

## The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail

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### SUMMARY

The oligomeric complex formed by major histocompatibility complex (MHC) class II  $\alpha$  and  $\beta$  chains and invariant chain (Ii) assembles in the endoplasmic reticulum and is then transported via the Golgi complex to compartments of the endocytic pathway. When Ii alone is expressed in CV1 cells it is sorted to endosomes. The Ii cytoplasmic tail has been found to be essential for targeting to these compartments. In order to characterize further the signals responsible for endosomal targeting, we have deleted various segments of the cytoplasmic tail. The Ii mutants were transiently expressed and the cellular location of the proteins was analyzed biochemically and morphologically. The cytoplasmic tail of Ii was found to contain two endosomal targeting sequences within its cytoplasmic tail; one targeting sequence was present within amino acid residues 12-29 and deletion of this segment revealed the presence of a second endosomal targeting sequence, located within the first 11 amino acid residues. The presence of a leucine-isoleucine

pair at positions 7 and 8 within this sequence was found to be essential for endosomal targeting.

In addition, the presence of this L-I motif lead to accumulation of Ii molecules in large endosomal vacuoles containing lysosomal marker proteins. Both wild type Ii and Ii mutant molecules containing only one endosomal targeting sequence were rapidly internalized from the plasma membrane.

When the Ii cytoplasmic tail was fused to the membrane-spanning region of neuraminidase, a resident plasma membrane protein, the resulting chimera (INA) was found in endocytic compartments containing lysosomal marker proteins. Thus the cytoplasmic tail of Ii is sufficient for targeting to the endocytic/lysosomal pathway.

Key words: cytoplasmic tail, endocytic pathway, invariant chain, endosomal targeting, MHC class II

### INTRODUCTION

MHC class II molecules are transmembrane proteins present at the plasma membrane. They consist of polymorphic  $\alpha$  and  $\beta$  chains, which are involved in the presentation of peptides, derived from exogenous antigens, to T helper cells (Unanue, 1984). Antigens are thought to be taken up by endocytosis, proteolytically processed and then bound to class II molecules in the endocytic pathway (Long and Jacobson, 1989; Koch et al., 1989).

Class II molecules assemble in the endoplasmic reticulum with a third polypeptide, the invariant chain (Ii) (Jones et al., 1978; Kvist et al., 1982), and are then transported to the endocytic pathway where Ii is proteolytically processed and dissociated from the class II molecules (Neeffjes et al., 1990; Pieters et al., 1991). The major form of Ii (P33) is a type II transmembrane protein of 216 amino acid residues

of which the 30 N-terminal residues are cytoplasmically exposed. In man, a 35 kDa form of Ii (P35), with a 15 amino acid residue extension at the N terminus is present, which results from initiation of translation at an AUG codon upstream of that used to generate the major translation form (O'Sullivan et al., 1987; Strubin et al., 1986). This N-terminal extension has been found to be responsible for retaining Ii in the endoplasmic reticulum (Lotteau et al., 1990). Both in mice and man an additional form of Ii (P41) arises through the use of an alternatively spliced exon (Strubin et al., 1986; Koch et al., 1987).

Ii has been proposed to perform two functions (Long and Jacobson, 1989; Koch et al., 1989). First, it prevents class II molecules from binding peptides prematurely in the ER (Roche and Cresswell, 1990; Teyton et al., 1990). Second, Ii targets the MHC class II complex to endosomes (Bakke and Dobberstein, 1990; Lotteau et al., 1990).

When the 33 kDa form of human Ii, P33, is expressed in CV1 cells in the absence of MHC class II molecules, it is found in organelles of the endocytic pathway. Very little Ii can be detected at the plasma membrane. Mutant Ii proteins from which 15 or more N-terminal amino acid residues are deleted accumulate at the plasma membrane when expressed in CV1 cells (Bakke and Dobberstein, 1990). In HeLa cells, expression of Ii mutant proteins from which the 29 N-terminal amino acid residues are deleted accumulate at the plasma membrane. From these findings it has been concluded that the cytoplasmic tail of Ii contains targeting information for the endocytic pathway (Bakke and Dobberstein, 1990; Lotteau et al., 1990).

The pathway by which Ii reaches endosomal compartments, when expressed in the absence of MHC class II molecules, is not known. Ii could follow the secretory route to the plasma membrane, and then be internalized into the endocytic pathway. Alternatively, Ii could be delivered directly from the *trans*-Golgi network (TGN) to the endosomes. Finally, it has been proposed that Ii may follow a route to endosomes bypassing the Golgi complex. This was concluded from the incomplete terminal glycosylation of Ii and its presence in endosomal/lysosomal structures (Lotteau et al., 1990).

Proteins that are known to be internalized from the plasma membrane, such as the LDL receptor (Goldstein et al., 1985), transferrin receptor (Collawn et al., 1990; Ktikakis et al., 1990) and the epidermal growth factor receptor (Prywes et al., 1986) contain a cytoplasmically located aromatic residue within a sequence that adopts a tight turn conformation (Bansal and Gierasch, 1991; Eberle et al., 1991). Ii does not contain any aromatic amino acid residue in its cytoplasmic tail.

Recently a second type of endosomal targeting signal has been identified. This signal centers around a di-leucine or a leucine-isoleucine motif (Letourneur and Klausner, 1992; Johnson and Kornfeld, 1992). This motif was shown to be responsible for endocytosis when transplanted onto the Tac antigen (Letourneur and Klausner, 1992). The cytoplasmic tail of the (cation-independent) mannose 6-phosphate receptor (CI-MPR) also contains a leucine-leucine pair that is involved in efficient Golgi sorting to the endosomal/lysosomal pathway (Johnson and Kornfeld, 1992).

The cytoplasmic tail of Ii can be replaced by that of -1,4-galactosyltransferase (GT), a *trans*-Golgi resident enzyme (Nilsson et al., 1991) without affecting the final location. The GT cytoplasmic tail contains a di-leucine motif implicated in endocytosis function. GT appears to contain at least two signals, one within the transmembrane segment that specifies location in *trans*-Golgi cisternae, and one in the cytoplasmic tail that retrieves GT from the plasma membrane.

To define more precisely the signals responsible for Ii targeting to and retention in endosomes, we deleted segments of the Ii cytoplasmic tail and determined the cellular location of the resulting mutant proteins. We found that two endosomal targeting signals are present within the Ii cytoplasmic tail, each of which, separately, was sufficient for endosomal targeting. Furthermore, Ii mutants that contained the first endosomal targeting signal, which we show

is a leucine-isoleucine pair, accumulated in large endosomal vacuoles.

To investigate whether the Ii cytoplasmic tail is sufficient to target a passenger protein to endosomes, we fused the invariant chain cytoplasmic tail to the transmembrane and luminal domain of influenza neuraminidase (NA), a resident plasma membrane molecule. We show that the Ii cytoplasmic tail was sufficient for targeting to the endocytic/lysosomal pathway. However, no accumulation of the chimeric INA molecule in large vacuoles was observed. This suggests that sequences either within the transmembrane and/or luminal domain of Ii also contribute to the precise endosomal location of Ii.

## MATERIALS AND METHODS

### Plasmid constructions

#### 12-15IipSV51L

12-15IipSV51L was constructed by amplifying a fragment in the 5' region of the coding sequence of the human Ii using the polymerase chain reaction (PCR) and C12IiCAT as a template (Lipp and Dobberstein, 1988). One of the primers that was used in the PCR reaction was identical to the coding bases 1-18 of Ii, and was extended at the 5' end to introduce *Nco*I, *Sma*I and *Xma*I sites (primer 1; 5'-CGCGCCCGGGCCATGGATGACCAGCGC-GAC-3'). The second primer was complementary to coding bases 12-61, excluding bases 34-45 (coding for amino acids E<sub>12</sub>QLP<sub>15</sub>), and was extended at the 5' end with a G+C-rich sequence (5'-GCGCGGCGCCGCCAGCATATTGTTGGAGATAAG-GTCGCC-3'). The amplified fragment was digested with *Xma*I and *Nar*I and ligated into plasmid IipSV51L (Bakke and Dobberstein, 1990), which had been digested with the same enzymes.

