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MHC Class II–Associated Invariant Chain Contains a Sorting Signal for Endosomal Compartments

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Summary

The invariant chain (Ii) is a transmembrane protein that associates with the MHC class II molecules in the endoplasmic reticulum. Expression of Ii in MHC class II–negative CV1 cells showed that it acquired complex-type oligosaccharide side chains and was retained in endosomal compartments. To search for a sorting signal, we made progressive deletions from the cytoplasmic N-terminus of Ii. Deleting 11 amino acid residues resulted in a protein that was still sorted and retained in endosomal vesicles, whereas deletion of 15 or more amino acid residues resulted in a protein that became resident in the plasma membrane. Amino acids 12–15 are thus essential for intracellular transport to endosomal compartments. As Ii is intracellularly associated with the MHC class II molecules, it is proposed that Ii determines the intracellular transport route of these molecules.

Introduction

The major histocompatibility complex (MHC) class II molecules are primarily expressed on the surface of B cells and macrophages and serve to present antigenic peptides to T helper cells. The class II molecules consist of the polymorphic α and β chains, which associate in the endoplasmic reticulum (ER) with the invariant chain (Ii) (Sung and Jones, 1981; Kvist et al., 1982). The human Ii is a membrane protein that exposes 30 N-terminal residues on the cytoplasmic side of the membrane, spans the membrane between residues 30 and 60, and has a large C-terminal domain on the exoplasmic side (Claesson et al., 1983; Strubin et al., 1984). This C-terminal domain has two N-linked carbohydrate units (Charron and McDevitt, 1980; Owen et al., 1981).

During intracellular transport of class II molecules to the cell surface, Ii is proteolytically processed and dissociates from the complex at an intracellular site (Kvist et al., 1982; Accolla et al., 1985; Cresswell et al., 1987). MHC class II molecules and Ii have been colocalized in intracellular vesicles, and biochemical and morphological studies suggest that these are endocytic vesicles (Cresswell, 1985; Guagliardi et al., 1990; Neefjes et al., 1990). The peptides that are presented by the class II molecules are thought to be derived from molecules taken up by endocytosis and proteolytically processed. The antigen would then be present in endosomes, which could be the location where it interacts with the class II molecules. Recently it has been found that the expression of Ii is required for the efficient processing/presentation of some antigens (Stockinger et al., 1989). The function of Ii in this process is not known, although recent models propose that its function could be 2-fold: to sort class II molecules to a specific intracellular compartment where antigen binding takes place, and to occupy the antigen binding site until this location is reached (Koch et al., 1989; Long, 1989).

Since the complex of MHC class II/Ii molecules is localized in endocytic compartments, it might contain a sorting/retention signal for these compartments. The signal might reside in either class II molecules, Ii, or both. The class II molecules are found on the cell surface even in the absence of Ii (Miller and Germain, 1986; Sekaly et al., 1986), whereas Ii is largely an intracellular molecule (Cresswell et al., 1987). It has been shown that Ii not in a complex with class II molecules remains in the ER for a long time (Cresswell et al., 1987), but nothing is known about the final cellular location of Ii and whether Ii carries sorting information for other intracellular compartments.

Many proteins have a common biosynthetic pathway that involves transport from the ER through the Golgi complex. The proteins remaining within organelles of this pathway must be distinguished from secretory proteins that are transported out of the cell via the secretory route and membrane proteins that end up on the plasma membrane. The more recent data support the view that transport of secreted proteins may occur by default, i.e., no specific secretion signal is needed (Pfeffer and Rothman, 1987). Accordingly, proteins that are resident within the secretory pathway (ER, Golgi) should contain a retention signal, such as the KDEL sequence, which retains soluble proteins within the ER (Munro and Pelham, 1987). A similar default pathway might exist for membrane proteins (Hiebert and Lamb, 1988), and it has also been found that removal of the ER retention signal from a viral membrane protein resulted in transport to the plasma membrane (Pääbo et al., 1987). Since Ii is retained at intracellular sites, one might expect that destruction of its sorting signal would result in plasma membrane expression.

Ii could reach the proposed endocytic compartment directly via the biosynthetic pathway (ER–Golgi–*trans* Golgi network) or follow a default pathway to the plasma membrane and then be reinternalized. Several plasma membrane receptors involved in endocytosis contain within their cytoplasmic tails an endocytosis sorting signal (for review see Breitfeld et al., 1989). A sorting signal to an endocytic compartment from the biosynthetic pathway has also been proposed to reside within the cytoplasmic tail of the mannose 6-phosphate receptor (Lobel et al., 1989). We show here that Ii is sorted to intracellular endosomal vesicles in the absence of class II molecules and that the N-terminal cytoplasmic tail plays an important role in this sorting.

Results

Expression of Ii and ΔIi Proteins in CV1 Cells

Complementary DNA coding for the 33 kd form of Ii and for N-terminal deleted forms of Ii were constructed and cloned into the pSVL vector as described in Experimental Procedures. In the mutant ΔIi proteins, 11, 15, 20, and 23 amino acids, respectively, were deleted as outlined in Figure 1. For the expression of these proteins, the plasmids were transfected into simian CV1 cells, the parent cell line of COS cells (Gluzman, 1981). COS cells have been reported to be devoid of detectable mRNA coding for either class II (Sekaly et al., 1986) or Ii (Sekaly et al., 1986; Miller and Germain, 1986). Using a panel of monoclonal and polyclonal antibodies to the human class II and Ii, we could not detect any specific antigen in the CV1 cell line neither by immunofluorescence nor by metabolic labeling and immunoprecipitation (data not shown). As a control for expression and processing of Ii we used the human melanoma cell line Mel JuSo, which also expresses endogenous MHC class II molecules (Johnson et al., 1982).

