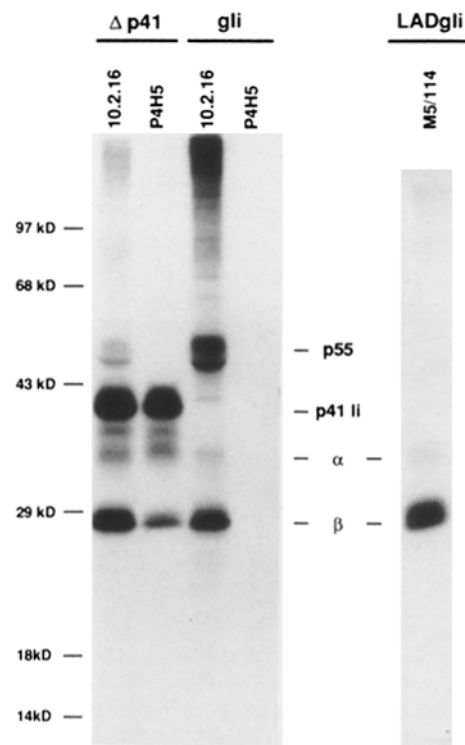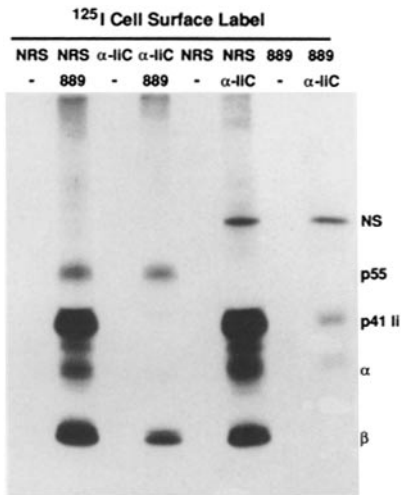
125I Cell Surface Label

NRS	NRS	α-IiC	α-IiC	NRS	NRS	889	889
-	889	-	889	-	α-IiC	-	α-IiC

NS
p55
p41 Ii
α
β

Figure 3. Only a subpopulation of class II expressed at the cell surface is associated with Δ2-17 p41. L cell transfectants expressing I-A^k plus Δ2-17 p41 Ii were surface labeled with ^{125}I. Ii-free class II complexes were detected by preclearing lysates with αIiC followed by immunoprecipitation with the class II–specific sera (889). Class II–free Ii complexes were detected by preclearing lysates with 889 followed by immunoprecipitation with the Ii-specific sera (α-IiC). To determine total class II or Ii, lysates were precleared with a cocktail of anti–class I mAb and preimmune rabbit sera (NRS) followed by immunoprecipitation with the class II–specific or with the Ii-specific antisera. The final preclear from each set was run in lanes 1, 3, 5, and 7. Class II α and β chains, Δ2-17 p41 Ii, and the p55 protein that coprecipitates with class II are indicated on the right. NS is a nonspecific protein that precipitates with the rabbit α-IiC antisera, but not with the mAb α-Ii, P4H5 (see Fig. 4).

I-A^k that is free of Ii at the cell surface appears to associate with a 55-kD protein (p55). In the preclear experiments (Fig. 3) none of the p55 associated with class II is seen in the anti-Ii precipitate and all of it remains associated with the Ii-free class II. Although p55 is readily detected in cell surface–labeled cells expressing no Ii (data not shown), wild type Ii,

or truncated (Fig. 2), it is not seen in biosynthetically labeled cells (see Fig. 6, below). This could indicate that p55 is derived from an extracellular source (i.e., serum), that p55 is synthesized at a slow rate, or that p55 is associated with a very small percentage of class II, but is extremely sensitive to cell surface labeling. Although the origin of p55 and the nature of p55 association with I-A^k are not known, several observations suggest that the interaction is specific. Association of p55 with class II is allele dependent; p55 is coprecipitated with I-A^k, but not I-A^d using several class II–specific antibodies, including one antisera that cross-reacts with I-A^k and I-A^d (Figs. 2 and 3). Immunoprecipitates of I-A^k from both L cell and EL4 cell transfectants contain p55 indicating that p55–I-A^k association is not cell type specific (data not shown). Finally, p55 does not coprecipitate with class I (see Fig. 6, below) or with transferrin receptor (data not shown). Class II–specific, allele-dependent interactions that are blocked by Ii are characteristic of class II–peptide binding. Because class II has been shown to form stable complexes with intact proteins (36), these results suggest that p55 may interact with the I-A^k–peptide site. Regardless of the nature of p55 association with class II, p55 does serve as a useful marker for class II that has dissociated from Ii (see Fig. 6, below).

