

# Isoforms of the Invariant Chain Regulate Transport of MHC Class II Molecules to Antigen Processing Compartments

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**Abstract.** Newly synthesized class II molecules of the major histocompatibility complex must be transported to endosomal compartments where antigens are processed for presentation to class II–restricted T cells. The invariant chain (Ii), which assembles with newly synthesized class II  $\alpha$ - and  $\beta$ -chains in the endoplasmic reticulum, carries one or more targeting signals for transport to endosomal compartments where Ii dissociates from  $\alpha\beta$ Ii complexes. Here we show that the transport route of  $\alpha\beta$ Ii complexes is regulated selectively by two forms of Ii (p33 and p35) that are generated by the use of alternative translation initiation sites. Using a novel quantitative surface arrival assay based on labeling with [6-<sup>3</sup>H]-D-galactose combined with biochemical modification at the cell surface with neuraminidase, we demonstrate that newly synthesized  $\alpha\beta$ Ii molecules containing the Ii-p33 isoform can be detected on the cell surface shortly after passage through the Golgi ap-

paratus/*trans*-Golgi network. A substantial amount of these  $\alpha\beta$ Ii complexes are targeted to early endosomes either directly from the *trans*-Golgi network or after internalization from the cell surface before their delivery to antigen processing compartments. The fraction of  $\alpha\beta$ Ii complexes containing the p35 isoform of Ii with a longer cytosolic domain was not detected at the cell surface as determined by iodination of intact cells and the lack of susceptibility to neuraminidase trimming on ice. However, treatment with neuraminidase at 37°C did reveal that some of the  $\alpha\beta$ Ii-p35 complexes traversed early endosomes. These results demonstrate that a fraction of newly synthesized class II molecules arrive at the cell surface as  $\alpha\beta$ Ii complexes before delivery to antigen processing compartments and that class II  $\alpha\beta$ Ii complexes associated with the two isoforms of Ii are sorted to these compartments by different transport routes.

**I**NTEGRAL membrane proteins can be targeted to endocytic compartments following either one of two known sorting pathways (Pearse and Robinson, 1990). AP-1 adaptors can recognize specific motifs in the cytoplasmic domains of membrane proteins in the *trans*-Golgi network (TGN), leading to the association of these proteins in clathrin-coated buds in the TGN and resulting in their direct transport to endosomal compartments. Alternatively, the cytoplasmic domains of membrane proteins can interact with plasma membrane-associated AP-2 adaptors and this interaction can also result in transport to endosomal compartments, however, in this case, the mechanism involves internalization in clathrin-coated vesicles and fusion of these vesicles with early endosomes. Identification of the specific endosomal targeting pathway used by a particular protein can be difficult, since cytoplasmic

targeting signals on proteins that are targeted to endosomes directly from the TGN can also be recognized at the plasma membrane and can lead to rapid internalization from the cell surface (Harter and Mellman, 1992; Ogata and Fukuda, 1994).

Major histocompatibility complex (MHC)<sup>1</sup> class II molecules are transmembrane glycoproteins that must reach endosomal compartments before they can exert their biological function. These molecules bind antigenic peptides derived from internalized antigen and display them on the cell surface for recognition by specific CD4<sup>+</sup> T lymphocytes (Germain, 1994). However, class II molecules cannot bind antigenic peptides until a “chaperone” subunit, termed class II-associated invariant chain (Ii), has dissociated from the  $\alpha\beta$ Ii complex in compartments of the endocytic pathway (Roche and Cresswell, 1990, 1991; Newcomb and Cresswell, 1993a). Ii is a type II integral

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1. *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; BSA, bovine serum albumin; [<sup>3</sup>H]galactose, [6-<sup>3</sup>H]galactose; Ii, class II-associated invariant chain; MHC, major histocompatibility complex; NANase, neuraminidase.

membrane protein with different isoforms generated by alternative splicing and by the use of two translation initiation sites that lead to the production of a 33-kD major form of Ii (Ii-p33) as well as a 35-kD minor form (Ii-p35, representing ~10% of all Ii; Strubin et al., 1986). Unlike the p33 isoform of Ii, the p35 isoform contains a di-arginine motif that is responsible for the retention of Ii-p35 in the ER (Schutze et al., 1994). However, class II  $\alpha\beta$ Ii complexes containing Ii-p35 can leave the ER and transport through the Golgi apparatus and into antigen processing compartments, presumably due to the masking of the ER retention motif (Newcomb and Cresswell, 1993b). Two dileucine-like motifs present in the cytoplasmic domain of all Ii isoforms mediate targeting of  $\alpha\beta$ Ii complexes to endosomes (Pieters et al., 1993; Bremnes et al., 1994; Odorizzi et al., 1994) where proteolytic enzymes degrade Ii (Blum and Cresswell, 1988; Newcomb and Cresswell, 1993b). Finally, ~2 h after biosynthesis, mature  $\alpha\beta$  heterodimers begin to accumulate on the cell surface (Cresswell and Blum, 1988; Neeffjes et al., 1990).

Intracellular class II molecules are predominantly located in a late endosomal compartment termed the MHC class II compartment, or MIIC, in human B-LCL (Peters et al., 1991). Since their initial identification in B-LCL, similar compartments have been identified in macrophages (Harding et al., 1990), melanoma cells (Pieters et al., 1991), and dendritic cells (Kleijmeer et al., 1995), whereas morphologically similar compartments with characteristics of earlier endosomes have been identified in mouse B cell lymphomas (Amigorena et al., 1994). MIIC are distinct from conventional early or late endosomes and are thought to represent a specialization of the prelysosomal system that has been adapted for efficient antigen processing and peptide loading onto newly synthesized class II molecules (Tulp et al., 1994; West et al., 1994; Rudensky et al., 1994; Qiu et al., 1994). Since most class II molecules in endocytic compartments localize to MIIC under steady-state conditions, it has been generally assumed that newly synthesized class II molecules are transported there directly from the TGN (Peters et al., 1991). However, it is also possible that transport to antigen processing compartments occurs via early endosomes, a pathway used by the mannose-6-phosphate receptor for delivery of certain lysosomal enzymes to late endosomes and lysosomes (Ludwig et al., 1991). Alternatively, newly synthesized class II molecules may be targeted to endosomal compartments following transport to the cell surface and rapid internalization. Support for this hypothesis was obtained in recent studies showing that the amino terminal endosomal targeting signal in the Ii cytoplasmic domain also functions as an efficient internalization signal, resulting in rapid endocytosis of class II  $\alpha\beta$ Ii complexes from the cell surface into endosomal compartments in which Ii dissociates from the complex (Roche et al., 1993). Since there are more than 500,000 mature  $\alpha\beta$  heterodimers per B-LCL and only ~18,000 of these are associated with Ii at any moment (Roche et al., 1993), the rapid passage of  $\alpha\beta$ Ii complexes through the cell surface can be difficult to detect using standard biochemical assays.

A recent study with concanamycin B-treated cells, in which transport from early to late endosomes was inhibited, concluded that the majority of newly synthesized

class II molecules were transported directly from the TGN to MIIC, even though a substantial amount of  $\alpha\beta$ Ii complexes accumulated at the cell surface after treatment with this drug (Bénaroch et al., 1995). It was not known whether these  $\alpha\beta$ Ii complexes arrived at the cell surface directly from the TGN or by escape from MIIC. Using a novel surface arrival assay, we now provide direct evidence that class II  $\alpha\beta$ Ii complexes can be rapidly transported from the TGN to the cell surface. A substantial amount of newly synthesized  $\alpha\beta$ Ii complexes containing Ii-p33 are targeted directly to the cell surface/early endosome before entering more acidic antigen processing compartments. By contrast,  $\alpha\beta$ Ii complexes containing Ii-p35 are not found to travel via the cell surface, but appear to be targeted directly to early/late endosomes before their delivery to antigen processing compartments.

