Efficient Endosomal Localization of Major Histocompatibility Complex Class II–Invariant Chain Complexes Requires Multimerization of the Invariant Chain Targeting Sequence

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Abstract. During biosynthesis, MHC class II–invariant chain complexes are transported into endosomal compartments where invariant chain (Ii) is degraded and class II encounters antigenic peptides. One of the signals that determines this intracellular transport route has been localized to the cytosolic domain of Ii. Deletion of this signal disrupts endosomal targeting and results in the stable expression of class II–Ii complexes at the surface. In this paper we have examined the role of Ii trimerization on the generation of this endosomal localization signal. In L cell transfectants expressing class II and both wild type Ii and a truncated form of Ii that lacks this endosomal localization signal, Ii was found to form multimers which could contain both wild type and truncated Ii. The multimers were not large aggregates but were found to be discrete complexes, probably the nine molecule class II–Ii complex that has been observed in human B cells. The co-expression of trun-

cated Ii allowed for cell surface expression of a subset of wild type Ii. This surface-expressed wild type Ii associated with truncated Ii in multimers at a 2:1 ratio, indicating that these trimers contain two truncated and one wild type Ii molecule. These data suggest a division in trafficking of Ii trimers: if two wild type Ii molecules are present, the complex is transported to and rapidly degraded in endosomes, whereas the presence of only one wild type Ii results in trafficking and expression of the heterotrimer on the cell surface. Following surface arrival, complexes containing only a single wild type Ii molecule are internalized more rapidly and have a shorter half-life than complexes containing only truncated Ii molecules. These data suggest that although a single Ii cytosolic domain can function as a plasma membrane internalization signal, multimerization of Ii is required for efficient Golgi complex to endosome targeting of class II–Ii complexes.

MAJOR histocompability complex (MHC)[1] class II molecules, involved in antigen presentation in the immune system, are heterodimers composed of a 34-kD α chain and a 28-kD β chain. During biosynthesis class II associates with a type II integral membrane glycoprotein called invariant chain (Ii) (Jones et al., 1979). Although many cell surface proteins are transported directly from the TGN to the plasma membrane, class II–Ii complexes are transported to and retained in a post-Golgi compartment where Ii is degraded by proteolysis prior to stable class II insertion in the plasma membrane (Neefjes et al., 1990). This post-Golgi compartment is clearly within the endocytic pathway (Nowell and Quaranta, 1985; Blum and Cresswell, 1988; Nguyen et al., 1989; Guagliardi et al., 1990; Lamb et al., 1991; Peters et al., 1991; Pieters et al., 1991), but appears to be distinct from lysosomes and both

early and late endosomes (Amigorena et al., 1994; Qiu et al., 1994; Tulp et al., 1994; West et al., 1994). Class II free of intact Ii, but possibly still associated with proteolytic NH_2-terminal fragments of Ii, is transiently accumulated in this compartment. Within this compartment, class II is thought to assemble with peptide and the complexes are eventually transported to the surface where interaction with T cell receptors present on CD4+ T cells can occur.

Although class II–Ii complexes are transported to a distinct endosomal/lysosomal compartment, the route taken and the mechanism used are not fully understood (for review see Sant and Miller, 1994). A number of endosomal localization signals have been implicated within the class II–Ii complex. Cells expressing class II but not Ii can function to present antigen suggesting that an endosomal localization signal may be present in class II (Sekaly et al., 1988; Peterson and Miller, 1990; Nadimi et al., 1991; Viville et al., 1993). In fact, mutations within the class II lumenal domain have been shown to affect intracellular localization (Chervonsky et al., 1994), suggesting that this region may contain a transport signal. Although class II alone can enter endosomal compartments, association with Ii can dra-

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1. *Abbreviations used in this paper:* CS, chondroitin sulfate; Ii, invariant chain; MHC, major histocompatibility complex.

matically increase the efficiency of class II localization to endosomes (Lamb and Cresswell, 1992; Arunachalam et al., 1993; Romagnoli et al., 1993; Simonsen et al., 1993). Ii-mediated endosomal localization appears to occur both by targeting (Odorizzi et al., 1994) and retention (Neefjes and Ploegh, 1992; Loss and Sant, 1993). Although the *trans*-membrane domain (Odorizzi et al., 1994) and the membrane proximal segment of the cytosolic domain of Ii (Swier, K., and J. Miller, manuscript in preparation) have recently been implicated in efficient delivery of Ii to a proteolytic endocytic compartment, the best defined endosomal localization signal in the class II–Ii complex is encoded by the two di-leucine motifs within amino acids 7–8 and 15–17 in the Ii cytosolic domain (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Pieters et al., 1993; Odorizzi et al., 1994). When this signal is present, Ii complexed with class II exits the ER, is transported to and retained in a post-Golgi compartment and is degraded with a short half-life (Blum and Cresswell, 1988; Nguyen and Humphreys, 1989; Neefjes et al., 1990; Pieters et al., 1991; Anderson et al., 1993). The route taken to the post-Golgi compartment may be direct from the TGN or may be via a brief cell surface intermediate (Wraight et al., 1990; Roche et al., 1993). When this endosomal localization signal is deleted from Ii, the resulting protein is found in large quantities at the cell surface and has a much longer half-life (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Anderson et al., 1993; Roche et al., 1993; Nijenhuis et al., 1994).

Ii is present in two alternatively spliced forms, a 31-kD protein (p31) and a 41-kD protein (p41) that contains an additional 64–amino acid sequence in its lumenal domain (Koch et al., 1987). Both forms are naturally occurring and can be expressed at different levels in various cell types (Kampgen et al., 1991). Functionally, both p31 and p41 can assemble with class II, facilitate class II folding and enhance endosomal localization (Anderson et al., 1993; Arunachalam et al., 1993; Romagnoli et al., 1993), but p41 is unique in its ability to enhance antigen presentation to a subset of T cells (Peterson and Miller, 1992).

Shortly after synthesis in the ER, Ii assembles to form multimeric complexes, consisting mostly of trimers (Marks et al., 1990) which may contain both the p31 and p41 forms of Ii (Arunachalam et al., 1993). Three class II heterodimers are then sequentially added to the pre-existing Ii trimer to form a large nine chain complex which exits the ER and transits through the Golgi complex (Roche et al., 1991; Lamb and Cresswell, 1992). This complex is thought to persist throughout transport into an endosomal compartment because proteolytic fragments of Ii have been found to exist in trimeric structures (Newcomb and Cresswell, 1993). In this report we address whether multimerization of Ii plays a role in determining the intracellular transport of class II–Ii complexes. We have found that at least two Ii cytosolic domains containing amino acids 2 through 17 are necessary for efficient endosomal localization. Complexes that contain only a single intact Ii endosomal localization signal appear to be transported to the cell surface. However, once at the surface a single Ii signal can mediate rapid internalization and degradation. Thus, multimerization of Ii may determine both the efficiency and route of endosomal targeting of the class II–Ii complex.

Materials and Methods

Cell Lines and Antibodies

Ltk− fibroblast cells transfected with class II (I- A^k) alone, with p31, or with Δ2-17p41 Ii have been described (Peterson and Miller, 1990; Anderson et al., 1993). The parental class II–positive cells were cotransfected with p31 and Δ2-17p41 to generate LAKp31Δ2-17p41 or were cotransfected with Δ2-17p31 and wild type p41 to generate LAKΔ2-17p31p41. Transfected cells were screened by flow cytometry after staining with 10.2-16, specific for I-A^k (Oi et al., 1978); P4H5, specific for the lumenal domain of murine Ii (Mehringer et al., 1991); and 16-1-11N, specific for class I K^k (Ozato et al., 1980). Cells were further screened by Western blotting with In-1, specific for the cytosolic domain of Ii (Koch et al., 1982).