Dissociation of Class II from Both Wild Type p41 and Δ2-17 p41 Involves Proteolytic Cleavage of Ii. The pool of Ii-free class II at the cell surface raises the possibility that even in the absence of the endosomal localization signal, class II–Ii dissociation can still occur. To determine where during class II biosynthesis Ii dissociation might take place, pulse–chase analysis of LAK, LAK-p41, and LAK–Δ2-17 p41 was performed (Fig. 4). Both wild type and truncated forms of Ii associate with class II early in biosynthesis. The difference in apparent molecular weight between the two forms of Ii appropriately reflects the size of the deletion. Based on the relative amount of Ii coprecipitating with anti–class II reagents, it appears that most, if not all, class II has associated with Ii early in
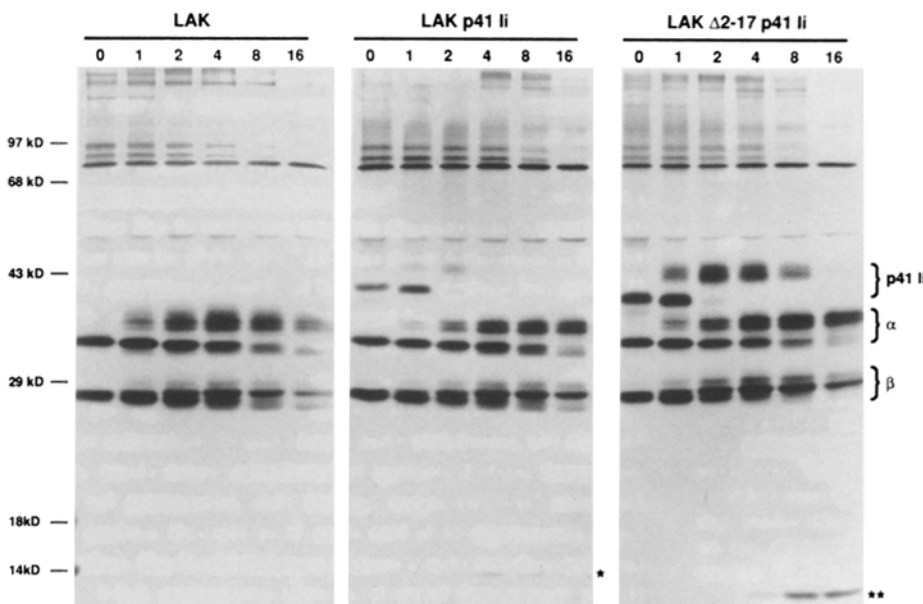

Figure 4. Dissociation of Δ2-17 p41 from class II is slower than wild type p41. L cell transfectants expressing I-A^k alone (LAK) or in combination with wild type p41 (LAK p41 Ii) or with Δ2-17 p41 Ii (LAK Δ2-17 p41 Ii) were pulse-labeled for 60 min with [³H]leucine and chased for 0, 1, 2, 4, 8, or 16 h. Class II was immunoprecipitated with 10.2.16 and precipitates were analyzed by SDS-PAGE. The position of molecular mass markers, class II α and β chains, Δ2-17 p41, and the Ii degradation fragments derived from wild type p41 (*) and Δ2-17 p41 (**) that coprecipitate with class II are indicated.

biosynthesis. Class II associated with the truncated form of p41 matures from the ER through the Golgi apparatus at a similar rate to class II associated with wild type p41 and fully processed forms of class II and Ii appear at the 1-h chase point. The majority of wild type p41 dissociates from class II shortly after mature glycosylated forms appear at the 1–2-h chase points. In contrast, Δ2-17 p41 remains associated with class II for an extended time and significant dissociation of Δ2-17 p41 from class II does not occur until 8 h. Thus, class II does completely dissociate from both wild type and Δ2-17 p41, although the process is considerably delayed in cells expressing the truncated form.