Two days after transfection the CV1 cells were labeled with [^{35}S]methionine for 4 hr. Antigens from the cell lysates were immunoprecipitated with an antibody recognizing a determinant within the C-terminal part of Ii (anti-IiC). Labeled proteins were analyzed by SDS–PAGE and visualized by fluorography. A single major polypeptide was detected with each plasmid used in the transfection (Figure 2). The apparent molecular weights of the proteins decreased corresponding to decreasing size of the cytoplasmic tails. Ii from transfected CV1 cells migrated to the same position as the major 33 kd form of Ii from Mel JuSo cells. Mel JuSo cells also express two other forms of Ii: a 35 kd protein from an upstream ATG initiation codon and a 41 kd protein with a C-terminal insert from alternative splicing (Strubin et al., 1986). A 25 kd protein appears in all lanes (Figure 2, p25). This protein is not seen using an antibody directed against the N-terminal portion of Ii (anti-IiN) (data not shown). Therefore, it is most likely a part of Ii lacking the N-terminus. A similar protein has been described in cells that also express class II (Thomas et al., 1988) and in cells transfected with Ii alone (Koch, 1988).

Glycosylation of Ii and ΔIi Proteins

Ii contains two N-linked oligosaccharides that can be modified to the complex type during passage through the Golgi compartment. Endoglycosidase H (endo H) is an enzyme that selectively cleaves off the N-linked oligosaccharides that have not been processed to complex type. Acquisition of endo H resistance is therefore commonly used to monitor glycoprotein passage through the initial part of Golgi (Kornfeld and Kornfeld, 1985).

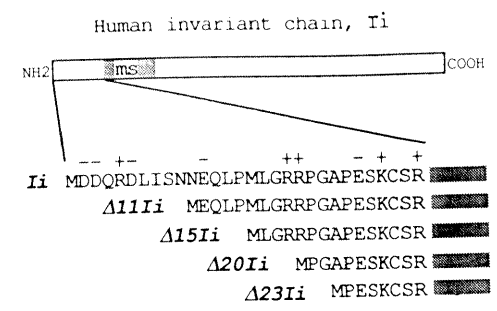

Figure 1. Amino Acid Sequences of the Cytoplasmic N-Terminal Domains of Ii and ΔIi Deletion Mutants

Figure 2. Expression of Ii and ΔIi Deletion Mutants in CV1 Cells

Transfected CV1 cells and Mel JuSo cells were labeled with [^{35}S]methionine for 4 hr. Proteins in the cell lysates were immunoprecipitated with anti-IiC, and the isolated proteins were separated by SDS–PAGE and visualized by fluorography. The migration of molecular size markers (kd) is indicated.

Transfected CV1 cells and Mel JuSo cells were labeled for 10 min with [^{35}S]methionine, and antigens were immunoprecipitated with anti-IiC. Precipitated proteins were treated with endo H before SDS–PAGE and fluorography. Figure 3A shows that Ii from Mel JuSo cells and CV1 cells as well as the ΔIi proteins are all sensitive to the endo H treatment. The molecular sizes were reduced by about 6 kd, indicating that the proteins are N-glycosylated by two oligosaccharide chains (Owen et al., 1981; Rudd et al., 1985). As potential glycosylation sites are only present on the C-terminal part of Ii, this shows that all proteins have the same topology across the ER membrane.

To characterize endo H resistance of Ii and mutants during intracellular transport, transfected CV1 cells were labeled continuously for 4 hr. Ii33 and p25 are indicated in Figure 3B. Digestion of the immunoprecipitated proteins with endo H resulted in three major proteins. As judged by their respective sizes they represent proteins without (arrowheads) or with one or two (arrows) endo H–resistant carbohydrate side chains. Note that p25 is also endo H sensitive and reduced by 6 kd after the endo H treatment (labeled p25EH). To quantify the amount of endo H–sensitive and –resistant proteins, the endo H–treated lanes in Figure 3B were analyzed by densitometric scanning as shown in Figure 3C. The largest peaks represent the endo H–sensitive protein (indicated by an arrowhead for Ii), and the two smaller peaks represent protein with one or two (see arrow for Ii) complex-type oligosaccharides. Common to all proteins is that a large proportion remains endo H sensitive, whereas a conspicuous difference is that the mutant proteins Δ15Ii, Δ20Ii, and Δ23Ii have a higher proportion of complex-type N-linked oligosaccharides than wild-type Ii and Δ11Ii.

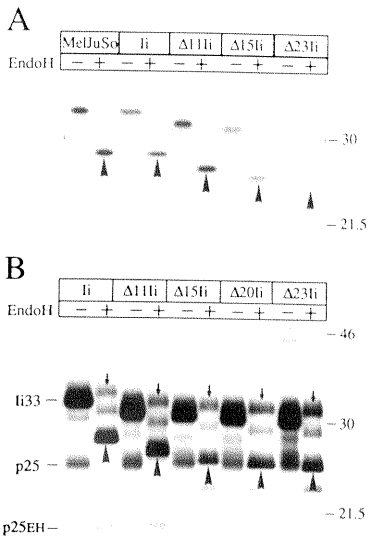

Figure 3. Endo H Resistance of Ii and ΔIi Mutants

Transfected CV1 cells were labeled with [^{35}S]methionine for 10 min (A) or 4 hr (B), and an aliquot of each immunoprecipitate was digested with endo H before SDS–PAGE and autoradiography. Endo H–sensitive proteins are indicated by arrowheads. Proteins with two endo H–resistant side chains are indicated with arrows. Ii33 and p25 are indicated (see Figure 2). p25EH indicates the endo H–sensitive form of p25. The migration of molecular size markers (in kd) is indicated on the right side. In (A) Ii of Mel JuSo cells is included as a control. (C) shows densitometric scans of the lanes representing endo H–treated samples in (B). For Ii the peaks indicated by an arrowhead and an arrow represent endo H–sensitive and endo H–resistant proteins, respectively (see [B]).