## Materials and Methods

### Cell Lines and Antibodies

The Epstein-Barr virus-transformed human B lymphoblastoid cell lines (B-LCL) 721.45 (Kavathas et al., 1980) and JY (Peters et al., 1991) were cultured in RPMI 1640 supplemented with 10% bovine serum and 5% FCS. Most experiments have been carried out using both B-LCL 721.45 and B-LCL JY and the results obtained were always similar. The figure legend indicates which cell line was used in the experiment shown. MHC class I molecules were isolated with mAb w6/32 (Amer. Type Culture Collection, Rockville, MD). MHC class II molecules were isolated using rabbit anti-DR  $\beta$ -chain serum (Karp et al., 1990) or mAb DA6.147 (Guy et al., 1982) as indicated. Total Ii was isolated with the mAb PIN.1.1 (Roche et al., 1991). Ii-p35 was isolated with affinity-purified rabbit anti-p35 antiserum Rlp35N (Roche et al., 1991; provided by Dr. Peter Cresswell, Yale University School of Medicine) or an affinity-purified antiserum generated against the p35 amino terminal sequence MHRRRSRS (2-amino-caprylic acid)<sub>2</sub> using conventional techniques.

### Metabolic Labeling of B-LCL

B-LCL were radiolabeled biosynthetically with [<sup>35</sup>S]methionine or surface iodinated with [<sup>125</sup>I] as described previously (Machamer and Cresswell, 1982; Roche et al., 1993). Before labeling with [6-<sup>3</sup>H]-D-galactose ([<sup>3</sup>H]galactose), B-LCL were cultured at 37°C in RPMI 1640 medium containing 10 mM Hepes (pH 7.4), 3% dialyzed FCS, and 0.1 mM D-mannose for 1–3 h to “starve” the cells of galactose. D-Mannose was added to eliminate the possibility that contaminants of [<sup>3</sup>H]-D-mannose in the [<sup>3</sup>H]galactose preparation or biosynthetic conversion of [<sup>3</sup>H]galactose to [<sup>3</sup>H]-D-mannose could label cellular glycoproteins. The cells (40 × 10<sup>6</sup>/ml) were then labeled at 37°C with 1 mCi [<sup>3</sup>H]galactose (20–50 Ci/mmol; DuPont New England Nuclear, Boston, MA or ICN Radiochemicals, Costa Mesa, CA) in RPMI containing 0.1 mM D-mannose, and 3% dialyzed FCS. In experiments in which neuraminidase (NANase) was incubated at 37°C shortly after pulse labeling, the labeling medium consisted of RPMI 1640 supplemented with 0.1 mM D-mannose, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 1 mg/ml bovine serum albumin (BSA). The labeling time is specified for each experiment. The chase was started by adding an excess of unlabeled D-galactose (0.2 mg/ml) to the culture. In the case of long chase times, the cells were pelleted directly after labeling and unused free radiolabel was removed. The cells were then chased in regular culture medium supplemented with 0.1 mM D-mannose and 0.02 mg/ml D-galactose.

### Desialylation with Neuraminidase

Radiolabeled B-LCL (5–10 × 10<sup>6</sup> cells/ml) were incubated alone or with 0.1 U/ml *Vibrio cholerae* NANase (Calbiochem-Novabiochem Corp., La Jolla, CA) in RPMI containing 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 0.1 mg/ml BSA either on ice for 2 h or for various times at 37°C as indicated. NANase activity on the cells was completely inhibited by adding the highly glycosylated NANase substrate fetuin (containing small amounts of *N*-acetylneuraminic acid; GIBCO/BRL, Gaithersburg, MD). The desialylated cells

were generally washed twice in ice-cold RPMI or Hank's Balanced Salt Solution containing 0.5 mg/ml fetuin.

### Immunoprecipitations

B-LCL (20–40 × 10<sup>6</sup>/ml) were lysed on ice for 1 h in 1% Triton X-100, 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 containing 1 mg/ml BSA, 0.5 mg/ml fetuin, 5 mM iodoacetamide, 0.1 mM *N*-*p*-tosyl-L-lysylchloromethyl ketone, 0.5 mM PMSF and 10 mM EDTA. Nuclei and cellular debris were removed by centrifugation. The lysates were precleared with rabbit anti-mouse immunoglobulin serum and protein A-agarose (Sigma Chem. Co., St. Louis, MO) or with an irrelevant isotype-matched mouse antibody (MOPC21; Sigma Chem. Co.) and rabbit anti-mouse immunoglobulin coated protein A-agarose. Lysates were then incubated for at least 2 h with specific antibodies at 4°C. Subsequently, the lysates were incubated with protein A-agarose or with rabbit anti-mouse immunoglobulin coated protein A-agarose for an additional hour at 4°C. The immunoprecipitates were washed three times with 10 mM Tris, 150 mM NaCl, pH 7.4 containing 0.1% Triton X-100 and once with 10 mM Tris, pH 7.4. The immunoprecipitates were analyzed on two-dimensional gel analysis (nonequilibrium pH gradient electrophoresis followed by reducing SDS-PAGE; Machamer and Cresswell, 1982) or quantitatively analyzed for their terminal carbohydrate content in the β-galactosidase assay.

### β-Galactosidase Assay

B-LCL were metabolically labeled with [<sup>3</sup>H]galactose and identical aliquots were either incubated with NANase or incubated in buffer alone before the isolation of either class I or class II molecules by immunoprecipitation. The immunoprecipitates were digested with 5 mg/ml pronase (Calbiochem) in 50 mM sodium acetate, pH 6.0, for 18 h at 37°C. The samples were then heated to 100°C for 10 min to inactivate the pronase, and passed through a Sephadex G-15 column equilibrated with 50 mM sodium acetate, 0.1 mg/ml BSA, pH 6. The two fractions containing the majority of the [<sup>3</sup>H]galactose labeled glycopeptides were pooled and digested with 5 mU of *Diplococcus pneumoniae* β-galactosidase (Boehringer Mannheim Corp., Indianapolis, IN) for 18 h at 37°C. The β-galactosidase digested samples were passed through a Sephadex G-15 column equilibrated in PBS/0.1 mg/ml BSA in order to separate the noncleaved [<sup>3</sup>H]galactose containing glycopeptide (peak 1) from the cleaved, free [<sup>3</sup>H]galactose (peak 2). Fractions were collected and counted in a liquid scintillation counter.

To specifically determine the effect of NANase on the release of terminal galactose by β-galactosidase, the amount of free [<sup>3</sup>H]galactose released from non-NANase-treated samples (expressed as a fraction of the total cpm) was subtracted from the amount of free [<sup>3</sup>H]galactose released from the NANase-treated samples (also expressed as a fraction of the total cpm) to arrive at a percentage of free [<sup>3</sup>H]galactose released as a consequence of NANase treatment. Since different glycoproteins possess different amounts of sialic acid residues and β-1,4 galactose linkages and therefore serve more or less well as β-galactosidase substrates, the maximum amount of cleavable galactose was determined by adding 0.1 U/ml NANase to the cell lysis buffer before immunoprecipitation and analysis in the β-galactosidase assay. All data were normalized using this value and are expressed as a fraction of the maximum amount cleavable and plotted as "relative percent terminal galactose."