In normal cells, class II–Ii dissociation requires proteolytic cleavage of Ii, a multi-step process that allows for the isolation of intermediate cleavage products in association with class II (1, 37, 38). These fragments are readily detected in L cell transfectants (asterisk in Fig. 4) without the addition of protease inhibitors. We are confident that these fragments represent Ii degradation products because they are not present in Ii-negative transfectants (Fig. 4) and similar sized fragments are detected on Western blots with In1, a monoclonal antibody that is specific for the cytosolic, NH$_2$ terminus of Ii (see 16). Furthermore, these fragments appear concomitantly with the loss of the intact p41 protein (Fig. 4). The turnover of Ii in the wild type transfectant is similar to that described previously (39) and indicates that class II has gained access to a late proteolytic compartment that results in the cleavage of Ii from class II (1, 37, 38). Dissociation of class II from the truncated Ii also appears to be proteolytic, as visualization of Ii subfragments associated with class II coincides with Ii dissociation (double asterisk in Fig. 4). The difference in apparent molecular weight of the Ii subfragments derived from wild type and Δ2-17 p41 Ii reflects the predicted size of the deletion, suggesting that the subfragments derived from Δ2-17 p41 are also derived from the NH$_2$ terminus of Ii. Because deletion of amino acids 2–17 eliminates the In1 epitope, we cannot directly test this possibility. Taken together, these data indicate that removal of the cytosolic tail of Ii delays class II–Ii dissociation, but that dissociation still occurs by cleavage of Ii while it is associated with class II.

Class II Associated with Δ2-17 p41 Ii Is Rapidly Transported from the trans-Golgi Apparatus to the Cell Surface. Post-Golgi apparatus transport of class II to the cell surface is delayed compared with other integral membrane proteins. During this delay class II is retained in an Ii-dependent manner in a cellular compartment that has access to endocytosed material (13, 14, 40, 41). Release of class II from endosomes to the cell surface requires proteolysis of Ii (35, 42). To determine whether class II associated with the truncated form of Ii was also delayed in transport to the cell surface, we took advantage of the sensitivity of Ii to various proteases, including trypsin. Pulse–chase labeled LAK-Δ2-17 p41 cells were treated with and without trypsin at 4°C before solubilization and lysates were precipitated with anti–class II antibodies. Sensitivity of Ii to trypsin treatment of intact cells indicates arrival of the class II–Ii complex at the cell surface.
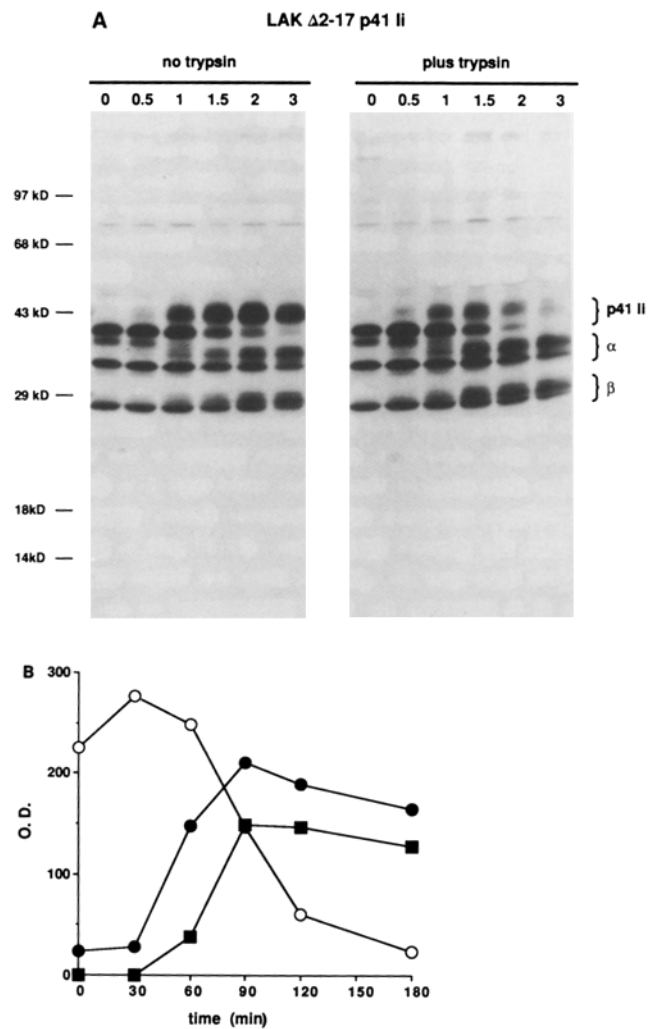
As can be seen in Fig. 5, mature glycosylated forms of α,