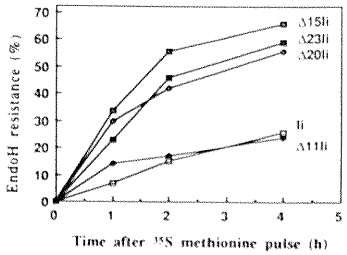

Figure 4. Kinetics of Acquisition of Endo H Resistance

Transfected CV1 cells were labeled for 15 min with [^{35}S]methionine and chased for the indicated times with medium containing an excess of cold methionine. The proteins were immunoprecipitated and treated with endo H before SDS–PAGE and fluorography. Endo H resistance was quantified by densitometric analysis of the respective bands in the autoradiograph.

Rate of Complex-Type Oligosaccharide Addition

As an intracellular sorting signal might influence transport through the ER and the Golgi apparatus, we monitored the time course for acquiring complex-type sugars. The transfected cells were labeled for 15 min with [^{35}S]methionine and chased in the presence of cold methionine for 1, 2, and 4 hr. Proteins from cell lysates were then immunoprecipitated, endo H digested, and analyzed by SDS–PAGE as above. The fluorographic profiles were quantitated by densitometry, and the endo H–resistant fractions were determined. The carbohydrate moieties on Ii and Δ11Ii were only slowly converted to complex types, and after 2 hr of chase only 17% of Ii and Δ11Ii were endo H resistant (Figure 4). A higher rate of conversion was seen for Δ15Ii, Δ20Ii, and Δ23Ii, and after 2 hr 45%–55% of these proteins were endo H resistant. The half-life of Ii in Raji cells expressing class II molecules has been found to be between 1 and 2 hr (Kvist et al., 1982). Quantifying the amount of Ii and ΔIi proteins from the above pulse chase experiments, we found for all proteins a half-life of about 1.5 hr (data not shown).

Localization of Ii in CV1 and Mel JuSo Cells

The localization of wild-type Ii in transfected CV1 cells was visualized by immunofluorescence microscopy using the monoclonal antibody LN2 (Figure 5A). Ii was seen in the perinuclear region and in distinct punctated structures in the cytoplasm. The same cells were also costained with the *trans* Golgi marker galactosyltransferase (GalTf) (Berger et al., 1987) as shown in Figure 5C. A similar distribution was found for Ii and GalTf in Mel JuSo cells, which also express class II molecules (Figures 5B and 5D, respectively). Clearly, Ii in both cell types is localized in the Golgi area and in distinct vesicles in the cytoplasm toward the periphery. Together with the finding that a fraction of Ii acquires endo H–resistant oligosaccharides (see Figures 2 and 3), this suggests that Ii is retained in a post-Golgi compartment in CV1 cells as well as in Mel JuSo cells. In Mel JuSo cells we have identified these compartments by immunoelectron microscopic studies as endosomes (unpublished data).

Intracellular and Cell Surface Localization of Ii and ΔIi Proteins

As discussed in the Introduction one might expect to find membrane proteins with a distorted intracellular sorting signal on the plasma membrane. To detect a possible cell surface location of ΔIi proteins, we labeled cells on ice by rabbit anti-liC antibody. The cells were then fixed with paraformaldehyde and permeabilized with Triton X-100. A monoclonal antibody (LN2) was used to visualize intracellular distribution of the ΔIi proteins in the same cells. As shown in Figures 6A and 6C, CV1 cells expressing wild-type Ii and Δ11Ii showed little cell surface expression of the proteins but intracellular localization in the perinuclear area and in vesicular structures more distant from the nucleus (Figures 6B and 6D). Cell surface appearance was

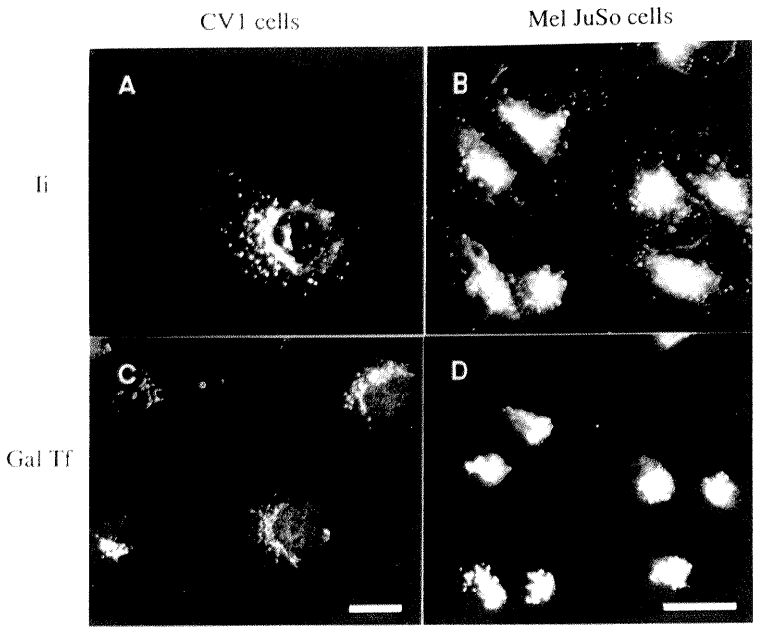

Figure 5. Localization of Ii and GalTf in Transfected CV1 Cells and Mel JuSo Cells