Radiolabeling

Cells were metabolically labeled with [^{3}H]leucine as described (Anderson and Miller, 1992). All biosynthetically labeled cells were prelabeled for 1 h in leucine-free media, pulse labeled for 30 min to 2 h and chased, where applicable, for various times in complete media containing unlabeled leucine. For cell-surface labeling, 5–8 × 10^6 cells (>95% viable) were labeled with ^{125}I by the lactoperoxidase method at 4°C. Viable cells were isolated on ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradients before and after iodination.

Immunoprecipitation and Electrophoresis

Labeled cells were solubilized at 2 × 10^6 cells per ml in 0.5% NP-40, 0.15 M NaCl, 0.05 M Tris-HCl, 5 mM EDTA, 200 μg/ml PMSF, 25 μg/ml aprotinin. Postnuclear lysates were precleared overnight with protein A-Sepharose CL-4B pro protein G–Sepharose fast flow (Pharmacia Fine Chemicals) at 4°C. Lysates were immunoprecipitated for 1 h with 10.2-16 or P4H5 previously bound to protein A–Sepharose or In-1 previously bound to protein G–Sepharose. Immunoprecipitates were washed in 0.2% NP-40, 0.15 M NaCl, 0.05 M Tris-HCl, 5 mM EDTA, eluted by boiling in 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 20% (vol/vol) glycerol, 2% 2-mercaptoethanol, and analyzed on SDS-10% polyacrylamide gels. Gels were processed with Enhance (New England Nuclear, Boston, MA), dried, and autoradiographed at −70°C. For two-dimensional gel electrophoresis, immunoprecipitates were eluted with 9.5 M urea, 2% (wt/vol) NP-40, 5% (vol/vol) ampholines, pH 3.5 to 10 (Pharmacia Fine Chemicals), 5% (vol/vol) 2-mercaptoethanol at room temperature for 1–2 h. Eluted immunoprecipitates were then loaded onto NEPHGE tube gels (O'Farrell et al., 1977) and overlaid with 3 M urea, 2.5% (vol/vol) ampholines pH 3.5–10, and run for 5 h at 500 V as described (O'Farrell et al., 1977). Gels were then extruded into SDS elution buffer and stored at −20°C. Thawed tube gels were run on 10% acrylamide-SDS gels and processed as above. For quantitation, optical densitometry was performed on autoradiographs using an AMBIS image acquisition and analysis system (San Diego, CA).

Biotinylation and Internalization

The internalization assay was performed essentially as published (Anderson et al., 1993). Briefly, cells were biotinylated on 100 mm tissue culture plates after washing with 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 Chemical Co., Rockford, IL) in PBS-CM for 30 min and the reaction was quenched by washing the cells with ice cold 50 mM glycine in PBS-CM. Plates to be chased were then floated on a 37°C water bath in a tissue culture incubator after adding pre-warmed 37°C media for the chase period. At the end of the chase period plates were removed from the incubator, the warm media was immediately removed, ice cold media was added and the plate was placed on ice. To determine the rate of internalization, the plates were then treated with 50 mM glutathione (Sigma Chemical Co., St. Louis, MO) in 75 mM NaCl, 10 mM EDTA, 1% BSA to remove biotin from protein remaining at the surface. To determine the half-life of material at the cell surface, the cells did not receive glutathione treatment. All plates were then washed with 5 mg/ml iodoacetimide (Sigma Chemical Co.) in PBS-CM. The cells were then solubilized and immunoprecipitated with 10.2-16, P4H5 and In-1 as described above. Immunoprecipitates were assayed by SDS-PAGE as above, then Western transferred at 250 mA for 1.5 h (Bio-Rad Laboratories, Cambridge, MA) onto nitrocellulose (Schleicher & Schuell, Keene,

NH) and dried overnight. The nitrocellulose was then blocked with 10% glycerol, 1 M glucose, 3% BSA, and 0.5% Tween 20 in PBS (TGG), blotted with peroxidase conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) and developed with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). For quantitation, optical densitometry was performed on autoradiographs using an AMBIS image acquisition and analysis system (San Diego, CA). Data are presented as percent internalized = (chase point − zero time point)/without glutathione. The zero time point is used as internal background and efficiency of glutathione treatment, and without glutathione is total label.

Results

Experimental Design

Ii has been shown to exist as trimers but the function of this association is unknown. Ii molecules contain a sequence in their cytosolic domain that has been implicated in targeting and/or retaining Ii within endosomal compartments. It is possible that Ii multimerization is necessary to generate a functional endosomal localization signal. To address this possibility we designed a system to detect and follow the transport of heterotrimeric complexes which contain fewer than three complete cytosolic domains. We examined the kinetics of proteolysis and the cell surface expression of these heterotrimers in cells expressing class II cotransfected with wild type p31 alone (which contains the endosomal localization signal), Δ2-17p41 alone (which does not contain the signal) or both p31 and Δ2-17p41 (Table I). The wild type p31 and Δ2-17p41 forms of Ii were used to allow us to discriminate between wild type and truncated Ii by using the difference in molecular weight provided by the alternatively spliced domain present in p41. In addition, two monoclonal antibodies specific for different regions of Ii were used to define relative expressions of mixed trimers. P4H5 recognizes an epitope in the lumenal domain of Ii that is present in all forms of Ii used. In-1 recognizes an epitope in the cytosolic domain of Ii that is present in wild type but not truncated Ii. Therefore, In-1 would only directly precipitate p31 Ii; coprecipitation of Δ2-17p41 would indicate the formation of heterotrimers. Assuming that trimers containing both p31 and Δ2-17p41 can form in Ltk− cells, LAKp31Δ2-17p41 cells (see Table I) would produce trimers of all four possible combinations (Fig. 1). By following the transport and cell surface expression of the different trimer species, and specifically those trimers containing both p31 and Δ2-17p41, we can determine whether 1, 2, or 3 cytosolic domains are necessary for efficient sorting of class II–Ii complexes into endosomal compartments.

Table I. Surface Expression of Class II and Ii

Name	Clone	Ii gene(s) transfected	GAM*	10.2-16	P4H5
LAKgpt+	64.1.3	gpt alone	6[‡]	154	8
LAKp31	62.1.1	p31	5	275	6
LAKΔ2-17p41	32.2.7.3	Δ2-17p41	7	379	110
LAKp31Δ2-17p41	3.1.3	p31 and Δ2-17p41	12	287	43
LAKΔ2-17p31p41	10.1.3	Δ2-17p31 and p41	8	720	37

* Goat anti–mouse secondary antibody.
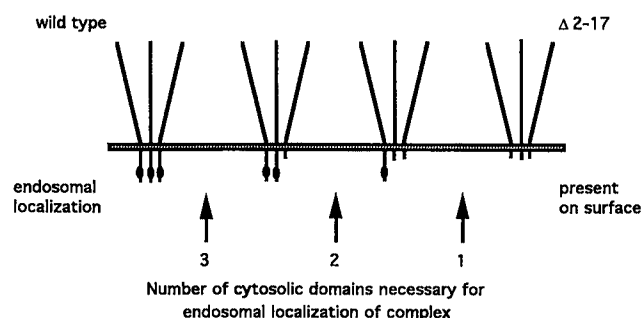[‡]Data are presented as mean fluorescence intensity.