#### 21-29IipSV51L

21-29IipSV51L was constructed using a similar strategy as was used for 12-15IipSV51L, except that the second primer was complementary to coding bases 43-61 (coding for amino acids P<sub>15</sub>MLGRR<sub>20</sub>) of Ii, and contained a *Sac*II site at the 5' end (5'-GCGGCCGCGCGCCGCCAGCATGGG-3'). The amplified fragment was then digested with *Xma*I and *Sac*II and ligated into IipSV51L, which had been digested with the same enzymes.

#### 13IipSV51L

13IipSV51L was constructed using IipSV51L as a template for PCR. Primer 1 was as described above. The second primer coded for amino acids S<sub>9</sub>-R<sub>20</sub> excluding Q<sub>13</sub> (5'-GCGCGGCGCCGCC-CCAGCATGGGCAGCTCATTGTTGGA-3'). The amplified fragment was digested with *Xma*I and *Nar*I, and ligated into IipSV51L that was digested with the same enzymes.

#### 16-29IipSV51L

16-29IipSV51L was constructed by amplifying a fragment using 21-29IipSV51L as a template and primer 1. The second primer was complementary to coding bases 26-50 of 21-29Ii, excluding bases 46-54 (coding for M<sub>16</sub>LG<sub>18</sub>; 5'-GGGCTCCGCG-GCGCCGGGCGAGTTGCTCATTGTTGG-3'). The amplified fragment was digested with *Xma*I and *Sac*II and ligated into IipSV51L digested with the same enzymes.

#### 12-29IipSV51L

The construction of 12-29IipSV51L was similar to that of 16-29IipSV51L, except that as a second primer an oligonucleotide was used complementary to coding bases 13-70 of 21-29Ii,

excluding bases 34-54 (coding for E<sub>12</sub>QLPMLG<sub>18</sub>; 5'-GGGCTC-CGCGGGCGCCGATTGTTGGAGATAAGGTCGCG-3'). The resulting fragment was digested with *Xma*I and *Sac*II and ligated into lipSV51L digested with the same enzymes.

#### 12-29lipSV51L<sup>LI-AA</sup>

This construct was made from 12-29lipSV51L by exchanging a *Xma*I-*Sac*II fragment for two annealed oligos encoding the first 11 amino acids of Ii with the leucine and isoleucine at positions 7 and 8 changed to alanine.

#### NAPSV51L

The cDNA for neuraminidase from human influenza virus strain A7/Victoria/3/75 (van Rompuy et al., 1982) was excised from pSV23NA (Huylebroeck et al., 1988) as a *Hind*III fragment, blunted by DNA polymerase I and ligated into the *Sma*I site of pSV51L.

#### INAPSV51L

A *Sac*II site was introduced at the cytoplasmic side of the transmembrane region of neuraminidase and a *Bam*HI site was introduced downstream of the neuraminidase coding region by PCR with two primers that introduced these two sites. The *Sac*II-*Bam*HI fragment was isolated and ligated into lipSV51L that was digested with the same enzymes.

The constructs 1-11Ii and 1-15Ii have been described previously as 11Ii and 15Ii (Bakke and Dobberstein, 1990). All constructs were verified in the mutated region by sequencing as described by Sanger (Sanger et al., 1977). General molecular biology techniques were performed according to Sambrook et al. (1989).

#### Cells and cell culture

The cell line CV1 originates from African green monkey kidney and was obtained from ATCC (no. CCL 70). COS cells are derived from CV1 cells that were transformed by an origin-defective mutant of SV40, which codes for the wild-type T antigen (Gluzman, 1981). COS cells were obtained from ATCC (no. CRL 1650). The cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DMEM-FCS).

#### Antibodies

The polyclonal antisera against fusion proteins of  $\beta$ -galactosidase and parts of Ii were described before (Lipp and Dobberstein, 1986; Wraight et al., 1990; Pieters et al., 1991). Antisera recognizing an Ii N-terminal portion were raised against a fusion protein containing the N-terminal 73 amino acids (Ii<sub>1-73</sub>) of Ii and  $\beta$ -galactosidase (anti-IiN); antisera recognizing an Ii C-terminal portion were raised against a fusion protein containing amino acids 73-216 of Ii (Ii<sub>73-216</sub>) and  $\beta$ -galactosidase (anti-IiC); VIC Y1 (Quaranta et al., 1984) is a mouse monoclonal antibody that recognizes an epitope within the N-terminal, cytosolic, amino acids of Ii (Wraight et al., 1990; Bakke et al., unpublished), and was a kind gift from Dr W. Knapp, Vienna, Austria. Clonab LN2 (Biotest AG, FRG) is a mouse monoclonal antibody recognizing an epitope in the C-terminal portion of Ii (within amino acids 157-216; Wraight et al., 1990). Rabbit anti-human transferrin was purchased from Boehringer Mannheim Biochemicals. Rabbit polyclonal antiserum against the (cation-independent) mannose-6-phosphate receptor (CI-MPR) (Griffiths et al., 1988) was a gift from Dr Hoflack, EMBL, Heidelberg, FRG. Rabbit polyclonal antiserum against human lysosome-associated membrane protein (h-LAMP; Carlsson et al., 1988) was a kind gift from Dr Fukuda, La Jolla, CA, USA. Antibodies against neuraminidase from influenza strain A/Victoria/3/75) were a kind gift from Dr Douglas, NIMR, Mill Hill, London).

#### Transient transfection in CV1 and COS cells

CV1 or COS cells were transiently transfected using DEAE-dextran-mediated transfection (Huylebroeck et al., 1988) or by using the transfection reagent DOTAP (Boehringer Mannheim). Briefly, 80% confluent monolayers of cells were trypsinized and seeded in 5 cm tissue culture dishes (Falcon) 8-16 hours prior to transfection. Five  $\mu$ g of plasmid DNA was used per dish. For DEAE-dextran-mediated transfection, the plasmid DNA was dissolved in 1.5 ml of Eagle's minimal essential medium (MEM) containing 25 mM HEPES (pH 7.2) (MEM-HEPES) and mixed with 1.5 ml of 1 mg/ml DEAE-dextran ( $M_r$  50,000; Pharmacia) in MEM-HEPES. After 30 minutes incubation at room temperature, this mixture was added to the cells and incubated for 60 minutes at room temperature. The transfection medium was removed, the cells were washed with DMEM and incubated for 4 hours in DMEM-FCS containing 0.1 mM chloroquine (Sigma) in 5% CO<sub>2</sub> at 37°C. The cells were washed in DMEM-FCS, and incubated in DMEM-FCS containing 0.1 mM sodium butyrate (Merck). After 12 hours incubation in 10% CO<sub>2</sub> at 37°C the cells were washed and fresh DMEM-FCS was added. For DOTAP-mediated transfection, 5  $\mu$ g plasmid DNA was dissolved in 100  $\mu$ l HBS (20 mM HEPES, pH 7.4, 150 mM NaCl) and mixed with 100  $\mu$ l 0.3 mg/ml DOTAP in HBS. After 10 minutes at room temperature, 4 ml DMEM was added, and cells incubated in this mixture at 10% CO<sub>2</sub> and 37°C. After 4 hours, 1% FCS was added and the cells were incubated for an additional 8 hours before the transfection medium was removed and fresh DMEM-FCS was added.

#### Metabolic labelling

Cells were grown in tissue culture dishes. Prior to labelling the medium was replaced by methionine-free or methionine- and cysteine-free medium. After 40 minutes this medium was replaced by methionine-free or methionine- and cysteine-free medium containing 0.1 mCi/ml [<sup>35</sup>S]methionine (Amersham, United Kingdom) or Tran-<sup>35</sup>S-label (ICN Biomedicals, Irvine, CA, USA). After 20 minutes, the radioactive medium was removed, the cells were washed twice in RPMI-FCS containing 2 mM methionine or 2 mM methionine and 2 mM cysteine and incubated at 37°C in the same medium.