The micrographs show the double immunofluorescence staining pattern for Ii (A) and GalTf (C) in methanol fixed CV1 cells. Note that only one of the cells in the micrograph (A) has been transfected. The corresponding double immunofluorescence micrographs for Mel JuSo cells are shown in (B) (Ii) and (D) (GalTf). Ii was labeled by a monoclonal antibody, LN2, and an FITC-conjugated second antibody. GalTf was visualized by a polyclonal antibody and Texas red–conjugated second antibodies. Bar = 20 μm.

detected for only 3% of the Ii-expressing cells, whereas the corresponding figure for Δ11Ii was 22% (see legend to Figure 6). A dramatic change in localization was found for Δ15Ii with 15 amino acids deleted from the N-terminus. A strong cell surface staining was detected in all cells transfected with this construct (Figure 6E). Intracellular distribution was also altered as vesicular structures in the periphery of the cells were not seen (Figure 6F). When proteins with further deletions were expressed (Δ20Ii, Δ23Ii), they showed a similar cell surface and intracellular appearance as Δ15Ii (Figures 6G–6J). To study possible effects caused by the level of expression of the proteins, we also stained cells early after removal of buturate (i.e., 24 hr after transfection). At this time point the cells express low levels of protein, but the intracellular and cell surface staining pattern for the various proteins was similar to that shown in Figure 6 (data not shown). We conclude from this and the data in Figures 3 and 4 that an intracellular sorting signal fails to function when amino acids 12 to 15 are removed from the N-terminus of Ii.

Immunoelectron Microscopic Localization of Ii and Δ11Ii in Endosomes

Immunoelectron microscopic analysis was used to identify more precisely the intracellular localization of Ii and Δ11Ii in CV1 cells. As Ii in cells expressing class II molecules is found in endosomes, we suspected that it might be found in the same organelles in CV1 cells. Transfected CV1 cells were therefore incubated with 5 nm BSA–gold for 1 hr, which enters organelles of the endocytic pathway (Griffiths et al., 1990). The cells were then prepared for immunoelectron microscopy and labeled with anti-IiC and 9 nm protein A–gold. As shown in Figure 7, both Ii and Δli colocalize in structures that also contain endocytosed marker. In addition to endosomal localization, Ii and Δli were also found in typical Golgi structures as shown for Δ11Ii (Figure 7C). We conclude from these observations that both Ii and Δ11Ii are sorted to endosomes or to compartments that fuse with endosomes.

Discussion

Our results demonstrate that Ii synthesized in MHC class II–negative CV1 cells is retained intracellularly. In addition to its presence in ER and Golgi, Ii is retained in endosomal compartments. This is concluded from the observations that Ii acquires complex-type N-linked oligosaccharides during transport and is localized in intracellular vesicles peripheral to the Golgi apparatus labeled with endocytosed marker. Sialic acid addition to Ii in the absence of class II molecules has also been shown after stable transfection of genomic DNA into rat fibroblasts (Koch, 1988). In cell lines expressing both the MHC class II molecules and Ii, these molecules have been localized in endocytic vesicles (Guagliardi et al., 1990). Thus, sorting of Ii to endosomal vesicles is independent of the presence of class II molecules.

Intracellular transport of the class II molecules and Ii has been investigated by several groups (for reviews see Cresswell et al., 1987; Koch et al., 1989; Long, 1989). It has been shown that Ii assembled with class II molecules is transported out of ER and acquires complex-type oligosaccharides less than 30 min after its synthesis (Machamer and Cresswell, 1982). Ii is produced in excess over class II molecules (Sung and Jones, 1981; Machamer and Cresswell, 1982), and Ii not assembled is retained in the ER for more than 3 hr (Kvist et al., 1982). Assembly of Ii with class II thus seems to facilitate the transport of Ii out