A LAK Δ2-17 p41 Ii

Figure 5. Class II is rapidly transported to the cell surface when associated with Δ2-17 p41. (*A*) L cell transfectants expressing I-A^k plus Δ2-17 p41 Ii were pulse labeled for 30 min with [^{3}H]leucine and chased for 0, 0.5, 1, 1.5, 2, and 3 h at 37°C. The cells were moved to 4°C and one half of the cells were incubated with trypsin at 4°C for 30 min. The cells were lysed, class II was immunoprecipitated with 10.2.16, and analyzed by SDS-PAGE. Class II α and β chains and Δ2-17 p41 are indicated on the right. The position of molecular mass markers are indicated on the left. (*B*) Quantitative analysis of class II-Δ2-17 p41 transport is displayed as O.D. of Ambis scanned autoradiographs. To compensate for minor differences in sample loading among lanes, the O.D. of Δ2-17 p41 has been normalized to the amount of class II β chain in each lane. (*Open circles*) Immature Δ2-17 p41 (from pulse chase without trypsin treatment); (*filled circles*) mature Δ2-17 p41 (from pulse chase without trypsin treatment); (*filled squares*) plasma membrane Δ2-17 p41 (amount of mature Δ2-17 p41 that is sensitive to trypsin treatment of intact cells determined by subtracting the amount of mature Δ2-17 p41 with trypsin treatment from the amount of mature Δ2-17 p41 without trypsin treatment).

β, and Δ2-17 p41 are first seen by 30 min of chase, and complete conversion of immature to mature proteins takes place over the next several hours ($t_{1/2} \simeq 50$ min). This slow maturation of class II–Ii complexes reflects variation in the efficiency of egress from the ER. Class II–Δ2-17 p41 complexes are

detected at the cell surface within 60 min, and by 3 h the vast majority of class II–Δ2-17 p41 complexes have arrived at the cell surface ($t_{1/2} \simeq 70$ min). These data indicate that once glycosylation is complete in the Golgi apparatus, class II associated with Ii lacking the cytosolic tail is rapidly transported to the cell surface ($t_{1/2}$ Golgi apparatus to plasma membrane $\simeq 20$ min). This is in marked contrast to L cell transfectants expressing wild type Ii, where transport of class II from the Golgi apparatus to the cell surface is delayed for ~4–6 hours (Fineschi, B., and J. Miller, manuscript in preparation).

Analysis of the kinetics of class II transport and Ii dissociation indicates that detectable loss of Δ2-17 p41 from the anti–class II precipitates and the appearance of Ii subfragments occur only after class II–Ii complexes appear at the cell surface. These data suggest that while wild type Ii is degraded in a post-Golgi network compartment before insertion of class II into the plasma membrane, Δ2-17 p41 is degraded slowly over time and this process does not begin until after the class II–Ii complex has been transported to the plasma membrane.

Internalization of Class II from the Cell Surface. The finding that in the Δ2-17 p41 transfectants Ii degradation occurs only after the class II–Ii complex has reached the cell surface suggests that this degradation occurs after internalization of the class II–Ii complex into an endosomal compartment. To measure class II internalization directly, cells were labeled with a reducible biotinylation reagent at 4°C, plasma membrane proteins were allowed to internalize at 37°C for 60 min, and endocytosed class II molecules were identified by protection from reduction by glutathione at 4°C. Significant internalization of class II, but not class I, molecules was observed (Fig. 6). Class II associated with both Δ2-17 p41 and with p55 were internalized at similar levels. Because class II only associates with p55 after it has dissociated from Ii (see Fig. 3, above), these results indicate that internalization of class II from the cell surface is not dependent on association with Δ2-17 p41. An endocytic rate constant of 0.52%/min was calculated for class II–Δ2-17 p41 complexes by kinetic analysis of internalization (Fig. 7). Assuming first order kinetics, it would take ~130 min to internalize 50% of the cell surface class II–Δ2-17 p41 complexes. As the half-life of mature Δ2-17 p41 is about 190 min, it would appear that this internalization rate can account for the slow degradation of Δ2-17 p41.

Taken together the biochemical analysis of class II transport in cells expressing Δ2-17 p41 suggests that deletion of the endosomal localization signal of Ii allows for the rapid transport of the class II–Ii complex to the plasma membrane. Once at the cell surface, the class II–Δ2-17 p41 complex is internalized over time, allowing access to the endosomal compartment, where Ii is degraded. Based on the significantly longer half-life of class II than Ii in Δ2-17 p41–positive cells (the half-life for class II degradation from biosynthesis is ~15 h and for Δ2-17 p41 is ~4 h), Ii-free class II likely recycles to the plasma membrane. This recycled population could account for the sizable fraction of Ii-free class II at the cell surface seen in Fig. 3.
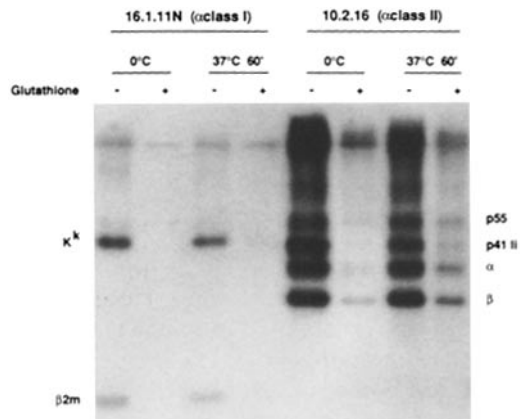