At the times indicated in the figure legends, the dishes were placed on ice, washed 3 $\times$  with ice-cold PBS and the cells were lysed in 20 mM Hepes, pH 7.5, containing 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100 and a cocktail of protease inhibitors consisting of 10  $\mu$ g/ml each of chymostatin, leupeptin, aprotinin, pepstatin and 20  $\mu$ M PMSF. After 15 minutes incubation on ice, the cell lysates were collected and centrifuged at 13,000 g for 15 minutes to remove cell debris.

#### Cell surface quantification

COS cells were grown in 10 cm dishes and transfected as described above. After 12 hours, cells were metabolically labelled for 12 hours using 0.1 mCi/ml Tran-<sup>35</sup>S-label. The cells were transferred to ice, washed with DMEM and incubated with anti-IiC in DMEM for 2 hours. Cells were then washed extensively with DMEM and PBS, and incubated for 15 minutes with an extract prepared from cells expressing Ii to block excess antibodies. Cells were then washed 4 $\times$  with PBS and PBS containing 5% FCS, followed by detergent lysis. From the lysates, immunocomplexes were either directly purified by the addition of Protein A-Sepharose beads (to precipitate cell surface Ii or Ii mutant proteins), or after the addition of anti-IiC antiserum (to precipitate total Ii or Ii mutant molecules). Immunoprecipitation and SDS-PAGE was carried out as described below.

#### Internalization

Internalization of anti-IiC antibodies was essentially performed as

described by Harter and Mellman (1992). Briefly, transfected COS cells were incubated with  $^{125}\text{I}$ -labeled anti-IiC antibodies for two hours at 4°C. Cells were washed extensively, and incubated in pre-warmed normal medium at 37°C for the times indicated. Cells were then cooled on ice and treated twice with 0.5 M acetic acid in 0.15 M NaCl (pH 2.5) for 5 minutes. Internalization was expressed as the fraction of antibody initially bound that was resistant to the low pH wash. Alternatively, the amount of anti-IiC antibodies that remained at the cell surface after the warm up period was quantified using FITC-conjugated second antibodies by FACSscan analysis. These two methods revealed quantitatively equivalent values.

### Immunoprecipitation and electrophoresis

Cell lysates from  $1 \times 10^6$  cells were incubated with antibody at 4°C for 12 hours, followed by the addition of 40  $\mu\text{l}$  Protein A-Sepharose (1:1 slurry) and further incubation for 2 hours. The beads were washed twice with 1 ml of low salt buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40 and 2 mM EDTA), twice with 1 ml of high salt buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% NP-40 and 2 mM EDTA), and twice with 1 ml of 10 mM Tris-HCl, pH 7.5. The antigens were eluted from the Protein A-Sepharose beads by the addition of sample buffer, denatured and subjected to SDS-PAGE (10–15%) according to Laemmli (1970), fluorography and autoradiography. For quantification, gels were exposed to a phosphorimager analysis screen and analyzed using the software supplied by the manufacturer.

### Endoglycosidase digestions

Antigens bound to 20  $\mu\text{l}$  of the Protein A-Sepharose beads (see above) were incubated with 5 mU endoglycosidase H (Boehringer Mannheim, Mannheim, FRG) in 100  $\mu\text{l}$  100 mM sodium acetate (pH 5.5), 9 mM  $\text{CaCl}_2$ , 150 mM NaCl containing protease inhibitors for 12 hours at 37°C. Thereafter, sample buffer was added to the beads and proteins were processed as described above.

### Immunofluorescence microscopy

Immunofluorescence labelling was essentially performed as described by Bakke and Dobberstein (1990). Briefly, viable cells grown on coverslips were incubated on ice with the appropriate antibody and a fluorescently labelled second antibody to label antigens on the plasma membrane. Then, the cells were fixed using 3% paraformaldehyde and permeabilized with Triton X-100. To label intracellular molecules, fixed and permeabilized cells were labelled using antibodies and fluorescein or Texas Red-conjugated second antibody as described in the figure legends. Alternatively, cells were fixed in methanol (4 minutes at  $-20^\circ\text{C}$ ). After labelling, the coverslips were mounted in Mowiol (Hoechst, FRG). Examination was performed using a Zeiss Axiophot fluorescence photomicroscope equipped with a  $\times 40$  objective and filters for fluorescein or Texas Red, or using a Bio-Rad MRC 600 CSLM.

## RESULTS

### Characterization of the compartments in which Ii accumulates when expressed in CV1 cells

Previously it has been shown that in transfected CV1 cells Ii can be localized in endosomes whereas very little Ii is found on the plasma membrane (Bakke and Dobberstein, 1990). Ten hours after transfection, Ii was seen in small vesicles in the perinuclear region (Bakke and Dobberstein, 1990; and Fig. 1A, panel A). After 20 and 30 hours Ii accu-

mulated in large vacuoles in all transfected cells (Fig. 1A, panels B and C). Accumulation of Ii in these vacuolar structures required the presence of amino acid residues 1–11 of the cytoplasmic tail, as Ii mutants lacking 11 or more residues from the amino terminus did not accumulate in these vacuoles but rather in small vesicular structures throughout the cell (Fig. 1A, panels D–F).

That the accumulation of Ii in large vacuoles is not an artefact of the expression system is further demonstrated by the fact that in cells expressing 1–11Ii vacuoles were never present, whereas in cells expressing Ii the large vacuoles could be detected using phase-contrast optics (Fig. 1B).

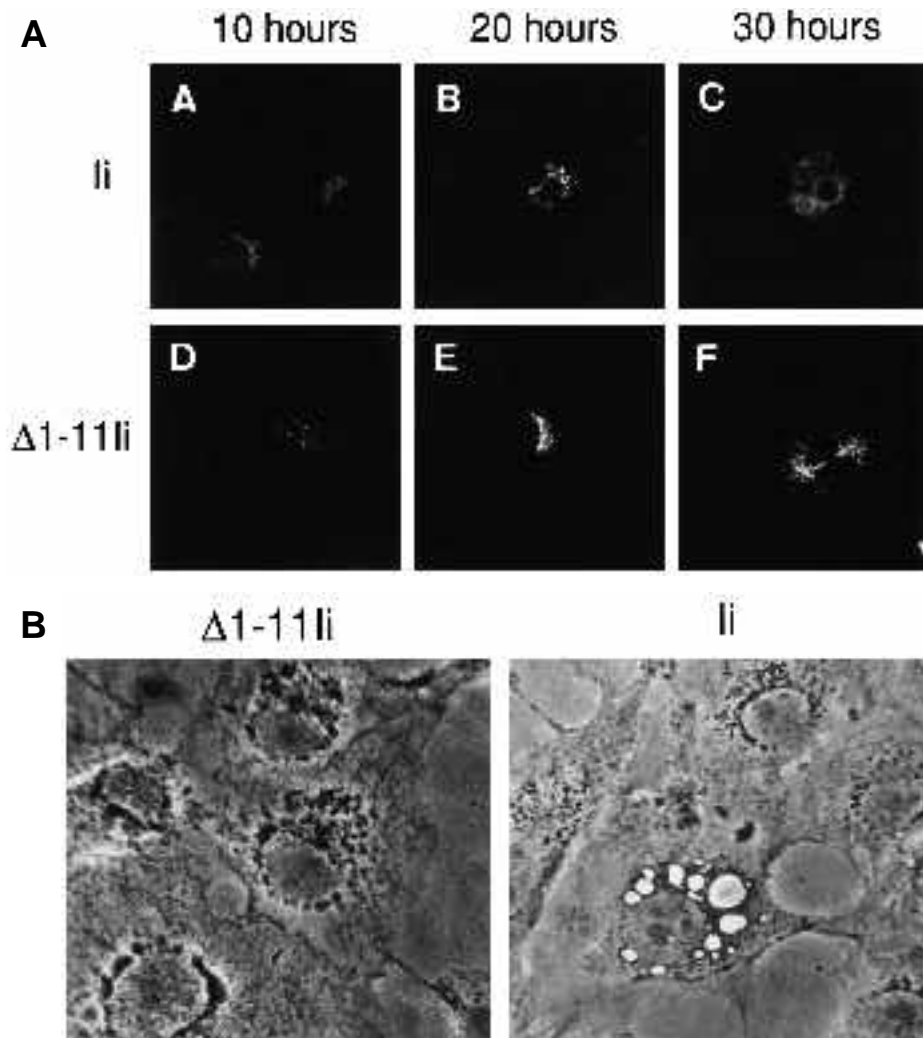
To identify these large vacuoles and to characterize further the site of Ii accumulation we double-labelled CV1 cells 30 hours after transfection with anti-Ii antibodies and antibodies against several markers for known intracellular compartments. For the endoplasmic reticulum (ER) we used a polyclonal antiserum raised against resident proteins of the rough endoplasmic reticulum (anti-RER antiserum; Louvard and Warren, 1982). As shown in Fig. 2A, panels A and B, the vacuoles, which were positive for Ii, were not labelled with the anti-RER antiserum. Ii also did not colocalize with protein disulfide isomerase (PDI; Freedman et al., 1984), another resident ER protein (Fig. 2A, panels C and D).