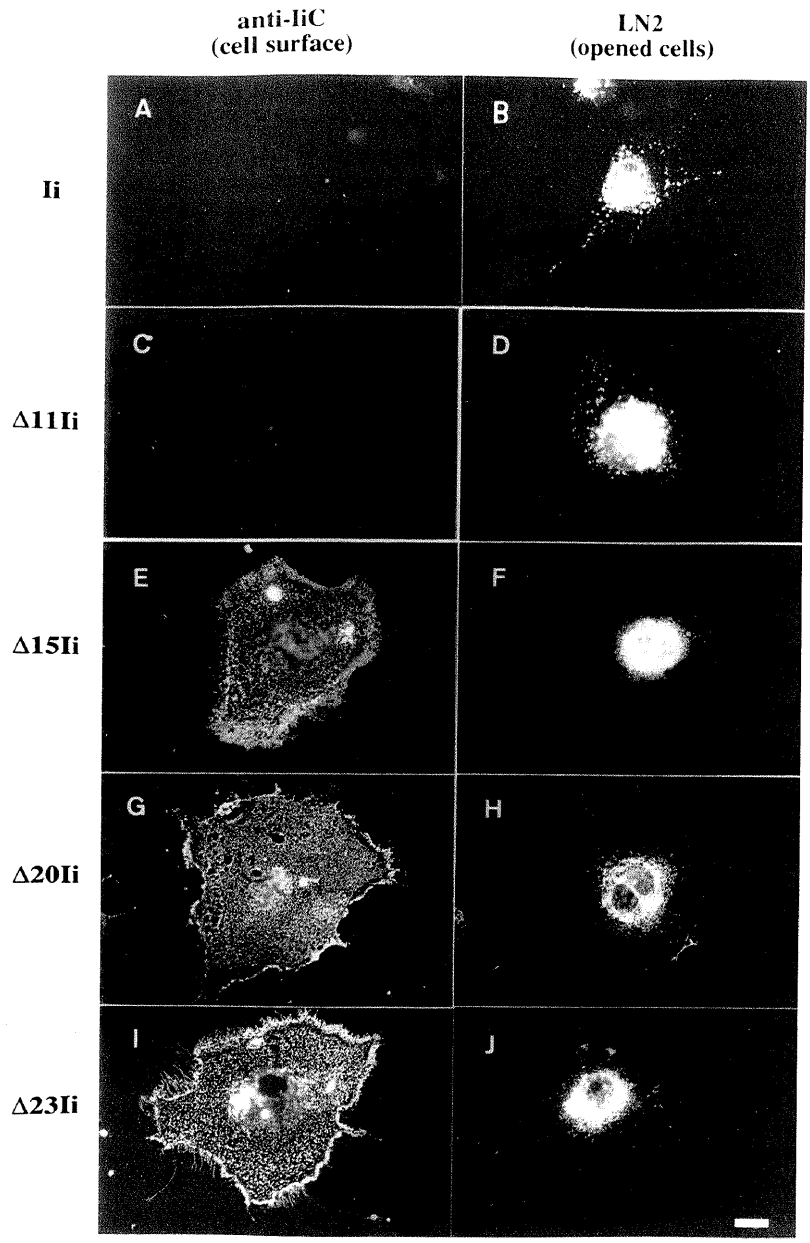

Figure 6. Cell Surface and Intracellular Localization of Ii and Δli Mutants in CV1 Cells

Transfected CV1 cells were first labeled on ice with rabbit polyclonal antibodies against Ii (anti-IiC) and a Texas red–conjugated second antibody. After paraformaldehyde fixation and permeabilization with Triton X-100 the cells were labeled with the mouse monoclonal antibody, LN2, and an FITC-conjugated second antibody. The left panels ([A], [C], [E], [G], and [I]) show cell surface localization (Texas red), and the right panels ([B], [D], [F], [H], and [J]) show the same cells labeled with LN2 (FITC) after permeabilization. Note the lack of surface staining by LN2 in (F), (H), and (J), indicating that the epitope is blocked by the polyclonal antibody. Bar = 20 μm. The percentage of transfected cells showing surface staining: Ii = 3%, Δ11Ii = 22%, Δ15Ii = 93%, Δ20Ii = 100%, Δ23Ii = 99%.

of the ER. We conclude from our data that Ii in CV1 cells is transported to the Golgi at a slow rate, as only 17% of the protein had acquired complex-type oligosaccharides 2 hr after synthesis. This shows that assembly of Ii with class II is not a prerequisite for its transport out of the ER. Slow passage to Golgi is not an inherent property of CV1 cells, as other membrane proteins like the influenza virus hemagglutinin acquired 80% endo H resistance within 20 min in these cells (Copeland et al., 1986).

The pathway taken by Ii to the endosomal compartment has not yet been identified. Although we do not detect Ii on the plasma membrane, we cannot exclude the possibility that Ii is first transported to the plasma membrane and then rapidly taken up into endosomes. Ii could also reach the endosomal compartment via the biosynthetic route from the *trans* Golgi network. This transport route has been demonstrated for the class II MHC molecules in human lymphoblastoid cells (Neefjes et al., 1990). They found that class II molecules were delayed intracellularly in endosomal compartments for 1 to 3 hr before reaching the plasma membrane.

We also demonstrate that the N-terminal cytoplasmic domain of Ii contains or effects an essential part of the intracellular sorting signal, since deletion of 15 or more amino acids leads to surface expression. Studies of the membrane receptors for low density lipoprotein (Davis et al., 1987), epidermal growth factor (Prywes et al., 1986), polymeric immunoglobulin (Mostov et al., 1986), Fc do-