## Results

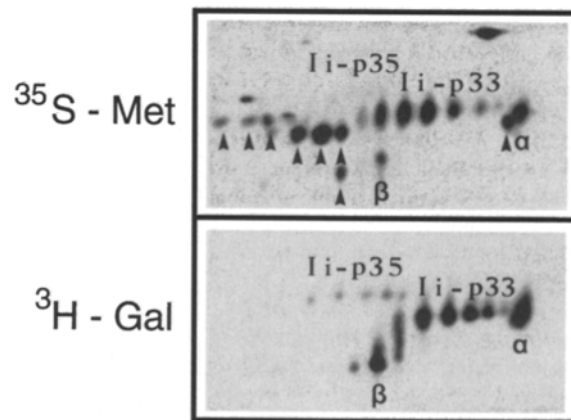
### Comparison of MHC Class II Molecules Labeled with [<sup>35</sup>S]Methionine or [<sup>3</sup>H]Galactose

To determine the kinetics of MHC class II molecule transport to the cell surface, we attempted to identify a biosynthetic pulse-chase labeling protocol that would not be affected by ER retention of free Ii. Conventional metabolic labeling for 4 h with [<sup>35</sup>S]methionine and immunoprecipitation with anti-class II mAb revealed the presence of both ER-retained, high mannose class II α-, β-, and Ii chains (noted by arrowheads) as well as post-ER, complex carbohydrate forms of class II α-, β-, and Ii chains (Fig. 1, upper panel). In addition, the large pool of free, ER-retained Ii

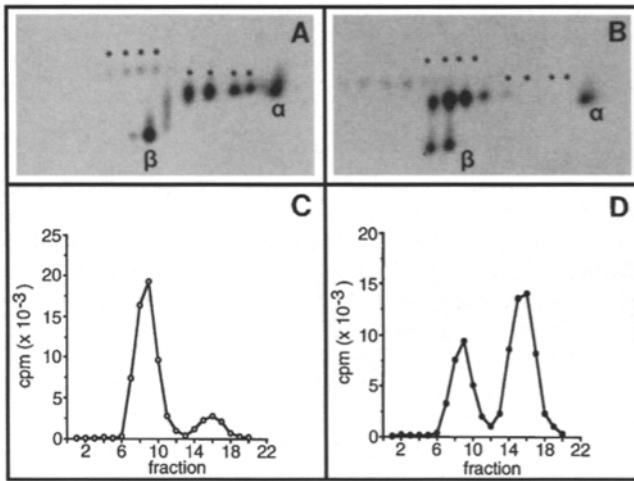
assembles slowly with nonradioactive class II α- and β-chains synthesized during a subsequent chase and only then acquires a complex carbohydrate (mature) phenotype. The net result of this process is that transport of radiolabeled class II molecules from the ER does not appear synchronous using this labeling protocol.

To circumvent these difficulties, we chose to label cells with [<sup>3</sup>H]galactose. As the enzyme β-1,4-galactosyltransferase resides in the *trans*-cisternae of the Golgi apparatus (Roth and Berger, 1982), glycoproteins will only be tagged with this radiolabel once they have reached this compartment. Therefore, all three subunits of the multimeric αβIi complex are labeled simultaneously, and the large excess of ER-retained Ii will not interfere with pulse/chase analyses. Immunoprecipitation of [<sup>3</sup>H]galactose-labeled class II molecules revealed only mature forms of α, β, and Ii containing complex carbohydrates (Fig. 1, lower panel), eliminating the possibility that [<sup>3</sup>H]galactose was biosynthetically converted to another carbohydrate that could label immature class II molecules in the ER. In addition to the predominant p33 form of Ii, the more basic p35 form of Ii is also labeled using this procedure, indicating that αβIi complexes containing a p35 form of Ii reach galactosyltransferase-containing compartments. Immunoprecipitates obtained using anti-DR and anti-Ii antisera were essentially identical, showing that free αβ heterodimers or free Ii are not labeled by this procedure (data not shown).

Cell surface arrival of glycoproteins can be determined by the susceptibility of their sialic acid residues to NANase digestion on ice (Neeffjes et al., 1990). To characterize the two-dimensional PAGE pattern of desialylated class II α-, β-, and Ii-chains, [<sup>3</sup>H]galactose-labeled B-LCL lysates were incubated alone (Fig. 2 A) or with NANase (Fig. 2 B) before isolation of class II molecules. NANase treatment of the B-LCL lysate resulted in a dramatic basic shift



**Figure 1.** Comparison of [<sup>35</sup>S]methionine- and [<sup>3</sup>H]galactose-labeled HLA-DR by two-dimensional PAGE. B-LCL were radiolabeled with [<sup>35</sup>S]methionine for 4 h or with [<sup>3</sup>H]galactose for 30 min. After radiolabeling, the cells were washed, lysed, and class II molecules were precipitated using the anti-DR mAb DA6.147. The immunoprecipitates were analyzed by nonequilibrium pH gradient electrophoresis from right to left (acidic to basic) followed by reducing SDS-PAGE. The positions of the DR α- and β-chains as well as the class II associated Ii-p33 and Ii-p35 isoforms are indicated. The migration of the nonsialylated forms of the α-, β-, and Ii chains is indicated by arrowheads.



**Figure 2.** Analysis of native and desialylated class II molecules by two-dimensional PAGE and the quantitative  $\beta$ -galactosidase assay. B-LCL 721.45 were radiolabeled with [ $^3\text{H}$ ]galactose for 30 min, divided into two equivalent samples and washed in ice cold RPMI. The cells were lysed for 1 h in lysis buffer alone (A and C) or in lysis buffer containing 0.1 U/ml NANase (B and D). MHC class II molecules were isolated using an anti-DR  $\beta$ -chain serum. After immunoprecipitation, 87% of the samples were analyzed by two dimensional PAGE (A and B) and 13% was used for quantitative analysis to determine the amount of terminal galactose residues present on each sample (C and D).

of the class II  $\beta$ -chain and Ii spots. (The use of NEPHGE in the first dimension does not allow resolution of the acidic DR  $\alpha$ -chain spots.) This experiment also confirms that the spots observed in the untreated samples corresponded to sialylated forms of the DR  $\beta$ -chain and Ii and demonstrates that labeling of immature, ER-localized class II molecules did not occur. (The relative position of each spot was determined by alignment with a partially desialylated sample.) An identical pattern was observed after extensive incubation with NANase or when  $^{125}\text{I}$ -labeled  $\alpha\beta\text{Ii}$  complexes were boiled before NANase treatment (data not shown). As noted previously, NANase treatment does not cause all class II  $\beta$ -chain or Ii spots to migrate at a single, distinct position, suggesting that other charge modifications not susceptible to NANase digestion account for this heterogeneity (Machamer and Cresswell, 1984).

#### ***A Quantitative Assay for the Arrival of MHC Class II Molecules at the Cell Surface***

In addition to visualizing sialylated vs desialylated  $\alpha\beta\text{Ii}$  complexes using an electrophoretic assay, we also adapted an assay to quantitate the extent of desialylation of  $\alpha\beta\text{Ii}$  complexes using the enzyme  $\beta$ -galactosidase (Duncan and Kornfeld, 1988). The assay is based upon the exquisite specificity of  $\beta$ -galactosidase for the cleavage of  $\beta$ -1,4 linked terminal galactose residues on complex carbohydrates. In most N-linked complex oligosaccharides, galactose is not the terminal carbohydrate residue, but is generally extended by one or more sialic acid residues (Kornfeld and Kornfeld, 1985). Since NANase can remove these sialic acid residues, NANase treatment of most complex

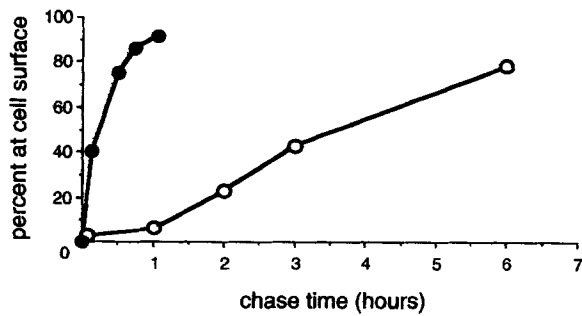
carbohydrates leads to the exposure of terminal galactose residues that become substrates for  $\beta$ -galactosidase. As a result, incubation of NANase-treated glycoprotein preparations with  $\beta$ -galactosidase allows quantitation of the amount of terminal galactose residues exposed and therefore acts as a quantifiable indicator for the extent of desialylation of surface exposed glycoproteins.