Glycoproteins acquire sialic acid in the *trans*-Golgi network (TGN). To label molecules that have been modified in the TGN we used wheat germ agglutinin (WGA) from *Triticum vulgare*. WGA is a lectin that binds to sialic acid and therefore labels proteins having reached or passed the TGN (Virtanen et al., 1980; Lippincott-Schwartz et al., 1989). As is shown in Fig. 2A, panels E and F, after blocking plasma membrane staining by preincubation of intact cells with unlabelled WGA, the Ii-positive large vacuolar structures can be labelled with FITC-WGA. This indicates that proteins resident in these structures have passed through the TGN.

In most cells the (cation-independent) mannose 6-phosphate receptor (CI-MPR) distributes in the TGN and late endosomes (Griffiths et al., 1988; Geuze et al., 1985; Geuze et al., 1988). As shown in Fig. 2B, panels A and B, Ii colocalized partially with the CI-MPR.

The Ii-positive vacuoles contained the lysosomal marker protein LAMP (lysosome-associated membrane protein; Carlsson et al., 1988) as is shown in Fig. 2B, panels C and D.

The large Ii-positive organelles can be reached by endocytic marker proteins. We used the iron-containing protein transferrin as a marker for endosomes. Iron-transferrin is internalized as a ligand of the transferrin receptor and transported to endocytic compartments where the iron is unloaded (Dautry-Versat et al., 1983). The complex of transferrin and receptor is then recycled to the cell surface. Transferrin and its receptor is widely used as a marker for endocytic compartments (Hopkins, 1983; Lippincott-Schwartz et al., 1991). CV1 cells expressing Ii were incubated for 2 hours with iron-saturated transferrin, fixed, permeabilized and double-labelled for immunofluorescence using anti-Ii antibodies and antibodies against transferrin. As is shown in Fig. 3A, the Ii-positive vacuoles were positive for transferrin.



**Fig. 1.** (A) Immunofluorescence analysis of the intracellular location of Ii (panels A-C) and  $\Delta 1-11$ Ii (panels D-F) in CV1 cells 10 (A, D), 20 (B, E) and 30 (C, F) hours after transfection. At the different times cells were fixed in paraformaldehyde, permeabilized using Triton X-100 and incubated with anti-Ii monoclonal antibody VIC Y1 or LN2. Antigens were visualized using FITC-conjugated second antibody. (B) Light micrograph using phase-contrast optics of transfected CV1 cells. Cells were grown on coverslips and transfected with Ii or  $\Delta 1-11$ Ii in pSV51L. After 30 hours, cells were analyzed morphologically using phase-contrast. Cells transfected with Ii contained large vacuolar structures (see right panel), whereas in cells transfected with  $\Delta 1-11$ Ii no large vacuoles could be detected (left panel).

The Ii-positive structures were also labelled with Texas Red-conjugated ovalbumin (OVA-TR) that was given to the cells for 45 minutes followed by a 45 minute chase in OVA-TR free medium (Fig. 3B).

Taken together, these results show that Ii in CV1 cells accumulated in vacuoles that were positive for marker proteins for endosomal and lysosomal compartments.

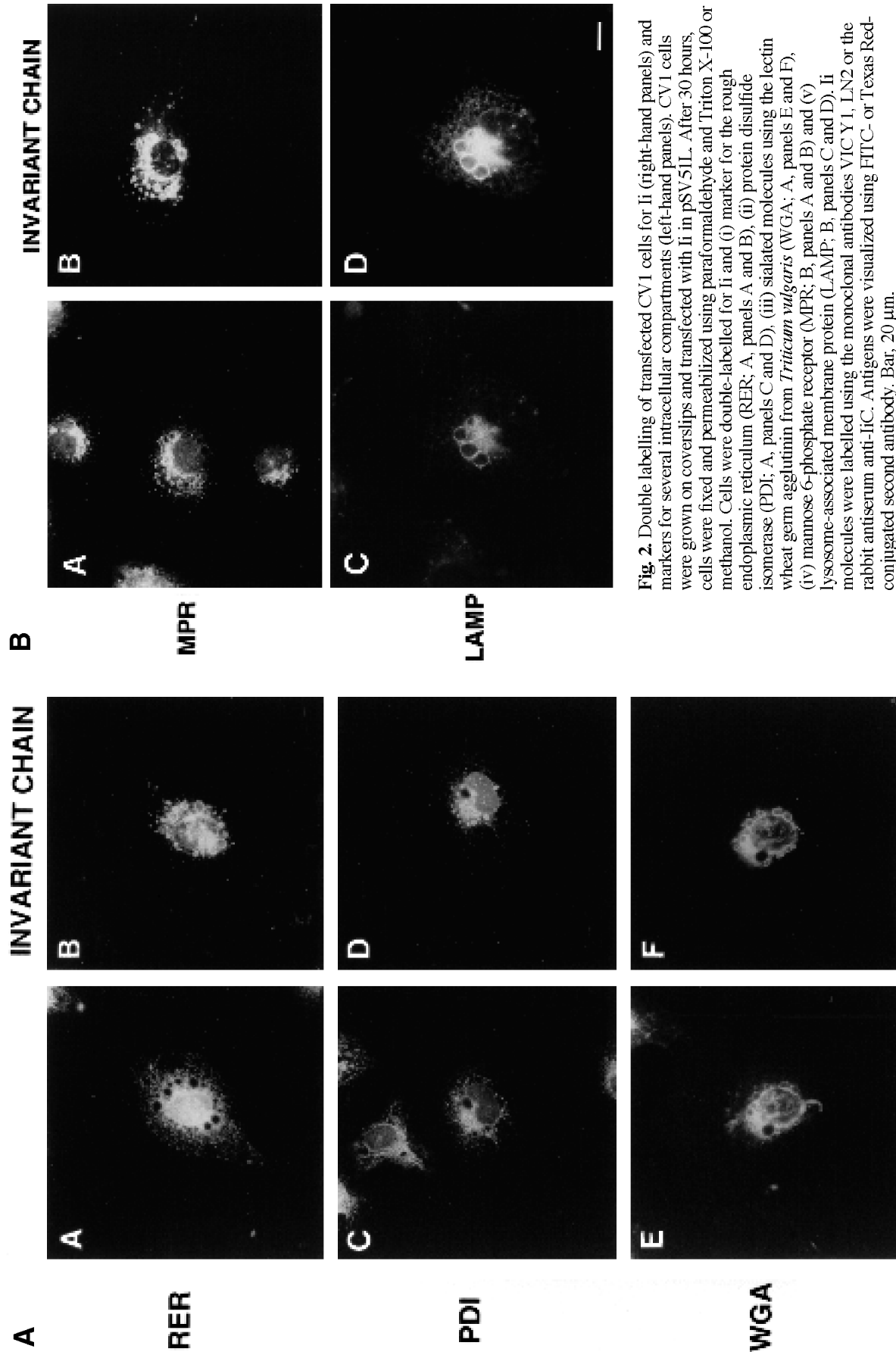
#### Ii deletion mutants

It has been shown previously that Ii and an Ii mutant in which the N-terminal 11 amino acid residues were deleted, is present in endocytic structures in CV1 cells. Deletion of 15 or more amino acid residues resulted in molecules that became resident at the plasma membrane (Bakke and Dobberstein, 1990). It was proposed that amino acids between residues 11 and 15 are important for targeting of Ii to endosomal compartments. To further characterize sorting information in the Ii cytoplasmic tail we made two types of deletions within the cytoplasmic tail (see Fig. 4); residues between amino acids 11 and 15 were deleted, as were increasing segments in the membrane proximal region.