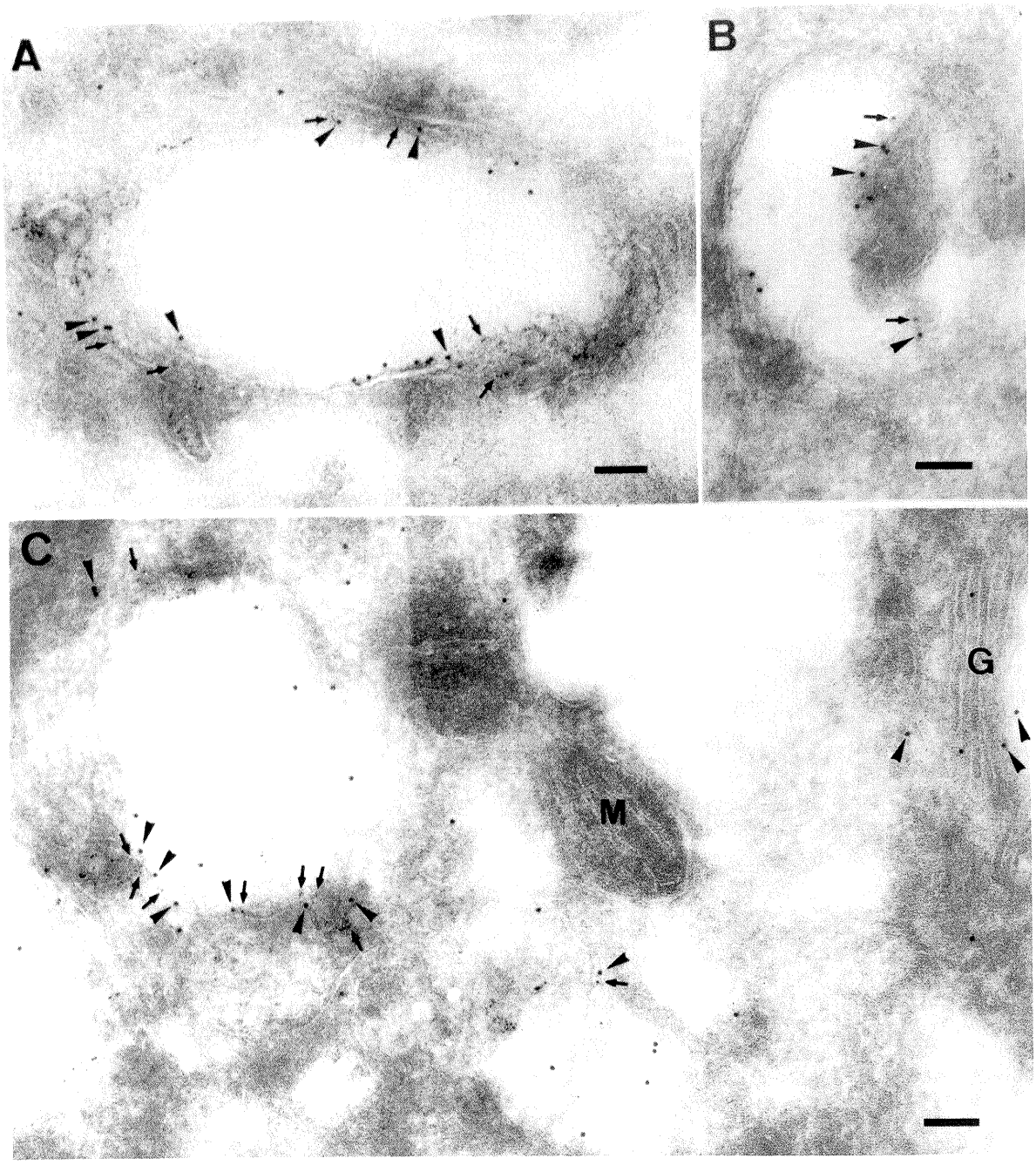

Figure 7. Immunoelectron Microscopy Localization of Ii and Δ11Ii in Transfected CV1 Cells

CV1 cells transfected with Ii and Δ11Ii were incubated with 5 nm BSA–gold for 1 hr to label the endosomal pathway. Sections were labeled with anti-IiC and 9 nm protein A–gold. In the micrographs the 5 nm gold endosomal marker is indicated by arrows, whereas the Ii and Δ11Ii (9 nm gold) are indicated by arrowheads. In cells transfected with Ii, structures that resemble early (A) and late (B) endosomes (Griffiths et al., 1990) are shown. (C) shows endosomes in a Δ11Ii transfected cell. Note that Golgi (G) is labeled with anti-IiC with no endocytosed marker, whereas mitochondria (M) are devoid of any label. Bar = 100 nm.

main of IgG (Miettinen et al., 1989), and transferrin (Rothenberger et al., 1987) have demonstrated that signals for rapid internalization are located within the cytoplasmic tail, and tyrosine residues play an important role (Davis et al., 1987; Jing et al., 1990). The cytoplasmic domain of the cation-independent mannose 6-phosphate receptor has been dissected into two signals, one for biosynthetic sorting of lysosomal enzymes and another for endocytosis (Lobel et al., 1989). ER retention signals have also been localized within the cytoplasmic domain of the adenoviral E3/19K glycoprotein (Pääbo et al., 1987; Nilsson et al., 1989); the signal here is localized within the six

extreme C-terminal amino acids and seems to be dependent on a lysine residue. Comparing the cytoplasmic portion of Ii with the proteins above, we find no tyrosine or any other obvious characteristics or elements of known signals.

It has been suggested that secreted proteins and membrane proteins follow a default pathway to the plasma membrane (Pfeffer and Rothman, 1987; Hiebert and Lamb, 1988). This would explain the transport of the adenoviral ER protein E3/19K to the plasma membrane after removal of the retention signal (Pääbo et al., 1987). Our data are compatible with such a model since deletions from the cytoplasmic tail of Ii resulted in plasma membrane localization of the truncated protein.

As a specific intracellular localization of a protein is essential for its function, one might expect the residues essential for sorting to be conserved between various species. The Ii cDNA sequences have been reported for three species: human (Long et al., 1983; Claesson et al., 1983), mouse (Singer et al., 1984), and rat (Henkes et al., 1988), and they display a high level of homology. Figure 8 shows the alignment of the amino acid sequences of the N-terminal parts of these proteins. The first 10 residues are identical (8 of these are also conserved in the chicken Ii [S. Ness, personal communication]), and one might suspect that such a conserved sequence would point to a crucial function within the Ii N-terminal tail. This region was, however, not essential for the intracellular sorting, as a protein lacking the first 11 residues was largely retained in intracellular compartments. Removal of four more residues did, on the other hand, result in a protein that was transported to the plasma membrane. These four amino acids (EQLP) are conserved between the three sequences but are not at all conserved in the chicken Ii (S. Ness, personal communication). From our data we can conclude that amino acids 12–15 are essential for retention of the molecule. The specific nature of the sorting signal remains to be determined.

Deletion of 15 or more amino acids from the N-terminus of Ii resulted in proteins that acquired complex-type oligosaccharides at a higher rate than Ii and Δ11Ii. Such a difference could be the result of a variety of effects: different stability at the plasma membrane as compared with endosomes, different exit rates from ER, different rates of passage through the sugar modifying compartments of the Golgi, or different degrees of post-Golgi modifications of the protein. Although we could measure a similar half-life for Ii and the mutants, the complexity of the kinetics does not allow us to distinguish between the different possibilities. The mutant proteins found on the plasma membrane do not, however, acquire complex oligosaccharides at the same rate as other plasma membrane proteins like the hemagglutinin virus protein (Copeland et al., 1986). In the case of Δ23Ii, only 45% of the protein was endo H resistant after 2 hr compared with 80% after 20 min for hemagglutinin. This may indicate that Δ23Ii carries additional information that causes ER delay. Although removal of 11 amino acids resulted in a protein that displayed many of the same characteristics as Ii, there were also differences. Ii was not found at the plasma membrane, whereas Δ11Ii could be detected at the plasma membrane in 22% of the transfected cells. This could indicate that the N-terminal 11 amino acids influence efficient sorting of Ii.