Figure 1. Types of Ii trimers in cells expressing both p31 and Δ2-17p41. Ltk− cells expressing class II and both wild type and truncated Ii could theoretically form four types of Ii trimers, varying by the number of complete cytosolic domains present in each trimer. Trimers containing only wild type Ii are sorted to an endosomal compartment where degradation occurs. Trimers containing only Δ2-17Ii are sorted to the cell surface. If multimerization of the endosomal localization signal determines the intracellular fate of the class II–Ii complex, the trimers containing both wild type and Δ2-17 Ii would be endosomally sorted with differing efficiencies.

Formation of Ii Multimers

The assembly of class II–Ii complexes was examined in class II–positive cells expressing p31, Δ2-17p41, or both p31 and Δ2-17p41. Cells were biosynthetically labeled with [³H]leucine to equivalently label α, β, and Ii. Lysates from the cell lines were immunoprecipitated with 10.2-16, P4H5, or In-1 (Fig. 2). In all cases, anti-class II coprecipitates the relevant Ii molecules expressed by the cell lines. Furthermore, the appearance of α, β, and Ii in an approximately equal molar ratio indicates that the majority of class II is associated with Ii in these cell lines. However, the anti-class II antibody 10.2-16 is specific for β chain, so in cell lines that express excess β chain, such as LAKΔ2-17p41, the apparent ratio of α to β may be distorted due to the precipitation of excess free β chain. As expected, wild type Ii is precipitated by both P4H5 and In-1 (Fig. 2 A). In contrast, Δ2-17p41 is only precipitated by P4H5, because the truncation eliminates the In-1 epitope (Fig. 2 B). Note that both P4H5 and In-1 preferentially precipitate class II–free Ii. However, in cells expressing class II and both wild type p31 and Δ2-17p41, an In-1 precipitate contains both wild type p31 and Δ2-17p41 (Fig. 2 C). Because In-1 cannot directly precipitate Δ2-17p41, this protein must be coprecipitated due to its association with p31. Therefore, the presence of Δ2-17p41 in an In-1 precipitate indicates that p31 and Δ2-17p41 can associate into multimeric complexes. This finding does not depend on the class II allele expressed because similar results were obtained with cells expressing I-A^k (Fig. 2) and I-A^d (data not shown).

To exclude the possibility that association of p31 and Δ2-17p41 occurred only after the cells were lysed, LAKp31 cells and LAKΔ2-17p41 cells were plated together, labeled, and lysed (Fig. 3). P4H5 precipitated both forms of Ii, but In-1 precipitated only wild type p31. This indicates that association of p31 and Δ2-17p41 in LAKp31Δ2-17p41 cells must occur during biosynthesis. To verify that these multimeric complexes represent the nine chain class II–Ii complex previously observed in human B cell lines (Marks

A LAKp31   B LAKΔ2-17p41   C LAKp31Δ2-17p41   D Long exposure of panel C
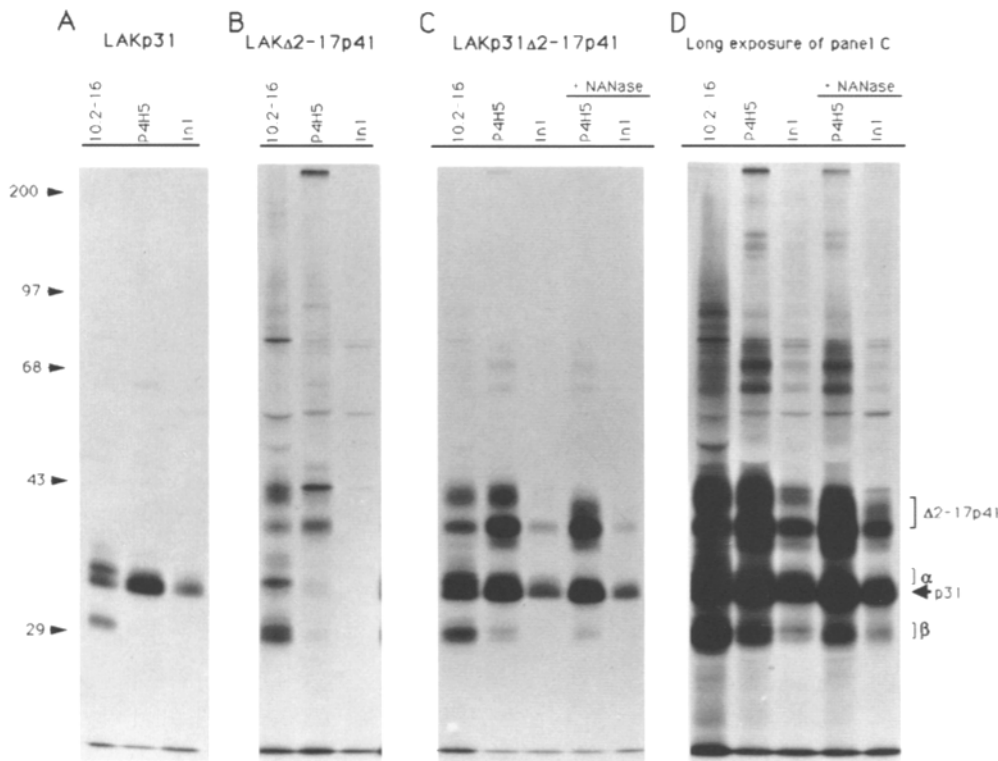
Figure 2. Ii multimers containing both p31 and Δ2-17p41 form in Ltk− cells. Ltk− cell transfectants expressing (*A*) I-A^k and p31 (*LAKp31*); (*B*) I-A^k and Δ2-17p41 (*LAKΔ2-17p41*); (*C*) I-A^k and both p31 and Δ2-17p41 (*LAKp31Δ2-17p41*) were labeled for 2 h with [^{3}H]leucine. Lysates were immunoprecipitated with 10.2–16 (class II specific), P4H5 (specific for lumenal domain of Ii), or In-1 (specific for cytosolic domain of Ii). In *C* and *D* duplicate P4H5 and In-1 precipitates of LAKp31Δ2-17p41 were treated with neurominidase (*NANase*) for 1 h. Panel D is a sixfold longer exposure of *C*. Δ2-17p41 is present both in an immature and a glycosylated, mature form of higher molecular weight. Neurominidase treatment affects only the mature form of Δ2-17p41, decreasing its molecular weight slightly. Precipitates were analyzed by 10% SDS-PAGE. Molecular mass markers are indicated on the left. Class II α and β chains, p31, and Δ2-17p41 are indicated on the right.

et al., 1990; Roche et al., 1991; Lamb and Cresswell, 1992), transfectants were biosynthetically labeled for 1 h, and then chased for 30 min to allow the complex to form. Cell lysates were run on a 5–20% sucrose gradient and fractions were precipitated with an anti-class II antibody. The class II–Ii complexes did not run as large aggregates, but rather as discrete complexes of ∼210 kD in both L cell transfectants and murine B cell lines (data not shown). Although this molecular mass estimate is lower than that seen previously in human B cell lines (Roche et al., 1991; Lamb and Cresswell, 1992), this probably reflects a difference in gradient conditions rather than inherent differences in the structure of the class II–Ii complexes.