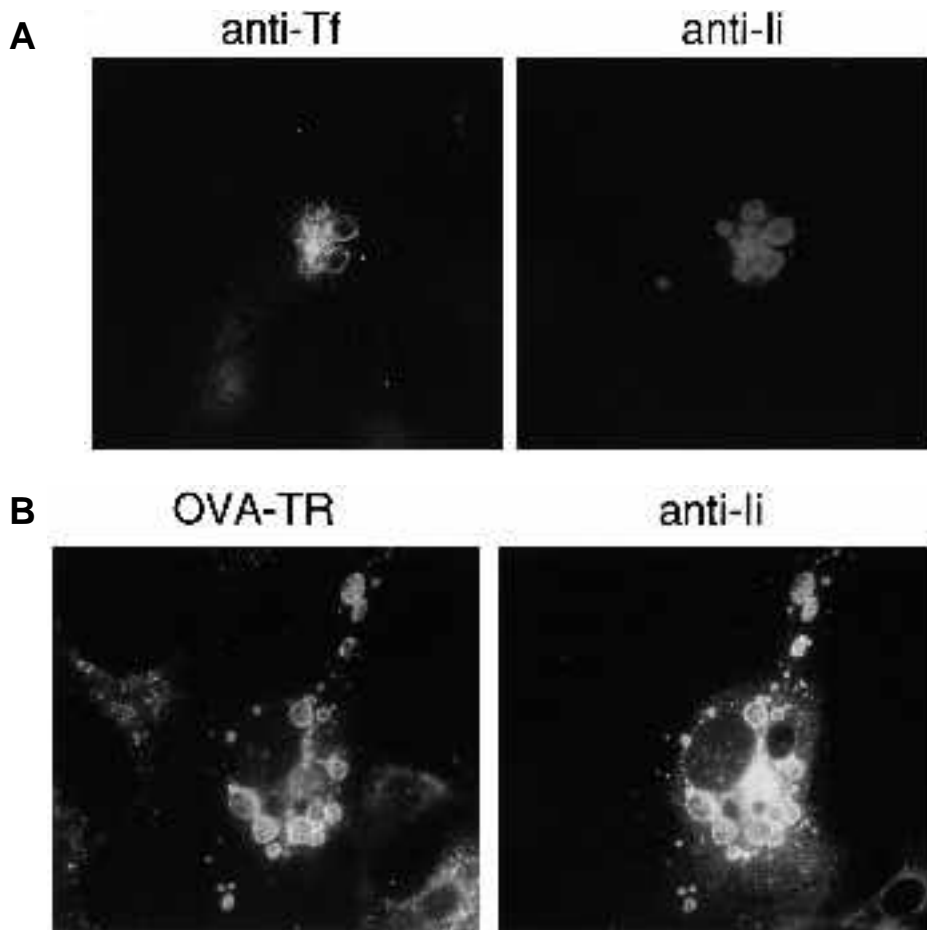
In two constructs ( $\Delta 12-15$ Ii and  $\Delta 13$ Ii) all four (E<sub>12</sub>QLP<sub>15</sub>) or only one (Q<sub>13</sub>) residue(s) of the sequence between residues 11 and 15 were deleted. Three other con-

structs ( $\Delta 21-29$ Ii,  $\Delta 16-29$ Ii and  $\Delta 12-29$ Ii) contained progressive deletions from the transmembrane region up to residue 11, in order to analyze the contribution of the membrane proximal region to the targeting function of the cytoplasmic tail.

Recombinant plasmids were transfected into CV1 cells and expression of the Ii mutants analyzed 20 hours after transfection. Newly synthesized proteins were metabolically labelled for 20 minutes using [<sup>35</sup>S]methionine, the cells lysed and antigens were immunoprecipitated using anti-IiC antiserum. Half of the immunoprecipitated material was digested with endoglycosidase H (endo H). After separation by SDS-PAGE and autoradiography in each case a single major polypeptide of the expected size was detected. Densitometric analysis of the autoradiographs revealed that all mutant proteins were expressed at equal levels. After endo H digestion, the molecular masses of Ii and all the mutant proteins were each reduced by about 6 kDa, indicative of the removal of two N-linked oligosaccharide side chains in each case (results not shown). As glycosylation sites on Ii are present on the luminal, C-terminal portion, this suggests that the Ii mutants have the same orientation across the membrane as Ii. To investigate the acquisition of complex-type carbohydrates on Ii mutant



**Fig. 2.** Double labelling of transfected CV1 cells for Ii (right-hand panels) and markers for several intracellular compartments (left-hand panels). CV1 cells were grown on coverslips and transfected with Ii in pSV51L. After 30 hours, cells were fixed and permeabilized using paraformaldehyde and Triton X-100 or methanol. Cells were double-labelled for Ii and (i) marker for the rough endoplasmic reticulum (RER; A, panels A and B), (ii) protein disulfide isomerase (PDI; A, panels C and D), (iii) sialated molecules using the lectin wheat germ agglutinin from *Triticum vulgare* (WGA; A, panels E and F), (iv) mannose 6-phosphate receptor (MPR; B, panels A and B) and (v) lysosome-associated membrane protein (LAMP; B, panels C and D). Ii molecules were labelled using the monoclonal antibodies VIC Y1, LN2 or the rabbit antiserum anti-IiC. Antigens were visualized using FITC- or Texas Red-conjugated second antibody. Bar, 20  $\mu$ m.



**Fig. 3.** Double labelling of transfected CV1 cells for Ii and markers for endocytic compartments. CV1 cells were grown on coverslips and transfected with Ii in pSV51L. (A) Cells expressing Ii for 30 hours were incubated in medium containing 50 µg/ml human transferrin (Tf) for 2 hours. After fixation and permeabilization antigens were labelled using rabbit anti-human transferrin antibody (anti-Tf) followed by Texas Red-conjugated second antibody, and anti-Ii mAb LN2 followed by FITC-conjugated second antibody. (B) Cells expressing Ii for 30 hours were incubated in medium containing 0.5 mg/ml OVA-TR for 45 minutes (left-hand panel), followed by a 45 minute chase in OVA-TR-free medium. After fixation and permeabilization antigens were labelled with the anti-Ii mAb LN2 followed by FITC-conjugated second antibody (right-hand panel).



**Fig. 4.** Outline of Ii and N-terminal, cytoplasmic amino acid sequences of the Ii deletion mutants. The construction of these mutants is described in Materials and Methods. The gray boxes indicate the transmembrane regions. Note the presence of two arginine residues in 16-29Ii and 12-29Ii that were introduced to ensure proper membrane insertion and orientation.

molecules, the above described labeling procedure was repeated, and the cells were chased for 2 hours in the presence of cold methionine before lysis. After immunoprecipitation of Ii molecules from the lysate and incubation of the immunoprecipitated material with endo H, the percentage of endoglycosidase H-resistant molecules was determined.