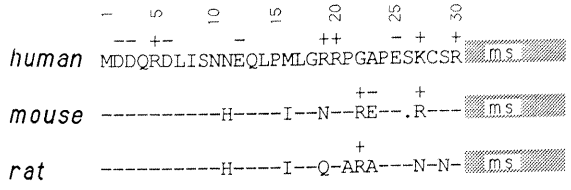

Figure 8. Comparison of the Cytoplasmic Tails of Ii from Human, Mouse, and Rat

N-terminal amino acid residues of the human Ii chain are compared with those of mouse and rat. Amino acids identical to the human sequence are indicated by dashes and gaps by a dot.

It has been proposed that sorting of the complex of Ii/class II molecules to an endosomal compartment is important for processing/presentation of some antigens by the class II molecules (Koch et al., 1989; Long, 1989). The endosomal compartment could be the site where Ii is released from the complex and where the class II molecules could bind to endocytosed and processed antigen. The class II molecules might then be transported to the plasma membrane, whereas Ii is retained intracellularly and is eventually degraded.

The class II molecules have been found to present mainly exogenous antigens to T cells and not antigens from the biosynthetic pathway (Babbit et al., 1985). The antigen binding groove of the class II molecules might be shielded until the class II molecules interact with endocytosed antigen. Ii is a likely candidate for such a function as it binds the class II molecules in the ER and is processed and released at a site where binding to exogenous peptide antigen could take place (Koch et al., 1989; Long, 1989). This model can now be tested by expressing class II molecules and mutated Ii that are transported to the cell surface. Ii and class II might still interact as they bind to each other within the luminal part of the proteins (Marks and Cresswell, 1986). The lack of intracellular retention might allow the complex to be transported to the plasma membrane via the constitutive transport route. It can then be tested whether Ii associated with class II molecules on the cell surface can prevent antigen presentation by the class II molecules.

Experimental Procedures

Expression Vector

pSV51 is a late replacement vector with a large polylinker behind the SV40 promotor (Huylebroeck et al., 1988). In pSVL the polylinker of pSV51 has been replaced by XbaI, SalI, SmaI, SacI, and BamHI sites (U. Hamann and K. Stanley, unpublished data). These vectors have been shown to give high transient expression of several proteins in simian cells (Huylebroeck et al., 1988).

Plasmid Constructions

pSVIi

The cDNA fragment coding for the 33 kd human Ii was isolated after SmaI–HincII digestion of plγ (Lipp and Dobberstein, 1986) and subcloned into the SmaI site of pGEM4 (Promega). A similar fragment was

thereafter isolated from this plasmid by EcoRI–BamHI, blunted with Klenow fragment, and cloned into the SmaI site of pSVL.

pΔ11li

A Sau3A–PstI fragment from plγ encoding the 72 N-terminal amino acids of li was subcloned into M13mp19. Around the nucleotides coding for the initiating methionine, a NcoI site was introduced by site-directed mutagenesis. In a second round of mutagenesis an additional NcoI site was introduced at amino acid 11, changing the nucleotides coding for an asparagine residue to methionine. The 33 bp NcoI fragment was then deleted. The li insert of the resulting plasmid was subcloned as a BamHI–PstI fragment together with the PstI fragment encoding the C-terminal part of plγ into pGEM3 (Promega). This construct was tested in a cell-free microsome-based translocation assay (Lipp and Dobberstein, 1986), and the resulting protein was found to maintain the membrane topology of li. Thereafter the construct was isolated by SmaI–SphI cleavage, blunted by polymerase, and subcloned into the SmaI site of pSVL. The site-directed mutagenesis was performed using a mutagenesis kit from Amersham.

pΔ15li

This construct was made from pSVIi by deleting a SalI–NarI fragment from the 5′ terminal end of li and replacing it with two annealed oligonucleotides encoding a protein starting with the methionine at position 16 of li.

pΔ20li and pΔ23li

These constructs were made by removing a SalI–SacII fragment encoding the 30 N-terminal amino acids of li and replacing this with annealed oligonucleotides coding for a methionine either at position 20 (pΔ20li) or position 23 (pΔ23li).

All constructs were verified by sequence analysis.

Cells and Cell Culture

The established CV1 cell line originates from African green monkey kidney (ATCC, number CCL 70). The Mel JuSo cell line is from a human malignant melanoma (Johnson et al., 1981) and was obtained from Dr. J. P. Johnson, University of Munich. The cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine calf serum (DMEM-FCS).

Antibodies

The polyclonal rabbit antibody, anti-liC, recognizes the lumenal C-terminal part of li, whereas anti-liN recognizes the N-terminal cytoplasmic segment as described previously (Lipp and Dobberstein, 1986). Clonab LN2 (Biotest AG, Federal Republic of Germany) is a mouse IgG1 monoclonal antibody that recognizes the C-terminal part of the human li (Wraight et al., 1990). Rabbit polyclonal antibody against the Golgi enzyme GalTf (anti-GalTf) was a gift from Dr Eric Berger, University of Zürich.