To follow the differential fate of Ii trimers containing both wild type and Δ2-17 Ii, it is crucial to first confirm that all types of trimers (see Fig. 1) form randomly during assembly in the ER. To address this issue, three cell lines producing wild type and Δ2-17 Ii in different ratios were biosynthetically labeled with [^{3}H]leucine for 30 min, chased 15 min, lysed, and immunoprecipitated with P4H5 and In-1 (Fig. 4). Using the P4H5 ratio of wild type to Δ2-17 Ii as the biosynthetic ratio, we could predict the In-1 ratio (wt/Δ) assuming random generation of trimers (Table II). The actual ratios for the cell lines examined are close to those expected if trimers are generated randomly (Fig. 4 and Table II). Together these data indicate that p31 and Δ2-17p41 can associate in the same Ii trimer during biosynthesis, that these trimers form randomly, and can associate with three class II heterodimers to form a nine chain complex.

Maturation and Transport of Δ2-17p41 Associated with p31

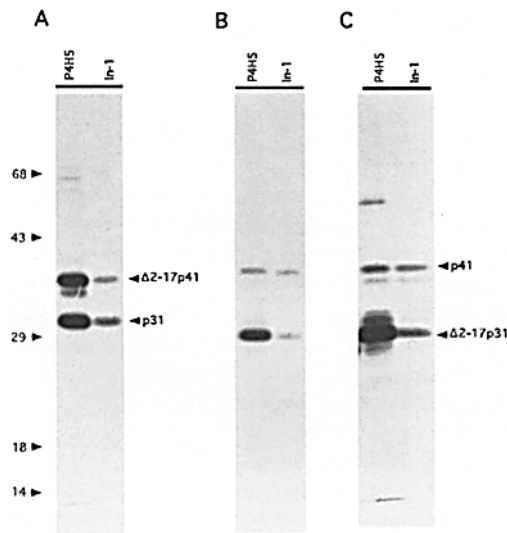To determine how long the class II–Ii multimers persist, In-1 and P4H5 precipitates from LAKp31Δ2-17p41 cells were treated with neuraminidase (Fig. 2, *C* and *D*), which cleaves the sialic acid residues added in the TGN from N-linked carbohydrate groups. Loss of sialic acid residues can be detected by a decrease in molecular weight of the treated protein on SDS-PAGE. Mature Δ2-17p41 coprecipitated by In-1, and thus in Ii multimers, is sensitive to neuraminidase indicating that it has been sialylated. This result shows that the class II–Ii complexes persist at least into the TGN. It is likely that the complexes remain intact during later transport events given the recent finding that Ii fragments remain trimeric in an endosomal compartment even after some proteolysis has occurred (Newcomb and Cresswell, 1993).

To more closely follow the transport of trimers containing both wild type and Δ2-17 Ii, LAKp31Δ2-17p41 cells were pulsed labeled, chased for various times, and lysates were then precipitated with 10.2-16 (specific for class II I-A^k) (Fig. 5 *A*). As expected, class II–Ii complexes form soon after synthesis, leave the ER and are transported
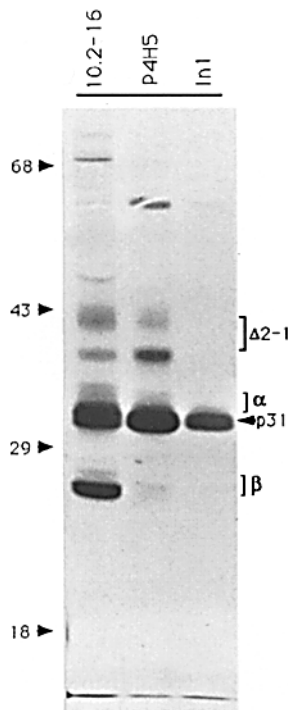
Figure 3. Ii multimers are not formed in cell lysates. LAKp31 cells and LAKΔ2-17p41 cells were grown in the same plate, pulsed for 2 h with [³H]leucine and lysed together. The lysate was immunoprecipitated with 10.2–16, P4H5 and In-1. Precipitates were analyzed by 10% SDS-PAGE. Molecular mass markers are indicated on the left. Class II α and β chains, p31, and Δ2-17p41 are indicated on the right.

through the Golgi complex resulting in maturation of α, β, and associated Ii. These complexes are eventually transported to a proteolytic endosomal compartment where Ii degradation occurs producing characteristic Ii fragments (data not shown). When the lysates were precipitated with

Table II. Ii Trimers Containing both Wild Type and Δ2-17 Ii Form Randomly

Cell line	Ratio of wild type:Δ2-17 Ii		
	P4H5*	In-1[‡]	
		Expected[§]	Actual
LAKp31Δ2-17p41 (3.1.3)	1:1	1:0.7	1:0.5
LAKΔ2-17p31p41 (10.1.3)	1:5	1:1.6	1:1.4
LAKΔ2-17p31p41 (10.2.5)	1:10	1:1.7	1:2.0

*P4H5 precipitates all Ii.
[‡]In-1 directly precipitates only wild type Ii, but may indirectly precipitate Δ2-17 that is associated with wild type Ii.
[§]Based on random association of Ii molecules into trimers and the relative biosynthesis rates of wild type and Δ2-17 Ii determined by the P4H5 precipitate.

In-1 to examine the maturation of class II and Δ2-17p41 associated with wild type p31, a similar pattern of maturation was observed (Fig. 5 *B*). In addition, it appeared that Δ2-17p41 associated with wild type p31 was degraded more rapidly than the bulk of Δ2-17p41 (Fig. 5, compare *B* with *A*). However, subsequent examination demonstrated that this difference is related to the degradation rate of class II–free Ii as compared to class II–associated Ii (data not shown). Despite many attempts to determine the fate of heterotrimers by pulse–chase analysis, several factors have precluded this determination. These factors include spreading of the biosynthetically labeled cohort during ER egress and Golgi transport, different transport and degradation rates of class II–Ii and Ii only complexes, and the preference of P4H5 and In-1 for precipitating class II–free Ii. Thus, although the pulse–chase analysis indicates that normal assembly, transport and maturation of class II–Ii oc-

Figure 4. Ii trimers containing wild type and truncated Ii are formed randomly. Ltk- cell transfectants expressing I-Aᵏ and both p31 and Δ2-17p41 (3.1.3, *A*), or I-Aᵏ and both Δ2-17p31 and wild type p41 (10.1.3, *B*; 10.2.5, *C*) were pulse labeled for 30 min with [³H]leucine and chased for 15 min to allow assembly of complexes. Ii was immunoprecipitated with P4H5 and In-1 and analyzed by SDS-PAGE. Molecular mass markers are indicated on the left and α and β chains, p31, Δ2-17p41, Δ2-17p31, and p41 are indicated on the right. Optical densitometry was performed from autoradiographs on the Ii bands to determine the ratios given in Table II.

LAKp31Δ2-17p41

Figure 5. Maturation of class II-Ii complexes in LAKp31Δ2-17p41 cells. Ltk- cells expressing I-Aᵏ and both p31 and Δ2-17p41 (3.1.3) were pulse–labeled for 1 h with [³H]leucine and chased for 0, 1, 2, 4, or 8 h. Class II (10.2–16) and Ii (In-1) precipitates were analyzed by SDS-PAGE. Molecular mass markers are indicated on the left and class II α and β chains, p31, and Δ2-17p41 are indicated on the right.