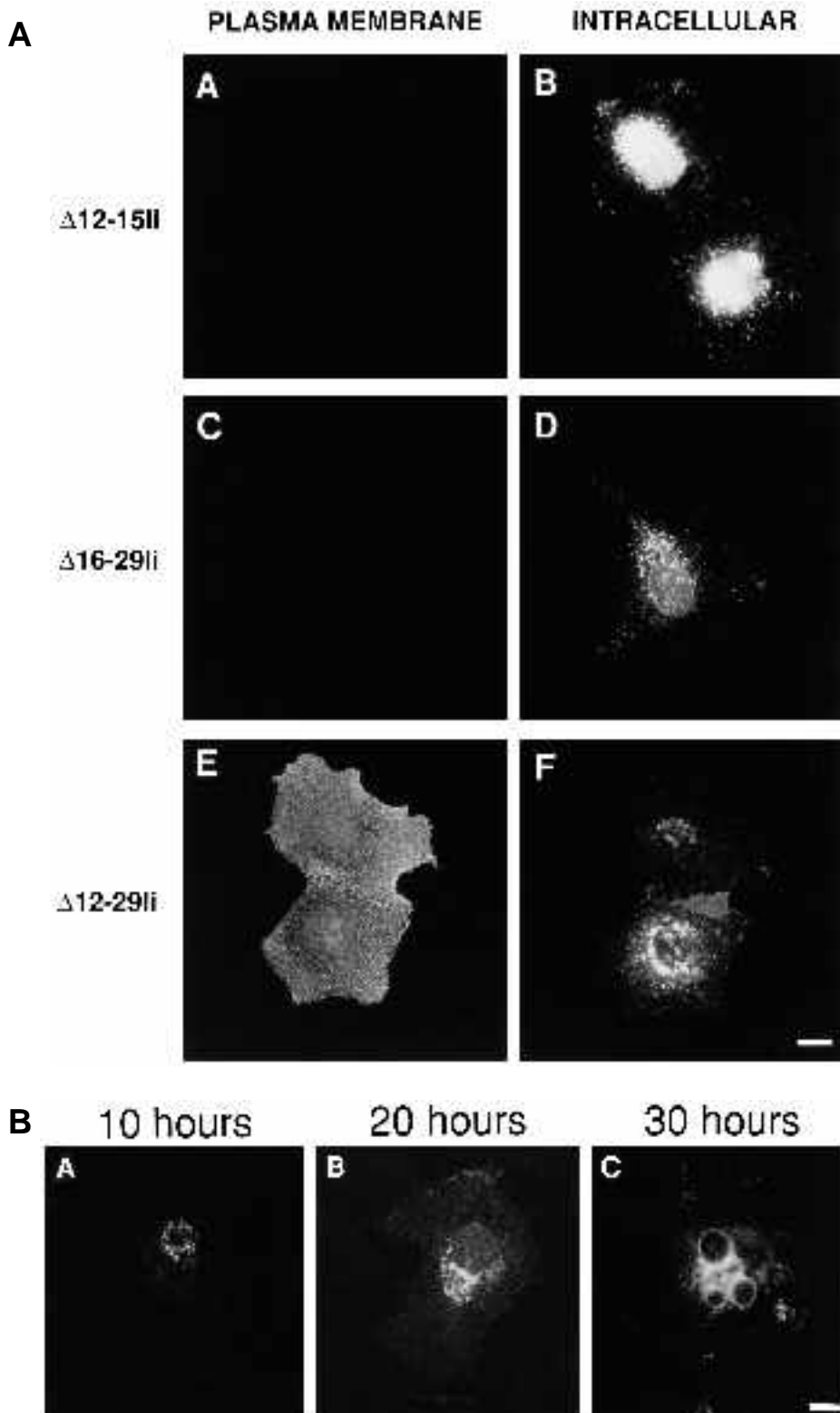
In all cases, 20-30% of the Ii mutant molecules were endo H resistant, indicating that the different Ii mutant molecules were transported at a similar rate.

#### Cellular localization of Ii mutant proteins

The location of the Ii mutants in transfected CV1 cells was analyzed by indirect immunofluorescence microscopy. Plasma membrane molecules were detected by incubation of cells on ice with rabbit anti-IiC antiserum followed by Texas Red-conjugated second antibody. To detect intracellular molecules, the same cells were fixed in paraformaldehyde, permeabilized with Triton X-100 and labelled with the monoclonal antibody LN2 followed by FITC-conjugated second antibody. LN2 recognizes a C-terminal epitope of Ii that is also recognized by anti-IiC (Wraight et al., 1990; Bakke and Dobberstein, 1990). Thus, Ii or Ii mutants localized at the plasma membrane will be labelled by anti-IiC only, not by LN2 (Bakke and Dobberstein, 1990).

12-15Ii, 13Ii, 21-29Ii and 16-29Ii were found largely in intracellular small vesicular structures throughout the cell (see Fig. 5A, A-D). In some cells low amounts of mutant proteins were present at the plasma membrane. After 30 hours of transfection, these mutant proteins accumulated in large endosomal vacuoles that labelled with the same marker proteins as the Ii-positive vacuoles (not shown).

A different phenotype was observed for mutant Ii pro-



**Fig. 5.** (A) Plasma membrane and intracellular localization of Ii mutant proteins in transfected CV1 cells. CV1 cells were grown on coverslips and transfected with the indicated constructs in pSV51L. After 20 hours, the cells were incubated on ice with anti-IiC antiserum and Texas Red-conjugated second antibody to label cell surface molecules only. After fixation with paraformaldehyde and permeabilization with Triton X-100 the intracellular antigens were labelled using the monoclonal anti-Ii antibody LN2 and FITC-conjugated second antibody. Panels A,C and E show plasma membrane labelling (Texas Red), and B, D and F show the same cells labelled with LN2 (FITC) after permeabilization. Bar, 20  $\mu$ m. (B) Intracellular localization of  $\Delta 12-29Ii$  analyzed after 10 (A), 20 (B) and 30 (C) hours of transfection. At the times indicated cells were incubated on ice with anti-IiC antiserum, fixed, permeabilized, and intracellular antigens were labelled using LN2 followed by FITC-conjugated second antibody. Bar, 15  $\mu$ m.

teins lacking amino acid residues 12-29.  $\Delta 12-29Ii$  was expressed in high amounts at the plasma membrane (Fig. 5A, panel E). In addition, this protein was located in a punctate, vesicular staining pattern throughout the cytoplasm (Fig. 5A, panel F). When the cellular location of  $\Delta 12-29Ii$  was analyzed after different times of transfection, it was

found to accumulate in large vacuolar structures (Fig. 5B, panels A-C).

Thus, the presence of the first 11 amino acid residues of the Ii cytoplasmic tail are sufficient to target the Ii mutant molecules to similar vacuolar structures to those in which wild type Ii is accumulated.