Figure 6. Class II is spontaneously internalized from the cell surface, independent of Ii association. L cell transfectants expressing I-A^k plus Δ2-17 p41 Ii were biotinylated with NHS-SS-biotin at 0°C and then either left at 0°C or incubated for 60 min at 37°C. Parallel plates of cells were then incubated at 0°C with or without glutathione to reduce biotin from the cell surface. Class I (16.1.11N) and class II (10.2.16) immunoprecipitates were electrophoresed on SDS/PAGE, transferred to nitrocellulose, and biotinylated molecules were detected with ^{125}I-avidin. Biotinylated cell surface molecules that were internalized during the 60-min incubation at 37°C are protected from reduction by glutathione treatment at 0°C and remain detectable by ^{125}I-avidin binding. The position of class I heavy chain (K^k) and light chain (β2m) are indicated on the left and the position of class II α and β chains, Δ2-17 p41 Ii, and p55 are indicated on the right.

Truncated p41 Ii Can Still Enhance Class II–restricted Antigen Presentation. Recently, Ii has been shown to facilitate presentation of only some antigens (15, 16) and this function is mostly restricted to the alternatively spliced p41 form of Ii (16). To determine if the endosomal localization signal in the cytosolic tail of Ii is critical for the ability of p41 to facilitate antigen presentation, cells expressing no Ii, wild type p41, or truncated p41 were assayed for presentation of Ii-
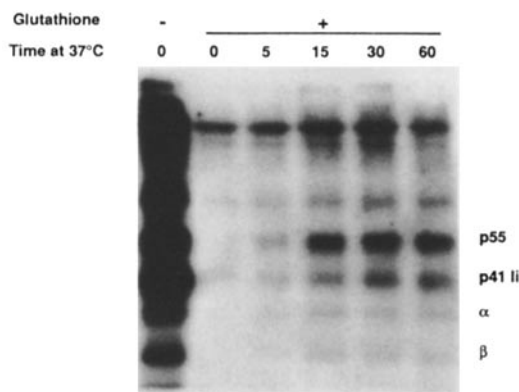

Figure 7. Class II–Δ2-17 p41 complexes are internalized slowly. L cell transfectants expressing I-A^k plus Δ2-17 p41 Ii were biotinylated with NHS-SS-biotin at 0°C and then either left at 0°C or incubated for 5, 15, 30, or 60 min at 37°C. Internalization was detected as described in Fig. 6 after immunoprecipitation with 10.2.16 (α-class II) mAb. The position of class II α and β chains, Δ2-17 p41 Ii, and p55 are indicated on the right. This experiment was repeated four times with similar results.

dependent and Ii-independent antigens to I-A^k-restricted T cells (Fig. 8, A–C). The T cell hybridoma TS12 is insensitive to enhancement of antigen presentation by wild type Ii, as both wild type Ii-positive transfectants and Ii-negative transfectants present RNase at equivalent levels to TS12 (Fig. 8 A). The Δ2-17 p41 Ii transfectant also presents RNase at equivalent levels to these cells, suggesting that this mutation has no apparent effect on the ability of these cells to process and present native RNase to TS12. Antigen presentation to the T cell hybridomas, 3A9 and SKK45.10, is enhanced by p41 Ii, as the coexpression of p41 Ii dramatically increases the efficiency of antigen presentation over the Ii-negative transfectant (Fig. 8, B and C). Interestingly, the Δ2-17 p41 Ii transfectant is able to present native hen egg lysozyme and KLH at levels equivalent to wild type p41 transfectants (Fig. 8, B and C). These results demonstrate that the p41 form of Ii can enhance antigen presentation independently of the endosomal localization signal located in the cytosolic tail.