Equivalent aliquots of the immunoprecipitates shown in Fig. 2, A and B were analyzed in the  $\beta$ -galactosidase assay. Gel filtration chromatography of  $\beta$ -galactosidase-treated samples demonstrated that the high molecular weight [ $^3\text{H}$ ]galactose containing glycopeptide (Fig. 2, C and D; peak 1) could be easily separated from free [ $^3\text{H}$ ]galactose (Fig. 2, C and D; peak 2) in this assay. For non-NANase treated  $^3\text{H}$ -labeled  $\alpha\beta\text{Ii}$  complexes,  $\sim 12\%$  of the total amount of cpm could be released by  $\beta$ -galactosidase (Fig. 2 C), representing the percentage of terminal galactose residues present in the labeled pool of molecules. By contrast, NANase digestion of identical samples that were completely denatured before  $\beta$ -galactosidase treatment resulted in the release of 69% of the total cpm (Fig. 2 D). It should be emphasized that results from independent experiments were remarkably reproducible. Less than quantitative release of [ $^3\text{H}$ ]galactose is presumably due to the restricted specificity of the NANase and  $\beta$ -galactosidase preparations for the cleavage of particular carbohydrate linkages as well as to the existence of galactose residues that receive additional complex carbohydrates not cleavable by NANase (Fukuda, 1991).

To test the validity of the quantitative NANase/ $\beta$ -galactosidase assay in analyzing the rate of cell surface arrival of MHC glycoproteins, B-LCL were labeled with [ $^3\text{H}$ ]galactose and chased for various times to allow processing and transport of MHC molecules. The cells were then either untreated or treated with NANase on ice and the surface arrival of MHC class I and class II molecules determined using the quantitative NANase/ $\beta$ -galactosidase assay. The data clearly show that MHC class I molecules were rapidly transported to the cell surface ( $t_{1/2} = 15\text{--}20$  min) from the TGN (Fig. 3, *filled symbols*). By contrast, it took  $\sim 2$  h of chase for the first detectable MHC class II molecules to accumulate at the cell surface and 3.5 h for half of the class II molecules to reach the cell surface (Fig. 3, *open symbols*). These data are in excellent agreement with previously published reports (Cresswell and Blum, 1988; Neeffes et al., 1990) and demonstrate that biosynthetic labeling with [ $^3\text{H}$ ]galactose combined with the NANase/ $\beta$ -galactosidase assay is able to accurately quantitate and distinguish cell surface arrival rates of different glycoproteins.

#### ***$\alpha\beta\text{Ii}$ Complexes Can Arrive at the Cell Surface Rapidly after Exit from the TGN***

We have previously shown that class II  $\alpha\beta\text{Ii}$  complexes are very rapidly internalized from the plasma membrane of B-LCL (Roche et al., 1993). While the experiments described above confirm that newly synthesized class II molecules accumulate on the plasma membrane slowly, they do not allow accurate identification of small populations of class II molecules that might appear rapidly but only transiently on the cell surface before internalization into



**Figure 3.** Kinetics of cell surface arrival of MHC class I and MHC class II molecules. B-LCL 721.45 were radiolabeled with [<sup>3</sup>H]galactose for 30 min, and chased for various times at 37°C. At each time point, cells were digested with NANase on ice. After 2 h incubation with NANase, 0.5 mg/ml fetuin was added, the cells were washed twice in RPMI/fetuin, and subsequently lysed in the presence of fetuin. Class II molecules were isolated using mAb DA6.147 (open symbols), whereas class I molecules were isolated using mAb w6/32 (filled symbols). After immunoprecipitation, samples were analyzed using the quantitative β-galactosidase assay, and the relative percent terminal galactose was calculated as described in the text.

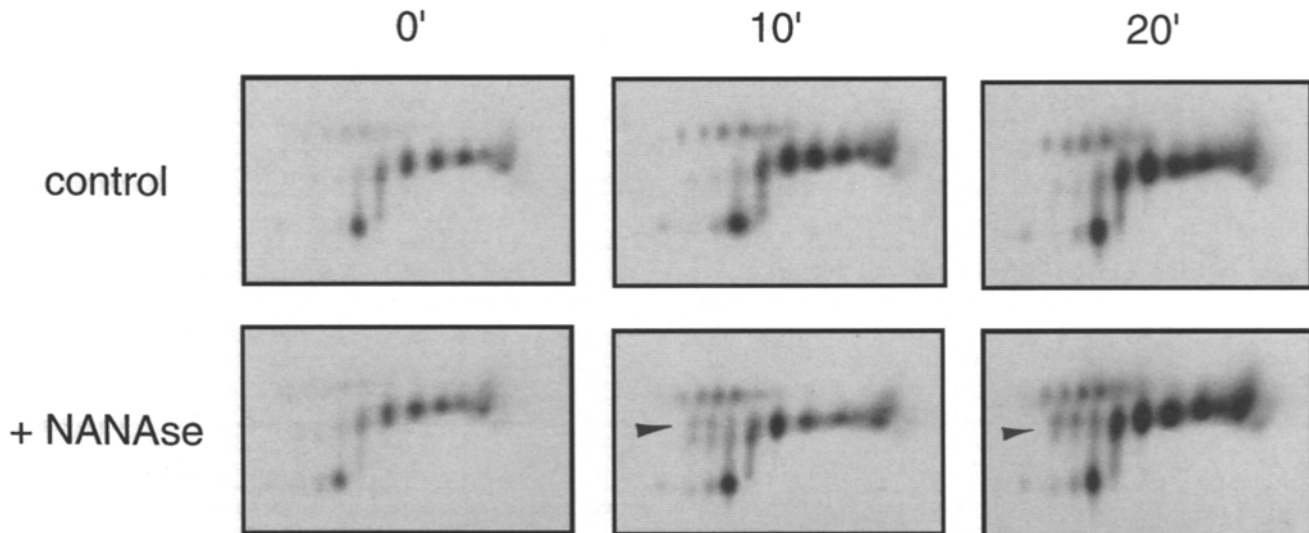
endosomes. To identify the rate at which newly synthesized αβIi complexes reach the cell surface from the Golgi apparatus, B-LCL were pulse-labeled with [<sup>3</sup>H]galactose for 15 min, chased for various times, and incubated with NANase on ice before cell lysis and the isolation of class II αβIi complexes (Fig. 4). After as little as 10 min of chase, extracellular NANase was capable of desialylating small amounts of [<sup>3</sup>H]galactose-labeled αβIi complexes, as evidenced by the appearance of new Ii spots migrating at a more basic position only following NANase treatment. Desialylation was more readily observed after 20 min of

chase. Note that despite the addition of excess unlabeled galactose, labeling with [<sup>3</sup>H]galactose continued during the first 20 min of chase. Quantitation using the NANase/β-galactosidase assay revealed that 5% of the total pool of labeled αβIi complexes was present on the cell surface after 20 min of chase (not shown). It is important to note that desialylation of αβIi was not observed without chase, confirming that the effect of NANase was dependent upon transport of αβIi out of the Golgi apparatus/TGN. These data demonstrate that a fraction of newly synthesized αβIi complexes can arrive rapidly at the cell surface from the Golgi apparatus.