A 10.2-16   B P4H5   C In-1
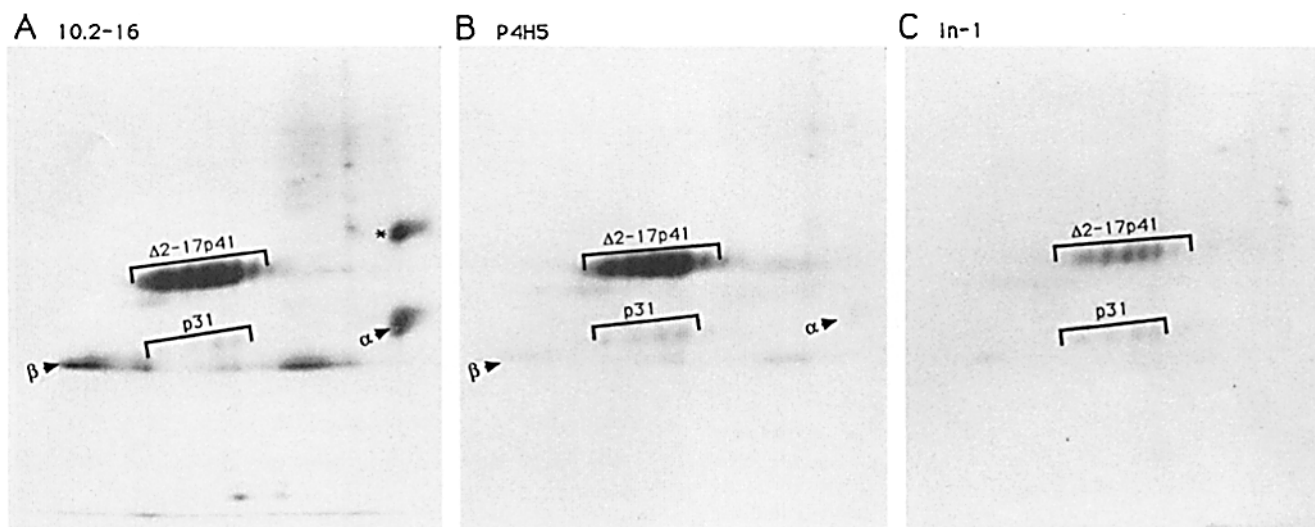
Figure 6. Wild type p31 Ii is present on the surface in cells expressing both wild type and truncated Ii. Ltk- cells expressing I-A^k and both p31 and Δ2-17p41 (3.1.3) were surface labeled with ^{125}I and viable cells were isolated on a ficoll gradient. Lysates were precipitated with 10.2-16 (A), P4H5 (B), or In-1 (C) and the precipitates were analyzed by two-dimensional gel electrophoresis. Class II α and β chains, p31, and Δ2-17p41 are indicated. Gels are oriented with the acidic end on the right side. The single asterisk (*) in A indicates p55, a protein that is stably associated with cell surface I-A^k molecules and is present in anti-class II, but not anti-Ii, precipitates (Anderson et al., 1993).

curs in these cells, it cannot examine the differential fate of class II–Ii complexes containing one or more wild type Ii molecules.

Wild Type p31 Ii Is Present on the Surface Associated with Δ2-17p41 Ii
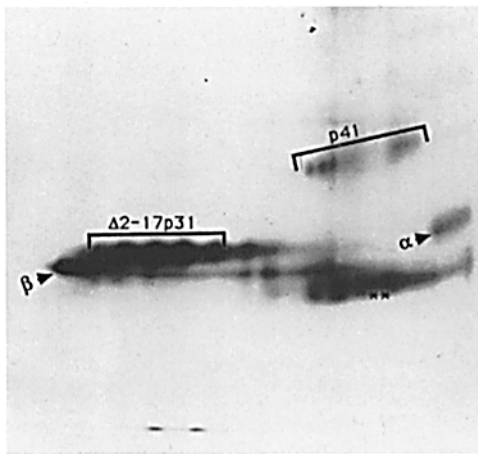
As a direct measure of a potential alteration in the fate of Ii trimers containing both wild type and Δ2-17 Ii, the ability of Δ2-17p41 to target associated wild type p31 molecules to the plasma membrane was determined. Ii was detected at the cell surface by flow cytometry in cells expressing Δ2-17p41, but not wild type p31 (Table I). Cells coexpressing both wild type and Δ2-17 Ii do express Ii on their surface (Table I). However, it could not be determined by flow cytometry whether both wild type p31 and Δ2-17p41 or only Δ2-17p41 are present on the plasma membrane.

To determine whether any wild type p31 could be detected on the surface, cells were surface iodinated and nonviable cells were removed by ficoll gradient to prevent detection of intracellular material. Lysates were precipitated with 10.2.16, P4H5, or In-1 and the precipitates were analyzed by two dimensional gel electrophoresis. After long exposures wild type p31 in class II–positive cells expressing only wild type Ii was occasionally but not reproducibly detected in very small quantities with anti-Ii antibodies, and never detected with an anti-class II antibody (data not shown). As reported (Anderson et al., 1993), in cells expressing class II and only Δ2-17p41 Ii, Δ2-17p41 is present on the surface associated with class II and can be precipitated with 10.2-16 and P4H5, but not with In-1 as Δ2-17p41 lacks the In-1 epitope (data not shown). In contrast to cells expressing only wild type p31, cells coexpressing p31 and Δ2-17p41 have both p31 and Δ2-17p41 reproducibly present on the cell surface (Fig. 6, A–C). They are clearly associated, since In-1 precipitates both p31, which
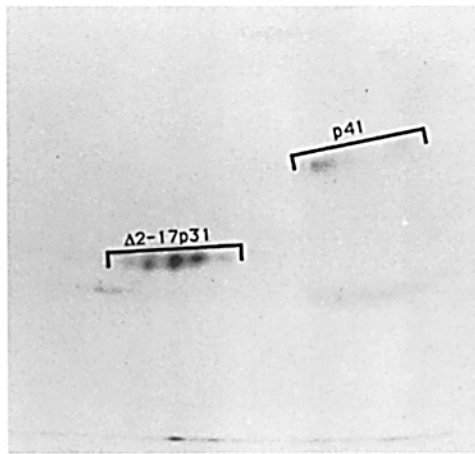
has the In-1 epitope, and Δ2-17p41, which does not. Likewise, at least a subset of these heterotrimers are associated with class II, as wild type p31 is detected in anti-class II precipitates (Fig. 6 A) and class II is present in long exposures of the In-1 precipitates (data not shown).

In the multimers containing both wild type and Δ2-17 Ii that are precipitated with In-1, the ratio of Δ2-17p41 to wild type p31 is approximately 2:1. This suggests that multimers containing only a single wild type Ii cytosolic domain are not diverted to an endosomal compartment, but instead travel to the plasma membrane by default. However, a caveat to using ^{125}I to determine molar ratios of cell surface proteins is that different proteins may be labeled at different efficiencies. In fact, p41 contains three additional tyrosine residues which could result in different iodination efficiencies of p41 and p31, creating an artifactual 2:1 ratio. This possibility was addressed in two ways. An independent labeling technique, biotinylation, was used to label surface proteins, and as with the iodinated cells, there was consistently more Δ2-17p41 than wild type p31 in In-1 precipitates (data not shown). In addition, new transfectants were generated which co-expressed class II with both Δ2-17p31 and wild type p41 (see Fig. 4). If the 2:1 ratio observed above reflected the labeling efficiency, then we would observe more p41 than Δ2-17p31 in the In-1 precipitate. If the 2:1 ratio reflects the intracellular sorting of trimers containing only a single wild type cytosolic domain, then we would again see more truncated wild type Ii, only this time the truncated Ii would be p31. These LAKΔ2-17p31p41 cells were surface iodinated, viable cells were isolated by ficoll separation, and lysates were immunoprecipitated with P4H5 and In-1 (Fig. 7). The ratio of Δ2-17p31 to wild type p41 in the In-1 precipitate in these cells was approximately 2:1. This is consistent with the original data from the LAKp31Δ2-17p41 cells and confirms that Ii heterotrimers on the surface contain two