When we extended these analyses to I-A^d-restricted T cells (Fig. 8, D–G), we found that Δ2-17 p41 inhibited antigen presentation of the Ii-independent epitope recognized by 3DO54.8 T cells (Fig. 8 D). Similarly, a truncated form of p31 Ii, Δ2-17 p31, can inhibit antigen presentation to the I-A^d-restricted T cells (Fig. 8, D and G), but not to the I-A^k-restricted T cells (data not shown). Thus, Δ2-17 p31

and Δ2-17 p41 have a generalized ability to inhibit antigen presentation to I-A^d-restricted, but not I-A^k-restricted, T cells that could reflect the antigens used or the specific T cells tested. Alternatively, these differences may reflect allelic control of the avidity of Ii-binding or internalization rates of class II-Ii complexes. When we tested the ability of Δ2-17 p41 to enhance antigen presentation of OVA to 3DO-18.3, in some (2/6) experiments Δ2-17 p41 Ii was able to enhance OVA presentation as well as wild type p41 (Fig. 8 E), however, in most (4/6) experiments, the truncated p41 was less efficient than wild type (Fig. 8 F). These results suggest that Δ2-17 p41 can enhance antigen presentation to I-A^d-restricted T cells, but that this effect may be masked by the ability of Δ2-17 Ii to inhibit antigen presentation, as seen for 3DO-54.8 cells. In fact, direct comparison of Δ2-17 p41 and Δ2-17 p31-positive cells shows a similar magnitude of p41-dependent enhancement of antigen presentation to I-A^d-restricted 3DO-18.3 T cells as does comparison of wild type p41- and p31-positive cells (Fig. 8 G). Because the inhibitory effect was not seen with the I-A^k-restricted T cells, Δ2-17 p41 Ii consistently was able to enhance antigen presentation at levels equivalent to wild type p41. Taken together, these results indicate that the cytosolic tail of p41 is not required for enhanced antigen presentation to class II–restricted T cells.
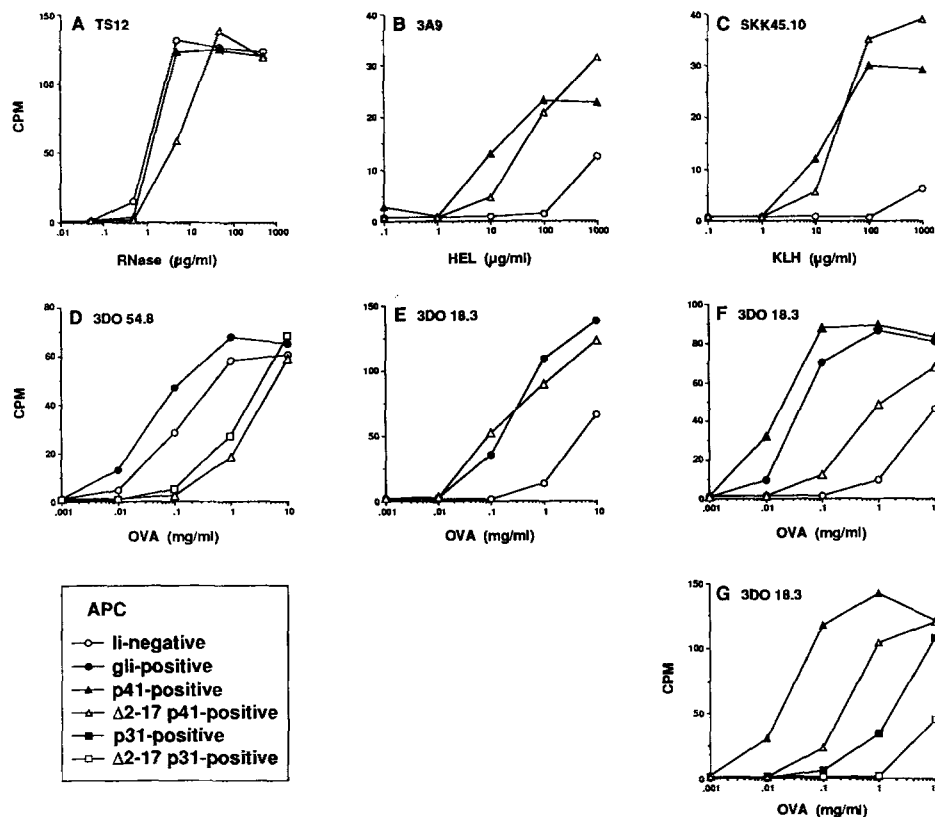