### Quantification of the cellular location

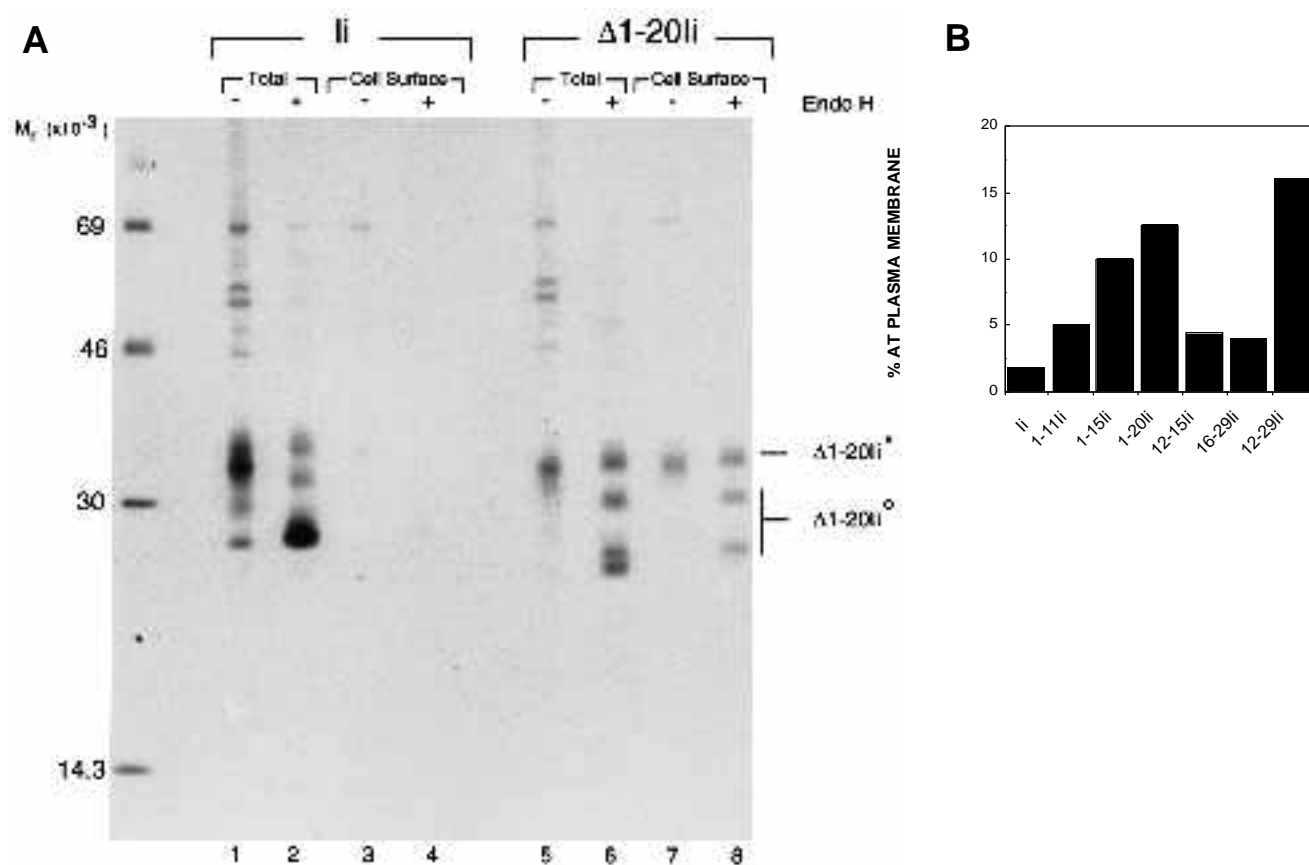
To quantify the surface expression of Ii and Ii mutant proteins we determined the percentage of cell surface expression of the different mutant proteins. For this part of the study, we made use of COS cells, a cell line that is derived from CV1 cells (Gluzman, 1981). The fraction of cells expressing mutant proteins was 40-50% for COS cells, compared to 5-10% for CV1 cells, possibly because COS cells are transformed with wild-type T antigen (Gluzman, 1981). The cellular locations of Ii and mutant proteins expressed in COS cells were identical to their location in CV1 cells. The morphology of COS cells we found to be less regular than the morphology of CV1 cells. Therefore, for the immunolocalization studies we used CV1 cells.

COS cells expressing Ii and Ii mutant proteins for 12 hours were metabolically labelled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 12 hours. Immunodetection of cell surface molecules was carried out by incubating the cells on ice with anti-IiC antiserum. Excess antibody was quenched by adding an extract prepared from cells expressing Ii. After lysis, the lysate was divided into 2 parts; from one, anti-

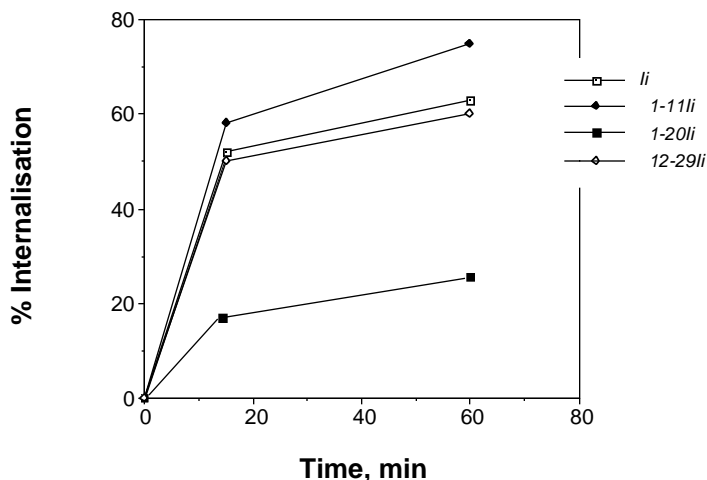
body-bound surface molecules were recovered by binding to Protein A-Sepharose (4/5th of the lysate). From the other, total Ii or mutant Ii molecules were recovered after incubation with anti-IiC antiserum and Protein A-Sepharose (1/5th of the lysate). Each immunoprecipitated sample was incubated in the presence or absence of endoglycosidase H, and polypeptides were separated by SDS-PAGE and visualized by autoradiography.

Fig. 6A shows the results for Ii and 1-20Ii. The amount of Ii present on the cell surface of Ii-transfected cells was found to be very low (Fig. 6A, lanes 1-4). In contrast, a considerable amount of 1-20Ii was found at the cell surface of transfected cells (Fig. 6A, lane 5-8).

1-20Ii proteins at the cell surface were found to contain carbohydrate side chains sensitive to endo H digestion. Three distinct bands were found after such treatment (Fig. 6A, lane 8); 1-20Ii proteins containing no (lower band), one (middle band) or two complex-type carbohydrates (upper band). Modification of carbohydrate moieties on polypeptides from the high-mannose form to the complex type takes place in the Golgi complex, and therefore is



**Fig. 6.** Quantification of Ii and Ii mutant molecules present at the plasma membrane of transfected COS cells. COS cells were transfected with Ii and Ii mutants in pSV51L as indicated. Twelve hours after transfection, cells were metabolically labelled with Tran-<sup>35</sup>S-label, and antigens were immunoprecipitated from the cell surface and from total lysates using anti-IiC antiserum as described in Materials and Methods. (A) Immunoprecipitation of total and cell surface localized Ii or 1-20Ii molecules from transfected COS cells. One half of the immunoprecipitated material was treated with endoglycosidase H (Endo H) before SDS-PAGE and autoradiography. Note the presence of both complex-type (\*) and high-mannose (o) carbohydrate forms of 1-20Ii at the cell surface. (B) Quantification of Ii and Ii mutant molecules localized at the cell surface. For quantification, gels were exposed to a phosphoimager screen and the percentage of cell surface immunoprecipitated molecules versus total was determined. Results represent the mean of three independent experiments ( $\pm$  5% of the values shown).



**Fig. 7.** Internalization of Ii and Ii deletion mutants in transfected COS cells. COS cells were transfected with Ii and Ii mutants in pSV51L as indicated. After 20 hours, cells were incubated on ice with  $^{125}\text{I}$ -labelled anti-IiC for 2 hours, washed and incubated at  $37^\circ\text{C}$  for the times indicated. At each time point, surface-bound antibodies were removed by low pH wash. Internalization is expressed as percentage of initially bound  $^{125}\text{I}$ -labelled anti-IiC present at the cell surface at each time point. Data points represent determinations in duplicate that differed by less than 10%.

usually taken as a marker for passage through the Golgi complex (Kornfeld and Kornfeld, 1985). Therefore, part of the mutant Ii molecules may have bypassed the TGN en route to the plasma membrane. We cannot, however, exclude the possibility that incomplete modification of the carbohydrate side chains may be due to the high expression of the Ii mutants in COS cells, or their inaccessibility to the carbohydrate processing enzymes.

To quantify the amount of Ii and Ii mutant proteins at the cell surface, the above described procedure was repeated for Ii, 1-11Ii, 1-15Ii, 1-20Ii, 12-15Ii, 16-29Ii and 12-29Ii. After exposure of the gel to a phosphorimager

screen, the percentage of molecules present at the plasma membrane relative to the total amount was calculated, and the result is presented in Fig. 6B. Ii was present in low amounts at the surface (2% of total). Ii mutants 1-11Ii, 12-15Ii and 16-29Ii were present in low, but slightly higher amounts than Ii at the plasma membrane (4-7% of total). High plasma membrane expression was found for 1-15Ii, 1-20Ii and 12-29Ii (10-16% of total).