Transient Expression in CV1 Cells

The procedure for transfection of the CV1 cells was similar to the one used by Huylebroeck et al. (1988). In brief, 70% confluent monolayers of cells were trypsinized and seeded 1:4 in 35 mm tissue culture wells (Nunc) 1 day before transfection. Four micrograms of plasmid DNA was dissolved in 0.2 ml of Eagle's minimal essential medium with 25 mM HEPES-KOH (pH 7.2) (MEM-HEPES) and mixed with 0.2 ml of 1 mg/ml DEAE-dextran (M_r 50,000, Pharmacia) in MEM-HEPES. After 30 min the DNA was added (0.4 ml per 36 mm well) and incubated for 45 min at room temperature. The monolayers were washed in MEM-HEPES, and DMEM-FCS containing 0.1 mM chloroquine (Sigma) was added. After 4 hr in 5% CO_2 at 37°C the cells were washed in DMEM-FCS and exposed to 0.1 mM sodium butyrate (Merck) in DMEM-FCS overnight before fresh medium was added. The cells were again washed in DMEM-FCS and incubated (37°C, 10% CO_2) overnight before the cells were fixed for microscopy or labeled with [^{35}S]methionine.

Metabolic Labeling and Pulse Chase Protocol

Two days after the start of transfection the cells in 35 mm dishes were incubated in prewarmed (37°C) methionine-free DMEM (with 0.5% FCS) for 20 min. The cells were then incubated in 0.5 ml of the same medium containing 0.08 mCi of [^{35}S]methionine for 15 min or continuously labeled for 4 hr. After the 15 min pulse labeling the cells were washed and further incubated in prewarmed chase medium (DMEM-FCS with 20 mM cold methionine) for the time periods indicated in the figure legends. After labeling and chase the cells were placed on ice, the medium collected, and the cells were lysed with ice-cold lysis buffer (1% Triton X-100, 50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1 mM phenylmethylsulfonylfluoride [PMSF]) for 20 min. Samples were centrifuged (10000 × g) for 3 min at 4°C to remove cell nuclei. The supernatants were either used directly for immunoprecipitation or frozen at −80°C.

Immunoprecipitations and Endo H Treatment

Thirty-five microliters of a 1:1 slurry of protein A–Sepharose (Pharmacia) equilibrated in buffer A (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.2% NP40, 2 mM EDTA) was added to the supernatants, adsorbed for 1 hr at 4°C, and the beads were removed by centrifugation. Then 1 μl of anti-liC (or anti-liN) antibody was added to a fraction of the supernatant and incubated for at least 4 hr at 4°C. Twenty microliters of the protein A–Sepharose slurry was added followed by a further 1 hr incubation. The beads were then washed twice in buffer A, twice in buffer A containing 500 mM NaCl, and twice in 10 mM Tris–HCl (pH 7.5). Beads with bound antigen were thereafter incubated in 1 mU of endo H (Miles Laboratories) dissolved in 50 μl of 0.15 M sodium citrate (pH 5.5), 0.1 mM PMSF for 12 hr at 37°C. Proteins bound to the beads were analyzed by electrophoresis on a 10%–15% gradient polyacrylamide gel. Fluorography of the gels were performed using Entensify as directed by the manufacturer (New England Nuclear, Boston). The autoradiograms were scanned using a densitometer (Quick Scan R&D, Helena laboratories, Texas, USA), and the endo H–resistant fractions were determined by planimetry. In the pulse chase experiment both endo H–resistant bands were included in the endo H–resistant fraction.

Antibody Labeling of Cell Surface and Intracellular Antigens for Immunofluorescence Microscopy

Glass coverslips (1 × 1 cm) were placed in the tissue culture wells before the cells were seeded. To label surface antigens the cells on coverslips were incubated with anti-liC for 1 hr on ice, washed in PBS with 2% FCS (PBS-FCS), and thereafter incubated with Texas red–conjugated goat anti-rabbit IgG antibodies (Jackson Immuno Research) for 45 min on ice. The cells were then washed in PBS and fixed for 20 min at room temperature in 3% paraformaldehyde. Remaining paraformaldehyde activity was blocked by 0.1 M glycine for 5 min. To expose intracellular antigens the cells were extracted with 0.1% Triton X-100 for 5 min and washed in PBS-FCS. The opened cells were then incubated with LN2 for 30 min, washed in PBS-FCS, and incubated with FITC-linked anti-mouse IgG (Zymed) for 20 min. Thereafter, the cells were washed in PBS and mounted in Mowiol on glass slides. In the studies using both LN2 and anti-GalTf the cells were fixed in methanol at −20°C for 5 min and washed with PBS-FCS. Methanol fixation was used since anti-GalTf stained poorly after the paraformaldehyde/Triton X-100 treatment. The cells were incubated with the two antibodies for 45 min, washed in PBS-FCS, and thereafter incubated with the labeled anti-rabbit and anti-mouse Ig antibodies for 30 min before the cells were washed in PBS and mounted in Mowiol. Unless specified, the treatments were performed at room temperature. The cells were examined using a Leitz Orthoplan fluorescence photomicroscope equipped with a 63× objective and filters for Texas red and FITC.

Determination of Fraction of Cell Surface Labeled Cells

The percentage of transfected cells displaying surface labeling was determined by first identifying cells that showed intracellular expression of li and Δli proteins (FITC labeled). The optical filter was then changed to visualize Texas red–conjugated antibodies on the cell surface of the same cell. At least 200 cells were analyzed for each construct.

Incubation with Endocytic Markers and Immunoelectron Microscopy

To label endosomes, the transfected CV1 cells were incubated with 5 nm BSA–gold for 1 hr at 37°C (Griffiths et al., 1989). At the end of the incubations the cells were removed by protease K (50 μg/ml) at 0°C and fixed with 8% paraformaldehyde in 250 mM HEPES (pH 7.35). The cells were prepared for cryoelectron microscopy as described previously (Griffiths et al., 1984), and the sections were labeled with anti-liC

and 9 nm protein A–gold. The anti-IiC serum was used at a dilution that showed no cross-interaction with proteins in nontransfected cells.

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