Figure 8. The endosomal localization signal is not required for p41-dependent enhancement of antigen presentation. L cell transfectants expressing I-A^k (A–C) or I-A^d (D–G) without Ii (open circles), with genomic Ii (gIi, filled circles), with wild type p41 (filled triangles), with Δ2-17 p41 (open triangles), with wild type p31 (filled squares), or with Δ2-17 p31 (open squares) were assayed for their ability to present various doses of antigen to class II–restricted T cell hybridomas. Data represent the incorporation of [³H]thymidine (cpm × 10³) by the IL-2-dependent T cell line, CTLL, when cultured in the presence of 25% culture supernatant from the antigen presentation assays. Wild type and truncated forms of p41 are indistinguishable in antigen presentation of both Ii independent (A) and p41-facilitated (B and C) antigens to I-A^k-restricted T cells. Although Δ2-17 p41 can facilitate I-A^d-restricted antigen presentation as well as wild type p41 in some experiments (2/6, E), in other cases (4/6) this enhancement is diminished (F). This correlates with the ability of both Δ2-17 p31 and Δ2-17 p41-positive transfectants to inhibit presentation of Ii-independent epitopes to I-A^d-restricted T cells (D) and Δ2-17 p31 to inhibit presentation of Ii-dependent epitopes to I-A^d-restricted T cells (G). The p41-dependent enhancement of antigen presentation is equivalent whether p41 is expressed as 10% of total Ii from alternative splicing of a genomic Ii construct or as 100% of total Ii from a p41 cDNA expression vector (F) (16). RNase, bovine ribonuclease; HEL, hen egg lysozyme; KLH, keyhole limpet hemocyanin; OVA, chicken ovalbumin.

Discussion

In the present study we have examined the role of the endosomal localization signal located in the cytosolic tail of Ii in class II transport and antigen presentation. We found that deletion of amino acids 2–17 from the cytosolic tail of p41 Ii dramatically altered the intracellular transport of class II–Ii complexes. Class II that is associated with wild type Ii exits the *trans*-Golgi apparatus and is transported to and retained for several hours within an endosomal compartment. Within this compartment Ii is degraded and class II is released to the cell surface. It is during passage through this proteolytic compartment that newly synthesized class II en route to the cell surface is thought to associate with antigenic peptides. Truncation of the Ii cytosolic tail results in the rapid transport of class II–Ii complexes from the *trans*-Golgi apparatus to the cell surface. Once at the cell surface the complex is internalized over time, Ii is degraded, and class II recycles to the plasma membrane. Surprisingly, altering the pathway of class II transport in this manner had little effect on the ability of p41 Ii to enhance antigen presentation. Thus, p41-dependent enhancement of antigen presentation does not require the endosomal localization signal encoded in the cytosolic tail of Ii.

Our biochemical data suggest that, when associated with Δ2-17 p41, class II intersects the endosomal antigen processing compartments after internalization from the cell surface. The transport rate to the cell surface of class II associated with truncated Ii is extremely rapid compared with class II associated with wild type Ii (41). Thus, in the absence of the Ii-encoded endosomal localization signal class II is either not sorted to or is not retained in the endosomal compartment during transport to the cell surface. Dissociation of both wild type and truncated p41 appears to occur by similar proteolytic events. However, the kinetics of Ii dissociation and of the appearance of class II–associated Ii proteolytic fragments is delayed in Δ2-17 p41–positive cell lines, and significant Ii dissociation occurs only after the class II–Δ2-17 p41 complex has arrived at the plasma membrane. These data suggest that Δ2-17 Ii gains access to endosomes via an internalization pathway from the cell surface which then results in the cleavage and turnover of the Δ2-17 Ii molecule. This was substantiated by a direct measure of class II internalization. Finally, the half-life of class II is much longer than that of Δ2-17 p41, arguing that the internalized class II returns to the cell surface after Δ2-17 Ii is degraded. The detection of a large population of steady state class II that is free of Ii at the cell surface supports this observation. Taken together, these analyses of the effects of Δ2-17 p41 on class II transport reveal a pathway of plasma membrane recycling for class II molecules.

Demonstration of class II internalization and recycling has been a controversial issue. Although some early studies were able to document internalization of class II (17–19), most of these studies relied on the use of antibodies to tag cell surface class II molecules, and it has been difficult to control for the possibility that antibody-binding modulates class II internalization. In several cases where ligand-independent internalization has been measured, little or no internalization has been

detected (41, 43, 44). However, Reid and Watts (45) have shown that class II endocytosis and recycling can occur in B cells, but the cycling time is extremely rapid (2–3 min) and an appreciable pool of internalized class II was not evident without the use of primaquine to neutralize endosomal compartments. In contrast, the L cell transfectants analyzed in the present study internalize class II more slowly and an appreciable pool of intracellular class II (10–20% of total) can be accumulated after internalization from the cell surface.