A. P4H5

B. In-1

Figure 7. Trimers containing only a single intact Ii cytosolic domain are present at the cell surface. Class II positive Ltk- transfectants expressing both Δ2-17p31 and wild type p41 (10.2.5) were surface labeled with ^{125}I and viable cells were isolated by ficoll gradient. Lysates were precipitated with P4H5 (*A*) or In-1 (*B*) and the precipitates were analyzed by two-dimensional electrophoresis. Class II α and β chains, Δ2-17p31 and wild type p41 are indicated. The **indicates a truncated form of Δ2-17p31 that probably represents p25

(Mehringer et al., 1991), and therefore contains only the lumenal domain of p31. Note the relative positions of wild type and Δ2-17 Ii in this figure and in Fig. 6. The change in positions is consistent with a change in pI due to deletion of amino acids 2 through 17. Gels are oriented with the acidic end on the right side.

Δ2-17 Ii molecules and one wild type Ii molecule. Thus, the presence of only a single intact Ii targeting sequence within the Ii trimer results in plasma membrane expression of class II–Ii complexes.

A Single Wild Type Ii Can Mediate Rapid Internalization

Based on these data Ii trimers on the surface should be composed of either three Δ2-17 Ii molecules or two Δ2-17 and one wild type Ii molecule. Given the 1:1 biosynthetic ratio of p31 and Δ2-17p41 in the LAKp31Δ2-17p41 cells (see Table II), the expected ratio of surface Ii would be one wild type to three Δ2-17. However, the actual ratio of wild type to Δ2-17 is about 1 to 10 in the P4H5 precipitate of surface Ii (Fig. 6). The underrepresentation of trimers containing a single wild type Ii on the surface raises the possibility that a single cytosolic domain is inefficiently capable of endosomal targeting, resulting in some single wild type Ii trimers being sorted directly from the TGN to an endosomal compartment, and the remainder being released to the plasma membrane. Alternatively, all the trimers containing either a single wild type or no wild type Ii may be transported to the cell surface, but a single wild type Ii might mediate faster internalization from the surface or increased degradation once internalized.

The second possibility was tested by directly measuring the internalization and half-life of the various class II–Ii complexes from the cell surface. Surface proteins on LAKp31Δ2-17p41 cells were biotinylated at 4°C and the cells were chased at 37°C for various times. At the end of the chase biotin was cleaved from molecules remaining on the surface and the percent internalized over time was quantitated for class II–associated Δ2-17 Ii (10.2-16 precipitate), total Δ2-17Ii (P4H5 precipitate), or wild type Ii-associated Δ2-17 Ii (In-1 precipitate) (Fig. 8). It is clear that trimers containing a single wild type Ii molecule are internalized at a faster rate than total Δ2-17 Ii molecules. Because trimers containing only Δ2-17p41 comprise over 75% of total Δ2-17p41 on the surface of these cells, these

data also indicate that trimers containing a single wild type Ii are internalized faster than trimers containing only Δ2-17p41. Similar results were seen with LAKΔ2-17p31p41 cells (data not shown). These data indicate that a single intact Ii cytosolic domain can mediate rapid internalization of class II–Ii complexes.

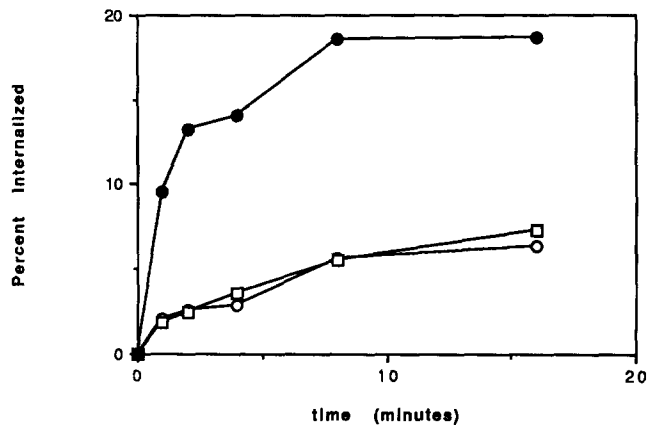To determine the fate of internalized class II–Ii complexes, the half-life of Ii trimers on the cell surface was as-

Figure 8. A single Ii cytosolic domain can mediate rapid internalization from the cell surface. Ltk− cell transfectants expressing I-A^k and both p31 and Δ2-17p41 were biotinylated at 4°C and chased at 37°C with pre-warmed media for 0, 1, 2, 4, 8, and 16 minutes. After each chase point the cells were chilled with ice-cold media and treated with glutathione to remove the biotin from proteins remaining on the surface. Immunoprecipitates were analyzed by SDS-PAGE, Western transferred, and blotted with peroxidase conjugated streptavidin. The Δ2-17p41 band was quantitated using optical densitometry of films. The open circle (○) is the 10.2-16 precipitate, and represents Δ2-17p41 associated with class II. The open square (□) is the P4H5 precipitate and represents total Δ2-17p41. The closed circle (●) is the In-1 precipitate and represents Δ2-17p41 associated with wild type p31. Data are given as the percent internalized, and are an average of four independent experiments.

sayed (Fig. 9). In this experiment, cells were labeled and chased as above, but biotin remaining on the surface was not removed. Thus, the experiment determines the rate of loss of biotin label by cellular processes, such as degradation of the labeled protein or reduction of the disulfide bond linking the biotin to the protein. Class II–associated Δ2-17 Ii, total Δ2-17Ii, and Δ2-17 associated with wild type Ii were immunoprecipitated and quantified (Fig. 9). The subset of Ii trimers that contain a single wild type Ii cytosolic domain are clearly turned over at a faster rate than complexes composed of Δ2-17 Ii only.

Taken together, these data indicate that some, if not all, trimers containing a single wild type Ii are transported to the cell surface, where they undergo internalization and degradation at a faster rate than trimers that contain only Δ2-17 Ii. Thus, the presence of a single intact Ii targeting sequence within the Ii trimer is not sufficient for efficient transport from the TGN to endosomes, but can mediate plasma membrane internalization and possibly endosomal retention and/or transport to an intracellular proteolytic compartment.