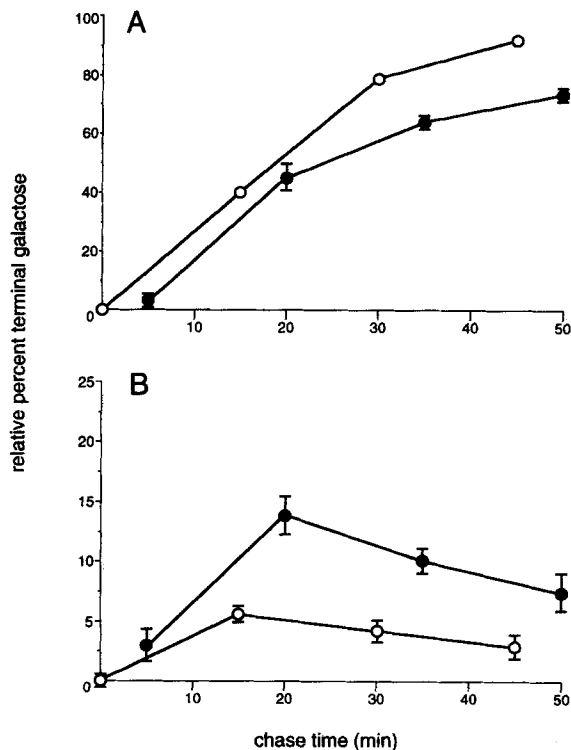
#### ***A Substantial amount of αβIi Complexes Reach the Cell Surface/Early Endosomes before Entry in Antigen Processing Compartments***

Since surface αβIi complexes are rapidly internalized, a large fraction of αβIi complexes would not be expected to be present at the cell surface at any given time. In an attempt to capture more of the class II molecules that transiently pass through the cell surface en route to antigen processing compartments, B-LCL were pulse labeled with [<sup>3</sup>H]galactose and chased for various times before a 5-min treatment of the cells with NANase at 37°C or with NANase on ice. Since this experimental design includes a 37°C incubation in the presence of NANase, we cannot exclude the possibility of NANase internalization and trimming of class II molecules in early endosomes. The short NANase treatment does, however, rule out cleavage of sialic acid residues in later endocytic compartments.

The results of the NANase/β-galactosidase assay for this experiment are shown in Fig. 5. As anticipated, MHC class I molecules were accessible to NANase on ice after even brief times of chase, demonstrating rapid arrival at the cell surface (Fig. 5 A, open symbols). Incubation with



**Figure 4.** Rapid transport of [<sup>3</sup>H]galactose-labeled MHC class II molecules to the cell surface. B-LCL 721.45 were radiolabeled with [<sup>3</sup>H]galactose for 15 min and chased for up to 20 min at 37°C. Samples chased 0 min (0'), 10 min (10'), and 20 min (20') were washed in ice cold RPMI and incubated in the absence (control) or presence (+NANase) of NANase for 2 h on ice. The cells were then washed extensively in RPMI medium containing 0.5 mg/ml fetuin, lysed, and MHC class II molecules isolated and analyzed by two-dimensional PAGE. Arrowheads indicate the mobility of the desialylated subunits. (The species migrating just below the desialylated Ii subunits most likely represent galactosylated but not yet sialylated Ii molecules and are therefore refractory to NANase trimming.)



**Figure 5.** Newly synthesized molecules intersect with NANase on the cell surface and/or early endosomes. B-LCL 721.45 were radiolabeled for 15 min with [<sup>3</sup>H]galactose and chased by adding 0.2 mg/ml unlabeled D-galactose to the samples for 0, 15, 30, or 45 min. After each chase point cells were split three ways; one sample served as a negative control; a second sample was digested with NANase for 2 h on ice (*open symbols*); a third sample was digested with NANase for 5 min at 37°C (*filled symbols*). MHC class I (*A*) and MHC class II molecules (*B*) were precipitated using mAb w6/32 and mAb DA6.147, respectively. The immunoprecipitates were analyzed in the  $\beta$ -galactosidase assay, and the relative percent terminal galactose was calculated as described in the text.

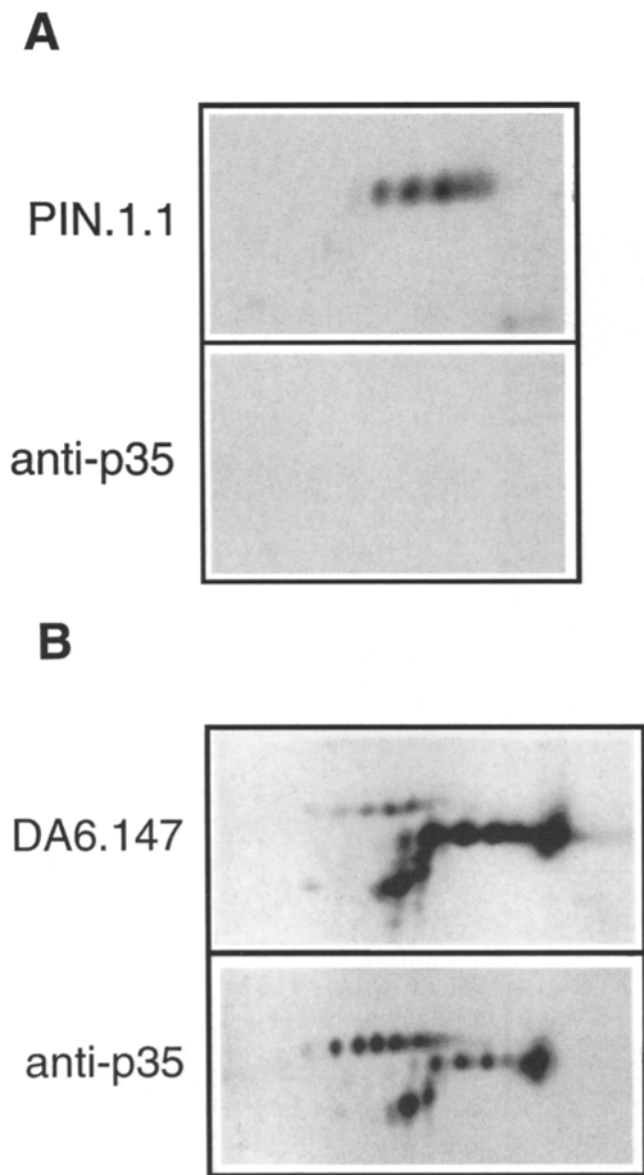
NANase for 5 min at 37°C did not result in complete desialylation of class I molecules, however, but it did result in desialylation to ~75% of the maximum obtained by incubation with NANase on ice (Fig. 5 *A*, *filled symbols*).

Class II molecules were isolated and analyzed in parallel experiments. At all chase points 3–5% of newly synthesized class II molecules were accessible to NANase on ice (Fig. 5 *B*, *open symbols*). Interestingly, treatment of the cells with NANase for only 5 min at 37°C revealed a flux of desialylated class II molecules which exceeded the amount cleavable on ice, reaching a peak of 14% after 15 min of chase despite the fact that desialylation for 5 min at 37°C is not completely efficient (Fig. 5 *B*, *filled symbols*). The increase in desialylation observed following NANase digestion at 37°C as opposed to NANase digestion on ice continued for up to 1 h of chase. These results suggest that a substantial fraction of newly synthesized MHC class II molecules intersect with NANase shortly after leaving the TGN and are targeted to the cell surface and/or early endosomes before their delivery to later, more acidic antigen processing compartments.