There is accumulating data showing that the majority of class II–peptide complexes at the cell surface are derived from newly synthesized class II binding peptides en route to the cell surface. The sensitivity of antigen presentation to protein synthesis inhibitors and brefeldin A (46, 47) implicates a role for newly synthesized class II. However, the lack of specificity of these reagents and the recent observation that brefeldin A can affect endocytosis (48, 49) raise the possibility that these drugs inhibit antigen presentation by modifying cellular functions other than class II biosynthesis. More recently, the observation that the ability of Langerhans cells to process and present antigen correlates with a transient induction of class II biosynthesis (50) and the ability of Ii to enhance presentation of some antigens (15, 16) argue that newly synthesized class II may be the principle component for antigen presentation. This hypothesis has been confirmed by biochemical analyses of class II–peptide association. Newly synthesized class II has been shown to form SDS-stable conformations, indicative of peptide association, while en route to the cell surface (42, 51). This process can be driven by both fluid phase uptake of antigen (51) and internalization of antigen through the immunoglobulin receptor on antigen-specific B cells (44). These data are consistent with a predominant role of de novo synthesized class II in antigen presentation.

The relative role of plasma membrane recycled class II in antigen presentation has been more controversial. One of the strongest arguments for a recycling pathway has been that the half-life of peptide–class II complexes is relatively short compared with the half-life of class II molecules (52, 53). This rapid turnover of peptide–class II complexes in living cells may allow for peptide exchange and reutilization of class II (54). In light of this possibility, dissociation of peptide–class II complexes appears to occur within an acidic compartment that might represent the peptide loading, endosomal compartment (53). In contrast, in EBV-transformed human B cells class II–peptide complexes are extremely stable and their turnover rate is similar to total class II, suggesting that in these cells there is no opportunity for peptide exchange (55). One possible explanation for the apparent disagreement as to whether class II can be recycled and whether this recycling component is functionally relevant is that this process may vary between cell types. Consistent with this possibility, little, if any, class II internalization has been detected in EBV-transformed human B cell lines (41, 43, 44), similar to those that express extremely stable class II–peptide complexes (55). In addition, expression of class II with Ii containing a cyto-

solic tail deletion in the human fibroblast cell line, M1, results in very stable expression of the class II–Ii complex at the cell surface and the vast majority of class II remains associated with Ii (5). This is in contrast to our finding in L cell transfectants that 30–45% of cell surface class II has dissociated from Ii. This may reflect differences in the ability of these cell types to internalize class II, allowing for endosomal degradation of Ii. In this report, we show that in the presence of Ii, endocytosed class II can enter the proper endocytic compartments for antigen binding. Although we know that Ii-free class II can still be internalized, it remains to be determined whether this pool of recycling Ii-free class II is capable of accessing antigenic peptides and whether this pathway exists in conventional antigen-presenting cells.

Previous studies from our laboratory have indicated that the p31 form of Ii, which contains a wild type endosomal localization signal, is not very effective at facilitating antigen presentation compared to Ii-negative cells (16). This observation along with the present study suggest that for some antigens the endosomal localization signal in the cytosolic tail of Ii is neither necessary nor sufficient to enhance antigen presentation. However, it is possible that under some circumstances, the ability of the endosomal localization signal to increase the relative concentration of class II in endosomes may be important for effective antigen presentation. For example, in cells that express low levels of class II or in B cells that internalize a bolus of antigen in a short time, the amount of class II available for peptide binding in endosomes may be the limiting factor during antigen presentation. In contrast, our L cell transfectants express high levels of class II and continuously internalize antigen by fluid phase uptake throughout the course of the antigen presentation assay. Under these conditions, the dwell time of class II within endosomal compartments may not be a critical factor in the efficiency of antigen presentation.

The results presented in this report suggest that the p41 form of Ii can facilitate antigen presentation regardless of the route that class II takes to the endosome. In wild type p41-positive cells the class II–Ii complex is sorted directly from the trans-Golgi apparatus to endosomes, where Ii is degraded and class II associates with peptide. Deletion of the endosomal localization signal in Ii results in the rapid transport of the class II–Δ2-17 p41 complex to the plasma membrane and Ii is degraded only after internalization of the complex. Thus, these data raise the possibility that, in addition to the conventional trans-Golgi apparatus to endosomal route, class II might form complexes with antigenic peptide when it enters the endosomal compartment after endocytosis from the cell surface.

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