Discussion

These studies were initiated to determine whether Ii multimerization is important for efficient endosomal localization of class II–Ii complexes. Ii assembles into trimers and then associates with three class II heterodimers to form a large complex in the ER which persists during transport through the Golgi complex and into an endosomal compartment. The cytosolic domain of Ii contains a sequence
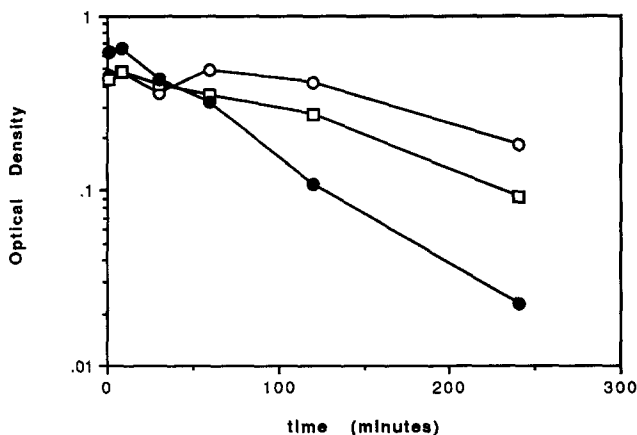
Figure 9. A single type Ii cytosolic domain increases the turnover rate of associated Ii molecules. LAKp31Δ2-17p41 cells were surface biotinylated at 4°C and chased for 1, 8, 30, 60, 120, and 240 min at 37°C with pre-warmed media. After each chase point the cells were lysed, immunoprecipitated and analyzed by SDS-PAGE followed by Western transfer and blotting with peroxidase conjugated streptavidin. The open circles (O) are the 10.2–16 immunoprecipitation, representing the Δ2-17p41 associated with class II. The open squares (□) are the P4H5 precipitate, representing total Δ2-17p41. The closed circles (●) are the In-1 precipitate, representing Δ2-17p41 associated with wild type p31. The Δ2-17p41 band was quantitated using optical densitometry on autoradiographs. Data presented are an average of three independent experiments.

that has been implicated in endosomal localization of class II–Ii complexes. We have found that the fate of a class II–Ii complex is determined by the number of complete Ii cytosolic domains it contains, and, therefore, that multimerization is necessary for efficient endosomal localization. Newly synthesized class II molecules associated with wild type Ii are transported to a post-Golgi endosomal compartment where Ii is rapidly degraded. When the endosomal localization sequence encoded by amino acids 2-17 is deleted from Ii, class II associated with this truncated Ii travels to the cell surface, resulting in a long half-life for Ii. However, class II–Ii complexes containing both wild type and truncated Ii may be transported either to the cell surface or to an endosomal compartment. The ratio of truncated to wild type Ii in heterotrimers detected on the surface by In-1 precipitation is approximately 2:1. This indicates that the heterotrimers present on the surface are composed of two truncated and one wild type Ii molecules, suggesting that one complete cytosolic domain is not sufficient for efficient endosomal localization. These results imply a division in the trafficking of heterotrimers containing both wild type and truncated Ii molecules which depends on the number of cytosolic domains present: complexes containing two or more wild type Ii molecules are transported to an endosomal compartment and are degraded, while complexes containing one or less wild type Ii molecules follow the constitutive route to the surface.

However, the ratio of Δ2-17 to wild type Ii on the surface indicates that trimers containing a single wild type Ii are handled differently than trimers containing only Δ2-17. This difference may occur at the TGN, where the presence of a single wild type Ii may result in inefficient transport to endosomes and missorting of a subset of complexes at the cell surface. Although at present we cannot formally exclude this possibility, our data favor an alternative interpretation, whereby a single wild type Ii cytosolic tail can alter the fate of Ii trimers after arrival at the cell surface. Trimers containing only Δ2-17 Ii are internalized slowly and are turned over with a long half-life, whereas the presence of a single wild-type Ii molecule results in faster internalization and more rapid turn over of the Ii complex. Although the rapid turn over of trimers containing a single wild type Ii molecule could be simply a consequence of rapid internalization, several observations suggest that a single Ii cytosolic domain may also directly mediate rapid turn over. The internalized pool of these complexes rapidly reaches a plateau (see Fig. 8), indicating rapid exit from the early endosomal compartment either by recycling or degradation. Recently, we have found that chimeric Ii molecules that contain the cytosolic domain of transferrin receptor are also rapidly internalized, but are degraded very slowly (Swier, K., and J. Miller, manuscript in preparation). Therefore, rapid cycling through the early endocytic pathway and back to the cell surface is not sufficient for Ii proteolysis. In contrast to the Ii/transferrin receptor chimeras, Ii complexes that contain a single Ii cytosolic tail are rapidly degraded without a significant lag time and with apparent first order kinetics (see Fig. 9). These data suggest that internalized Ii trimers that contain a single intact Ii are rapidly targeted to proteolytic compartments. Thus, although a single Ii cytosolic domain may not function for efficient TGN to endosomal sorting, it ap-

pears to mediate rapid internalization from the cell surface and may still function as an endosomal retention and, possibly, a late endosomal sorting signal.

The Ii cytosolic domain contains two di-leucine–like motifs that are important for endosomal targeting (Pieters et al., 1993; Odorizzi et al., 1994). The mechanism by which di-leucine motifs direct intracellular sorting is not understood (Trowbridge et al., 1993). Endosomal targeting can also be mediated by a well defined tyrosine-based motif that is thought to interact with adaptor proteins associated with clathrin coated pits at the plasma membrane (Trowbridge et al., 1993). Although di-leucine–like and tyrosine-based motifs have been found in many proteins, their function may vary, either mediating direct transport from the TGN to an endosome or internalization of cell surface protein to the endosome. For example, the CD3γ chain contains both a tyrosine-based signal and a di-leucine signal, both of which function independently as internalization signals from the surface. However, when both of these signals are present, the protein is directly transported from the TGN to the endosomal pathway (Letourneur and Klausner, 1992). In other proteins, however, multimerization of these signals does not appear to create an additional function, although each signal functions independently (Johnson and Kornfeld, 1992; Chen et al., 1993; Hunziker and Fumey, 1994). There also appears to be some disagreement on the independent function of di-leucine signals. In some proteins a single di-leucine motif may mediate internalization (Letourneur and Klausner, 1992; Aiken et al., 1994; Ogata and Fukuda, 1994), while in other proteins the di-leucine signal may mediate sorting from the TGN to an endosome (Johnson and Kornfeld, 1992; Chen et al., 1993). This had led some to speculate that there are different di-leucine signals that function at different sites in the cell (Dietrich et al., 1994), specifically a membrane distal signal that functions at the TGN and a membrane proximal phosphorylation-dependent site that functions at the cell surface. Ii contains two di-leucine motifs, a potential membrane distal signal at amino acids 7–8 and membrane proximal signal at amino acids 15–17. Consistent with the presence of the membrane proximal site Ii has been shown to be phosphorylated at a serine residue in its cytosolic domain (Spiro and Quaranta, 1989). This raises the possibility that one of the Ii di-leucine–like motifs could function at the TGN, requiring multimerization for sorting, while the other could function as an internalization signal. Alternatively, the two signals could interact to mediate either or both of these sorting events. Finally, there may be additional signals within the Ii cytosolic tail (Swier, K., and J. Miller, manuscript in preparation) or within the transmembrane region of Ii (Odorizzi et al., 1994) that further modulate the function or efficiency of endosomal sorting of Ii.

The generally accepted route of class II–Ii transport involves direct trafficking of class II–Ii complexes from the TGN to an endosomal compartment. This is based on the rapidity of Ii degradation following maturation in the Golgi, the difficulty in detecting wild type class II–Ii complexes on the surface, and the ability to transfer endosomal targeting onto heterologous proteins with sequences from the Ii cytosolic domain (Blum and Cresswell, 1988; Neefjes et al., 1990; Anderson et al., 1993; Odorizzi et al., 1994).