### *$\alpha\beta$ Ii Complexes Containing p33 or p35 Isoforms Are Targeted Differentially into Endosomal Compartments*

Very long exposures of the two-dimensional gels shown in Fig. 4 revealed that whereas the p33 form of class II-associated Ii was sensitive to NANase digestion on ice, the p35 form of class II-associated Ii was not, suggesting that class II molecules containing the p35 form of Ii were not present at the cell surface. To test this directly, B-LCL were radiolabeled with <sup>125</sup>I on ice and surface  $\alpha\beta$ Ii complexes were isolated using an isoform-independent anti-Ii antibody. Immunoprecipitation with this antibody revealed the existence of surface  $\alpha\beta$ Ii complexes containing sialylated Ii-p33 but did not reveal any  $\alpha\beta$ Ii complexes containing Ii-p35. (It should be noted that the class II  $\alpha$ - and  $\beta$ -chains do not label well with this labeling procedure; Roche et al., 1992.) To exclude the possibility that the p35 isoform of Ii is not accessible for iodination (even though the extracellular domain of Ii-p35 is identical to the extracellular domain of Ii-p33), we isolated  $\alpha\beta$ Ii complexes using an Ii-p35-specific antiserum. Previous studies have demonstrated that class II  $\alpha\beta$ Ii complexes exist as nonamers which can contain both Ii-p33 and Ii-p35 isoforms (Roche et al., 1991). For this reason, even if the Ii-p35 isoform present in cell surface  $\alpha\beta$ Ii complexes could not be labeled with <sup>125</sup>I, other iodinated components of the nonameric  $\alpha\beta$ Ii complex should be labeled and would be detected. However, Fig. 6 (*A*) demonstrates that no iodinated components of class II  $\alpha\beta$ Ii complexes could be detected after immunoprecipitation with the anti-Ii-p35 antiserum. This was not due to a nonfunctional antibody because this antiserum effectively precipitated [<sup>3</sup>H]galactose-labeled  $\alpha\beta$ Ii complexes (Fig. 6 *B*). Note that these complexes also contain the Ii-p33 isoform (due to the presence of mixed Ii trimers in the  $\alpha\beta$ Ii nonamer). In addition, intracellular Ii-p35 could be clearly labeled by iodination of nonviable, permeabilized cells (data not shown). These data, together with NANase digestion of [<sup>3</sup>H]galactose-labeled  $\alpha\beta$ Ii complexes, indicate that Ii-p35 containing  $\alpha\beta$ Ii complexes are not present at the cell surface.

The specific labeling of Ii-p35 containing class II  $\alpha\beta$ Ii complexes in the Golgi apparatus/TGN with [<sup>3</sup>H]galactose and the identification of sialic acid residues on these complexes, together with our failure to detect Ii-p35 containing class II  $\alpha\beta$ Ii complexes on the cell surface, demonstrates that these complexes are present in the Golgi apparatus and suggests that they are either retained there or that they are transported to endocytic compartments using a pathway distinct from that used by Ii-p33 containing  $\alpha\beta$ Ii complexes. To determine whether Ii-p35 containing class II  $\alpha\beta$ Ii complexes were transported to endosomal compartments, B-LCL pulse-labeled with [<sup>3</sup>H]galactose were chased for various times in the absence or presence of the endosomal protease inhibitor leupeptin. The majority of both Ii-p33 and p35 isoforms were degraded and dissociated from class II  $\alpha\beta$ Ii complexes within 90 min of chase in the absence of leupeptin (Fig. 7, *left panels*). By contrast, the dissociation of both Ii-p33 and p35 isoforms from  $\alpha\beta$ Ii complexes was significantly inhibited by inclusion of leupeptin in the chase medium, and both remain clearly associated with class II  $\alpha$ - and  $\beta$ -chains even after 180 min of chase (Fig. 7, *right panels*). These results dem-



**Figure 6.**  $\alpha\beta$ Ii-p35 complexes are not detectable at the cell surface. (A) B-LCL 721.45 were iodinated on ice, washed, and  $\alpha\beta$ Ii complexes were isolated from the lysates using mAb PIN.1.1 (recognizing all isoforms of Ii) or a rabbit anti-p35 antiserum (recognizing the p35 form of Ii only). The precipitates were analyzed using two-dimensional PAGE. (B) B-LCL 721.45 were radiolabeled with [ $^3$ H]galactose for 1 h and  $\alpha\beta$ Ii complexes were isolated from lysates using the anti-DR mAb DA6.147 (recognizing class II molecules associated with all isoforms of Ii) or a rabbit anti-p35 antiserum (recognizing class II molecules specifically associated with the p35 form of Ii).

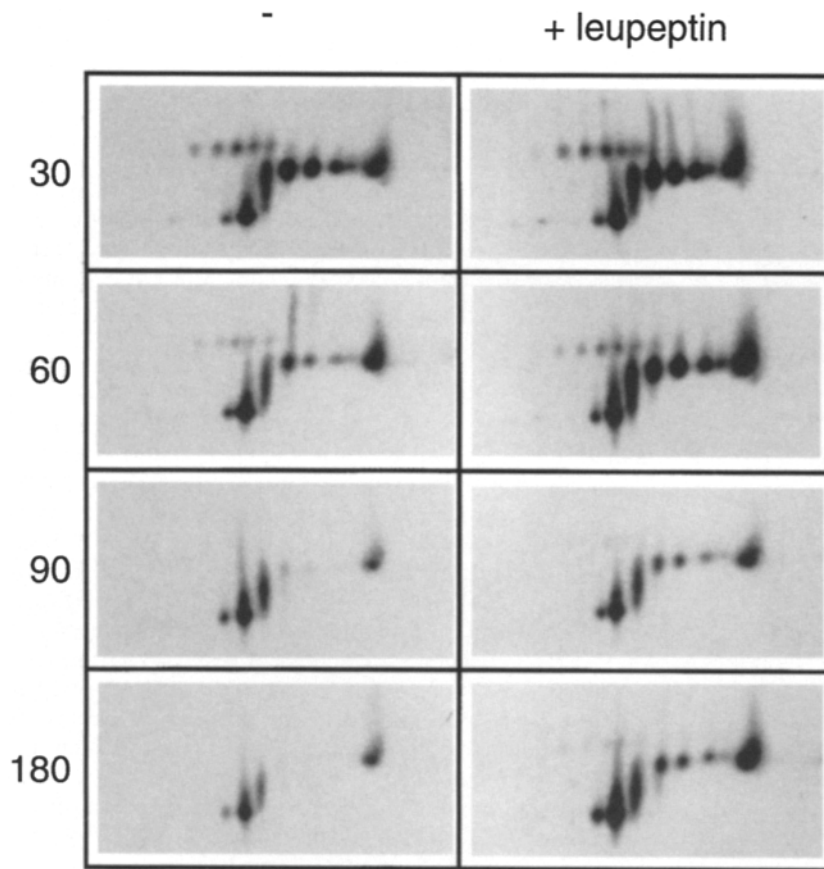
onstrate that the  $\alpha\beta$ Ii complexes labeled in the TGN with [ $^3$ H]galactose are targeted into the endosomal compartments and are not retained in or retrieved from the Golgi apparatus/TGN. These results are also in agreement with previous reports showing that leupeptin-induced peptides derived from Ii-p35 can be generated in B-LCL (Newcomb and Cresswell, 1993b).

To confirm that Ii-p35 containing  $\alpha\beta$ Ii complexes arrive

in endosomes without passage through the cell surface, B-LCL were pulse-labeled with [ $^3$ H]galactose for 15 min and Ii-p35 containing  $\alpha\beta$ Ii complexes present at the cell surface at various times were isolated and analyzed using the NANase/ $\beta$ -galactosidase assay. In parallel experiments, the incubation was allowed to continue for 5 min in the presence of NANase at 37°C before  $\beta$ -galactosidase treatment and quantitative analysis of [ $^3$ H]galactose release. The time course of NANase sensitivity of Ii-p35  $\alpha\beta$ Ii complexes on ice vs 37°C is shown in Fig. 8. Unlike the results obtained with the entire pool of  $\alpha\beta$ Ii complexes (Fig. 5), virtually no  $\alpha\beta$ Ii complexes containing Ii-p35 molecules could be desialylated when the cells were incubated with NANase on ice (*open symbols*). These results are consistent with our inability to detect Ii-p35 on the plasma membrane as shown in Fig. 6. Incubation with NANase during the last 5 min of chase (*filled symbols*) showed that some Ii-p35 containing  $\alpha\beta$ Ii complexes could be desialylated. Since desialylation was not detectable by NANase treatment on ice, this result suggests that NANase had internalized under these conditions and had cleaved Ii-p35 in early endocytic compartments. It should be emphasized that in this study the percentage of  $\alpha\beta$ Ii-p35 on the cell surface is expressed relative to the total amount of  $\alpha\beta$ Ii-p35 present in the sample and not to the total amount of all  $\alpha\beta$ Ii complexes synthesized in the cells. Taken together, the lack of NANase cleavage on ice and the lack of surface iodination of Ii-p35 containing  $\alpha\beta$ Ii complexes show that Ii-p33 and Ii-p35 containing  $\alpha\beta$ Ii complexes are targeted differently from the TGN to endosomal compartments.

### Discussion

We have demonstrated that the isoform of Ii associated with class II  $\alpha\beta$ -dimers regulates the transport route taken by class II  $\alpha\beta$ Ii complexes to antigen processing compartments. This was made possible by the use of an assay designed to quantitate cell surface arrival of newly synthesized MHC glycoproteins. The sensitivity of the assay made it possible to detect a transient population of MHC class II molecules on the surface of B-LCL that would have been difficult to detect using previously described assays. The main feature of this assay involves the use of [ $^3$ H]galactose to biosynthetically "tag" glycoproteins in the Golgi apparatus/TGN, thereby avoiding the labeling of unassembled Ii and class II  $\alpha\beta$ -dimers. In most APCs Ii is present in a large excess over class II  $\alpha$ - and  $\beta$ -chains (Kvist et al., 1982; Roche et al., 1992), and these free Ii molecules are for the most part ER retained (Marks et al., 1990). Therefore, pulse-chase labeling studies using conventional biosynthetic labels such as [ $^{35}$ S]methionine or [ $^3$ H]leucine are confounded by the fact that at early times of chase a disproportionate percentage of radiolabel is present on Ii, whereas at later times of chase newly synthesized (nonradioactive) class II  $\alpha$ - and  $\beta$ -chains associate with ER-retained Ii, become transport competent, and leave the ER. The combination of these factors leads to nonsynchronous class II transport and makes analysis of transient populations of molecules difficult to detect. On the other hand, labeling assembled  $\alpha\beta$ Ii complexes in the Golgi apparatus/TGN with [ $^3$ H]galactose circumvents these



**Figure 7.** Dissociation of both Ii isoforms p33 and p35 from  $\alpha\beta$  heterodimers is inhibited by leupeptin. B-LCL 721.45 were radiolabeled with [ $^3$ H]galactose for 45 min in the absence or presence of leupeptin. The cells were pelleted by centrifugation to remove free radiolabel, and chased in regular medium containing 0.02 mg/ml unlabeled D-galactose and 0.1 mM D-mannose in the absence (-) or presence (+) of 1 mM leupeptin. After incubation for 30, 60, 90, or 180 min at 37°C, the cells were washed in ice cold RPMI, lysed, and MHC class II molecules were isolated using mAb DA6.147. After immunoprecipitation, samples were analyzed using two-dimensional PAGE.

problems, as there is no pool of unassembled molecules to interfere with the pulse-chase study.

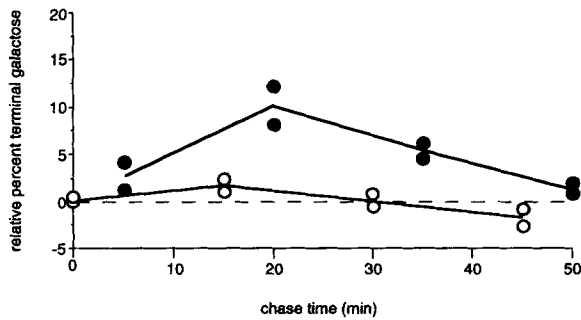
Using this labeling system, we have demonstrated that MHC class II  $\alpha\beta$ Ii complexes can be detected at the surface of B-LCL within 10 min of chase from the Golgi apparatus/TGN. This rate of transport rivals that of class I MHC molecules, glycoproteins thought to arrive at the cell surface by the "default" pathway. However, unlike class I molecules, newly synthesized MHC class II molecules are rapidly internalized once they arrive at the cell surface by the recognition of an internalization signal in the cytosolic domain of Ii (Roche et al., 1993). For this reason,  $\alpha\beta$ Ii complexes exist only transiently on the cell surface and are therefore present there at low levels under steady-state conditions. Indeed, we have previously shown that ~3% of all surface class II molecules are associated with Ii (Roche et al., 1993). However, due to their rapid uptake into the endocytic pathway, even this small population of molecules at the surface can represent a significant fraction of the total biosynthetic pool of newly synthesized class II  $\alpha\beta$ Ii complexes that passes through the cell surface en route to antigen processing compartments.

The newly synthesized  $\alpha\beta$ Ii complexes detected in this study may have arrived at the cell surface directly from the TGN. Alternatively, the  $\alpha\beta$ Ii complexes may have passed rapidly through a recycling endosome before subsequent transport to the cell surface, as shown for the transferrin receptor (Futter et al., 1995). However, recycling of internalized class II  $\alpha\beta$ Ii complexes is very inefficient: over a 30–60-min period most of the surface Ii is degraded and

dissociates from internalized class II molecules (Roche et al., 1993 and present study). Nevertheless, we have recently measured that about half of the  $\alpha\beta$ Ii complexes remaining after 30 min of internalization reappear at the cell surface in 10 min (data not shown), indicating that limited recycling of surface  $\alpha\beta$ Ii complexes does occur in B-LCL.