However, the Ii cytosolic domain also contains a plasma membrane internalization signal (Roche et al., 1993; Odorizzi et al., 1994; and this report) and a potential endosomal retention signal (Neefjes and Ploegh, 1992; Loss and Sant, 1993). The presence of these additional signals has led to the suggestion that class II–Ii complexes are not targeted directly from the TGN to an endosome, but rather pass through a transient cell surface intermediate during transport to the endosome (Roche et al., 1993). In our studies, we have been unable to reproducibly detect wild type Ii complexes at the cell surface, although we can readily detect Ii trimers containing a single wild type Ii molecule. However, it is possible that multimerization of Ii increases the rate of internalization from the surface. In this case extremely rapid internalization of wild type Ii complexes coupled with an efficient endosomal retention signal to inhibit recycling would make the detection of a plasma membrane intermediate very difficult. The issue of a cell surface intermediate in sorting from the TGN to the endosomal pathway can be difficult to resolve. This is illustrated by the unresolved controversy concerning the intracellular route of lysosomal glycoproteins (Williams and Fukuda, 1990; Harter and Mellman, 1992; Mathews et al., 1992). Often high levels of expression are required to detect the transitory cell surface intermediate. In that case, an artifactual cell surface intermediate can occur by saturation of its specific transport mechanism, resulting in surplus protein expressed on the surface (Harter and Mellman, 1992). In the Ii trimer system, this saturation could occur more quickly due to the presence of three signals and transport mechanism binding sites, when only two signals appear to be required. However, we do not believe that saturation can account for the detection of wild type Δ2-17 Ii trimers on the cell surface described in this report, because cells expressing greater levels of wild type Ii, as detected by Western blotting, do not express Ii on the cell surface (data not shown).

This study has demonstrated that multimerization of Ii is important in endosomal localization of class II–Ii complexes. Multimerization of proteins and signals appears to be a recurrent theme in cellular transport. For instance, transport of proteins by mannose-6-phosphate is greatly enhanced when the mannose is diphosphorylated (Tong et al., 1989). Likewise, many plasma membrane proteins are oligomeric (Hurtley and Helenius, 1989), raising the possibility that the formation of homomeric complexes may regulate sorting of other proteins as well. One interesting possibility where multimerization may regulate protein sorting is during down-modulation of protein hormone receptors. Many of these proteins contain tyrosine-based internalization signals, however these proteins have a low intrinsic internalization rate in resting cells (Chang et al., 1993; Herbst et al., 1994). Hormone binding induces a rapid receptor down modulation that is mediated in part by internalization and degradation (Lund et al., 1990a,b; Chang et al., 1991, 1993; Jackle et al., 1991; Herbst et al., 1994). Although ligand binding induces many changes to the receptor, including activation of the tyrosine kinase signaling domain, it also induces dimerization (Chang et al., 1991; Sorkin and Carpenter, 1991). Dimerization has been shown to be critical for effective activation of the kinase and for cell signaling. The data presented in this re-

port suggest the possibility that dimerization may also activate the internalization signal, leading to receptor downmodulation. Multimerization of proteins into heteromeric complexes, such as class II–Ii complexes or the T cell antigen receptor (Klausner et al., 1990), can result in the accumulation of a wide array of sorting signals. Clearly, many of these signals are in place to regulate the fate of partial complexes and assembly with other chains has been shown to mask otherwise potent ER retention signals (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1989, 1990; Lotteau et al., 1990; Lamb and Cresswell, 1992) and Golgi retention and degradation signals within partial complexes (Klausner et al., 1990; Letourneur and Klausner, 1992). As discussed above, interaction between different sorting signals within heteromeric complexes may also increase the efficiency of targeting and can create unique sorting signals. Finally, one interesting possibility to consider is that different sorting signals function at different points within the intracellular transport pathway. This is especially relevant to the class II–Ii complex, not only because sorting occurs at distinct intracellular sites, but also because during intracellular transport, Ii is removed from class II, possibly revealing signals within Ii proteolytic fragments (Fineschi, B., L. S. Arneson, M. Naujokas, and J. Miller, manuscript in preparation; Newcomb and Cresswell, 1993; Pieters et al., 1991) or within class II (Chervonsky et al., 1994), that might regulate later events, such as sorting of Ii-free, peptide bound class II from late endosomes to the cell surface.

Data presented in this paper also suggest a possible model to account for the biosynthesis of the chondroitin sulfate form of Ii (Ii–CS). A small proportion (2–5%) of Ii is modified by the addition of chondroitin sulfate and expressed on the cell surface with a relatively short half-life (Sant et al., 1985a,b). It has not been determined whether the addition of xylose in the ER or the addition of chondroitin sulfate in the trans-Golgi determines whether a specific Ii molecule will become modified by glycosaminoglycan addition. Preliminary data suggests that the population of class II–Ii complexes modified by chondroitin sulfate are segregated from the bulk of class II–Ii complexes early after biosynthesis, in that they rapidly exit the ER, transit through the Golgi and arrive at the cell surface (Arneson, L. S., and J. Miller, unpublished results). Although Ii rapidly assembles into trimers immediately after biosynthesis in the ER, a small amount of Ii may escape trimerization, possibly due to association with a class II heterodimer before contact with another Ii molecule. This class II–Ii monomer may undergo modification and transport steps different from the class II–Ii nine chain complex, that allow for chondroitin sulfate addition. Because these class II–Ii–CS molecules contain only one Ii cytosolic domain the complex would transit to the cell surface through the default pathway allowing for functional expression of Ii–CS as a T cell interaction molecule at the plasma membrane (Naujokas et al., 1993). Once at the cell surface the single Ii cytosolic domain would mediate rapid internalization and degradation resulting in a short half-life for the complex (Sant et al., 1985b). Thus, the subset of Ii modified by chondroitin sulfate could represent single class II–Ii monomers that are accessible to glycosaminoglycan addition and are transported to the surface because they did not participate in Ii trimerization in the ER.

Multimerization of Ii has been shown to mediate two other effects on class II–Ii transport and function. Alternative initiation of Ii translation in human B cells results in a p33 Ii molecule with additional amino acids on its amino terminus (p35). This region has been shown to contain an ER retention signal (Lotteau et al., 1990). When Ii trimerizes in the ER, any trimer containing p35 is effectively retained within the ER, indicating that a single ER retention signal can determine the fate of an entire Ii trimer (Marks et al., 1990; Lamb and Cresswell, 1992). Likewise, the p41 form of Ii appears to mediate a dominant effect on antigen presentation and Ii degradation. A number of studies have also implicated a role for Ii in antigen presentation (Stockinger et al., 1989; Bertolino et al., 1991; Nadimi et al., 1991; Peterson and Miller, 1992; Bikoff et al., 1993; Viville et al., 1993). Interestingly, it has been shown that this effect is mediated primarily by the alternatively spliced p41 form of Ii (Peterson and Miller, 1992). The function can be imparted by p41 equally well when it is expressed as 100% of total Ii or as only 10% of total Ii. Although the mechanism by which this enhancement occurs is not completely understood, recent data indicate that p41 is processed differently than p31 (Fineschi, B., L. S. Arneson, M. Naujokas, and J. Miller, manuscript in preparation; Arunachalam et al., 1993). Interestingly, co-expression of p31 and p41 can alter the proteolytic processing of p31 into a p41-like fashion, possibly through a direct effect of p41 on p31 molecules if associated within the same multimer (Fineschi, B., L. S. Arneson, M. Naujokas, and J. Miller, manuscript in preparation). It is not known whether this results from a p41 specific conformational effect on associated p31 molecules or from a positive transport signal in the lumenal p41 specific segment. Nevertheless, the ability of Ii to form multimers can have a dramatic effect on the intracellular transport and ultimate fate of class II–Ii complexes. This in turn can have functional consequences on the expression of Ii–CS accessory molecules and the generation of class II–antigenic peptide complexes, both of which play a critical role in T cell activation.

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