Surprisingly, and in contrast to the results obtained with  $\alpha\beta$ Ii complexes containing the p33 form of Ii, class II molecules associated with the p35 form of Ii did not appear on the plasma membrane. This was demonstrated both by the absence of Ii-p35 in immunoprecipitations from surface-iodinated cells and by the lack of NANase trimming of [ $^3$ H]galactose-labeled Ii-p35 molecules. These  $\alpha\beta$ Ii-p35 complexes were not simply retained in or retrieved from the Golgi apparatus since their dissociation from class II molecules was inhibited by the endosomal proteinase inhibitor leupeptin. Unlike the results obtained on ice, incubation with NANase at 37°C for 5 min did reveal some sialic acid trimming, presumably as a consequence of internalization of NANase into early endosomes during the incubation. We conclude from these data that the transport route taken by class II  $\alpha\beta$ Ii complexes is regulated by the isoform of Ii: in the absence of a p35 form in the ( $\alpha\beta$ Ii) $_3$  complex, surface arrival is possible (either directly from the TGN or via recycling endosomes), whereas the presence of an Ii-p35 cytosolic tail prevents transport of the  $\alpha\beta$ Ii complex to the cell surface. Since Ii trimers in the nonameric  $\alpha\beta$ Ii complex can contain a mixture of Ii-p33 and Ii-p35 (Marks et al., 1990; Roche et al., 1991; Lamb and Cresswell, 1992), these results also suggest that the





**Figure 8.** Ii-p35 containing  $\alpha\beta$ Ii complexes do not travel via the cell surface, but are targeted directly to early/late endosomes. B-LCL JY were radiolabeled for 15 min with [ $^3$ H]galactose and were chased by adding 0.2 mg/ml unlabeled D-galactose to the cells for 0, 15, 30, or 45 min. After each chase point the cells were split three ways; one sample served as a negative control; a second sample was digested with NANase for 2 h on ice (*open symbols*); a third sample was digested with NANase for 5 min at 37°C (*filled symbols*). Ii-p35 containing  $\alpha\beta$ Ii complexes were isolated using an affinity-purified rabbit anti-p35 antibody. The immunoprecipitates were analyzed in the  $\beta$ -galactosidase assay and the relative percent terminal galactose was determined.

targeting signal in the Ii-p35 tail dominates over the signal present in the Ii-p33 tail.

The fraction of the biosynthetic pool of class II molecules that are transported to the cell surface before arrival in antigen processing compartments cannot be accurately quantitated for several reasons. The very short half-life of cell surface  $\alpha\beta$ Ii complexes makes it difficult to detect them quantitatively, since at any time during a pulse-chase experiment most of the labeled molecules will be either still inside the cells or already internalized. Furthermore, true chase conditions cannot be achieved with galactose; as a result, labeled molecules that have not yet reached the surface continue to be produced during short chase times. Finally, the extent of recycling of internalized  $\alpha\beta$ Ii complexes will affect the estimate of how many newly synthesized molecules reach the cell surface. Nevertheless, a highly reproducible 5% of the biosynthetic pool of class II  $\alpha\beta$ Ii complexes was detected at the cell surface shortly after egress from the Golgi apparatus/TGN. Considering that this value was determined by treating cells with NANase on ice, a much greater fraction of the new class II molecules must pass through the cell surface. Indeed, treatments with NANase at 37°C for 5 min revealed that ~15% of the total pool of radiolabeled  $\alpha\beta$ Ii complexes was accessible to NANase digestion. This short incubation time was chosen to exclude the possibility of trimming class II molecules that are transported through late endosomes. Previous studies have shown that at least 30 min is required for fluid-phase markers to reach prelysosomal antigen processing compartments such as the originally defined MIIC (Peters et al., 1991, 1995). Therefore, a substantial fraction of the biosynthetic pool of class II molecules passes through the plasma membrane/early endosome compartment before their delivery to antigen processing compartments.

Active transport through the cell surface was also revealed in a study using chimeric proteins composed of the cytosolic tail and transmembrane domains of Ii and the ex-

tracellular domain of the transferrin receptor (Odorizzi et al., 1994). This chimeric protein led to uptake of transferrin that was even more efficient than that mediated by wild-type transferrin receptor. From the measurements of steady-state surface levels, the percentage of internalized molecules at steady state, the rate of synthesis of these molecules, and their rate of degradation, the authors calculated that ~20% of these chimeric molecules trafficked via the cell surface (Odorizzi et al., 1994). However, this estimate is based on the accurate determination of these four independent variables, so that even a twofold error in any given variable will dramatically alter the estimate of trafficking via the cell surface. Nevertheless, it is important to reiterate that ~150,000  $\alpha\beta$ Ii complexes are internalized in a B-LCL every hour (Roche et al., 1993) and that all of the [ $^3$ H]galactose-labeled  $\alpha\beta$ Ii complexes synthesized in the experiments described here lose Ii and give rise to mature class II  $\alpha\beta$  dimers. Therefore, irrespective of whether these internalized complexes represent a minority or a majority of the biosynthetic pool (a distinction that cannot be made accurately with existing techniques), they represent a very large number of molecules as compared to the ~300 surface class II-peptide complexes required to stimulate T cells (Harding and Unanue, 1990).

The demonstration that a substantial fraction of newly synthesized class II  $\alpha\beta$ Ii complexes is transported to the plasma membrane/early endosomes before delivery to antigen processing compartments such as the MIIC is in excellent agreement with several recent studies. Germain's laboratory has shown that Ii is targeted predominantly to early endosome-like structures in the absence of class II molecules (Romagnoli et al., 1993) and that peptide loading onto newly synthesized class II molecules can occur in multiple endocytic compartments (Castellino and Germain, 1995). Pieters et al. (1991) have localized class II molecules and Ii in early endosomes, multivesicular bodies, and late endosomes/prelysosomes in human melanoma cells. Marič et al. (1994) found intact Ii in conventional endosomes before the generation of Ii fragments in more lysosome-like compartments in B-LCL. Finally, Amigorena et al. (1995) found the p10 fragment of Ii (but not intact Ii) in the murine B cell antigen processing compartment "CIIV," suggesting that most class II  $\alpha\beta$ Ii-p10 complexes reach this compartment only after arriving in endosomes as  $\alpha\beta$ Ii complexes. On the other hand, the predominant localization of class II  $\alpha\beta$ Ii in MIIC at steady state is also compatible with the results presented here. Steady-state measurements cannot determine pathways of transport, although they were invaluable for the identification of processing compartments in antigen-presenting cells. By analogy, the mannose-6-phosphate receptor has been localized to late endosomes by immunoelectron microscopy (Klumperman et al., 1993), and until recently, it was thought to arrive in this compartment directly from the TGN. However, kinetic studies demonstrated that this receptor was in fact targeted to early endosomes before delivery in late endosomes (Ludwig et al., 1991). Taken together, this suggests that the accumulation of class II molecules in MIIC represents the fact that egress from this compartment is the rate-limiting step in class II molecule transport along the biosynthetic pathway.

The strength of the endosomal targeting signal in the Ii

cytoplasmic tail is dependent on Ii trimerization (Arneson and Miller, 1995). The choice between the intracellular or the surface route may also be controlled by the strength of the cytoplasmic tail signal. The cytoplasmic tail of the CD3 $\gamma$  chain carries two targeting signals, one tyrosine-based, and another made up of a di-leucine motif (Letourneur and Klausner, 1992). Either signal alone leads to transport to the cell surface and retrieval by internalization, whereas the presence of both signals results in transport to lysosomes without detectable surface expression (Letourneur and Klausner, 1992). The cytoplasmic tail of Ii is devoid of tyrosine but contains a di-leucine-like motif that is essential for internalization (Pieters et al., 1993; Bremnes et al., 1994; Odorizzi et al., 1994). The very basic 16-amino acid extension on the p35 cytoplasmic tail may strengthen the signal(s) present on the p33 cytoplasmic tail. Studies on this basic amino acid extension will be complicated by the fact that it carries a strong ER-retention motif (Schutze et al., 1994) that can be overcome by assembly of Ii with class II molecules. The identification of adaptor or receptor molecules that interact with the p33 and the p35 forms of the Ii cytoplasmic tail will clarify the role of the p35 isoform and the mechanism by which it prevents surface arrival of class II  $\alpha\beta$ Ii complexes. Finally, the novel assay described here should prove very useful to study the transport of class II molecules in different cell types and the transport of other endosomal/lysosomal proteins in general.

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