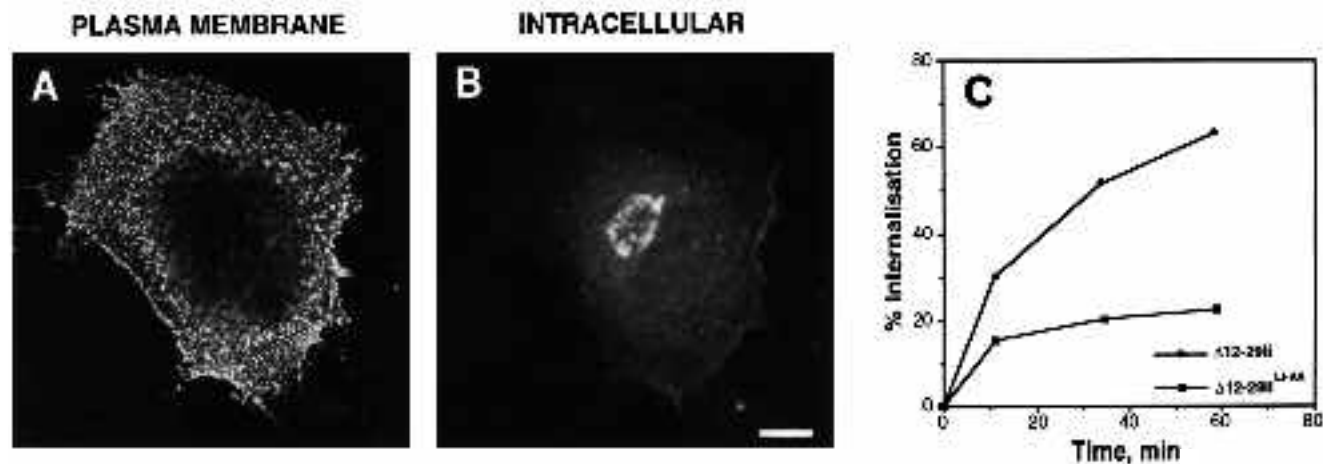
#### Internalization of Ii and Ii mutant molecules

Targeting of Ii to endosomes may occur directly intracellularly or by internalization from the plasma membrane. As some of the wild-type Ii and the Ii cytoplasmic deletion mutants were detected at the plasma membrane (Fig. 6B), we investigated whether Ii was internalized from the plasma membrane. Internalization was monitored after binding of  $^{125}\text{I}$ -labelled anti-IiC antibodies at  $4^\circ\text{C}$  to the surface of transfected COS cells expressing Ii and mutant Ii molecules (Fig. 7). Ii was found to be rapidly internalized from the cell surface, in contrast to 1-20Ii. The cytoplasmic deletion mutants 1-11Ii and 12-29Ii were both internalized from the cell surface with an efficiency similar to that of wild-type Ii (Fig. 7).

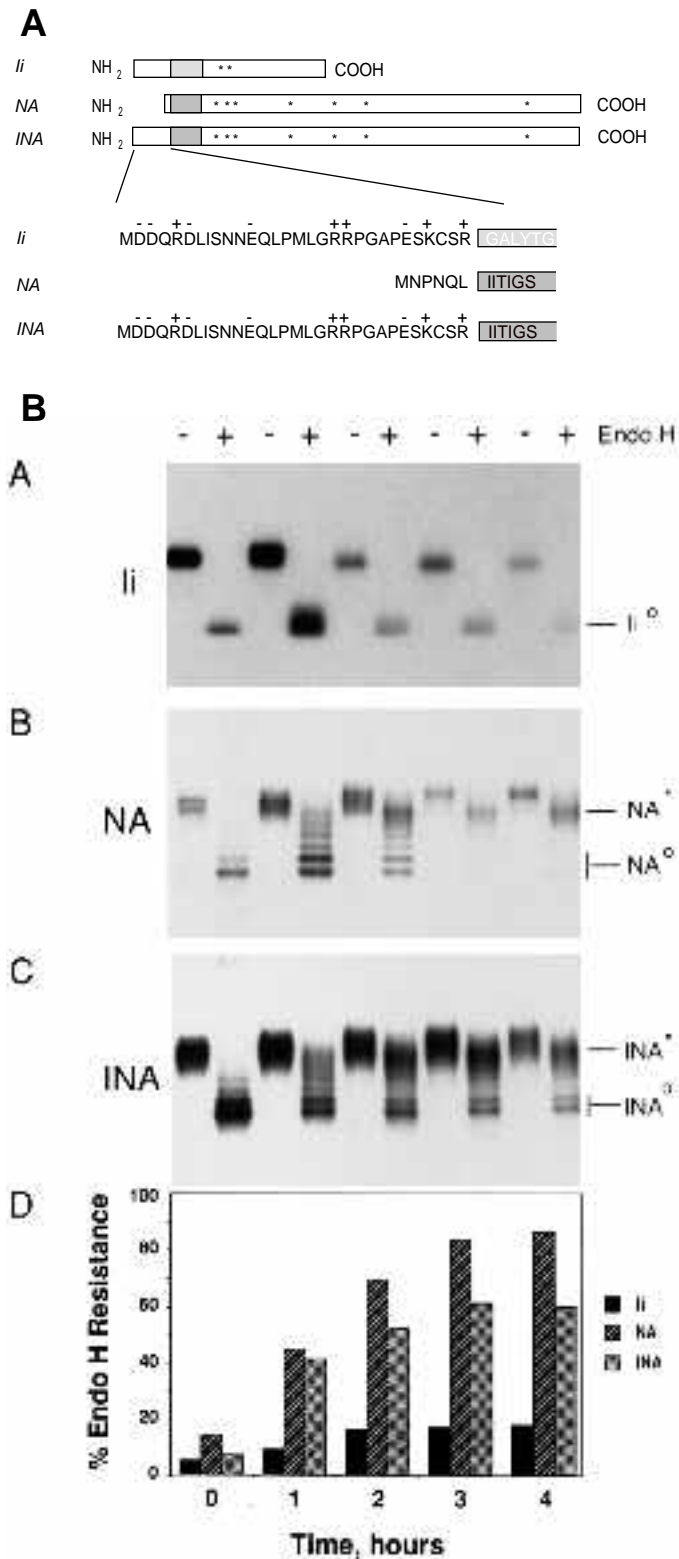
#### Identification of a leucine-isoleucine pair as an endosomal targeting signal in the Ii cytoplasmic tail

The first 11 amino acid residues of the Ii cytoplasmic tail are sufficient for Ii localization in endocytic structures (Fig. 5), function as an endocytosis signal (Fig. 7), and are also responsible for the accumulation of Ii molecules in large vacuolar structures (Fig. 5B). This sequence contains a leucine-isoleucine pair at position 7 and 8 that has been implicated both in internalization (Letourneur and Klausner, 1992) and in sorting from the Golgi to endosomes/lysosomes (Johnson and Kornfeld, 1992).

To investigate the contribution of the leucine-isoleucine motif in endosomal targeting of Ii directly, these two amino acid residues were changed to alanine in the mutant 12-



**Fig. 8.** (A,B) Plasma membrane and intracellular localization of  $12\text{-}29\text{Ii}^{\text{LI-AA}}$ . CV1 cells were grown on coverslips and transfected with  $12\text{-}29\text{Ii}^{\text{LI-AA}}$  in pSV51L. Plasma membrane (A) and intracellular labeling (B) was performed as described in the legend to Fig. 5. Bar, 20  $\mu\text{m}$ . (C) Internalization of 12-29Ii and  $12\text{-}29\text{Ii}^{\text{LI-AA}}$  in transfected COS cells (see legend to Fig. 7). Internalization is expressed as the percentage of anti-IiC antibodies present at the cell surface at each time point as determined using FITC-conjugated second antibody. Shown is the mean ( $\pm 10\%$ ) of duplicate experiments.



29Ii. The resulting mutant 12-29Ii<sup>LI-AA</sup> was transfected into CV1 cells and the cellular localization was analyzed 30 hours after transfection by indirect immunofluorescence microscopy. As shown in Fig. 8, panels A and B, this mutant protein is present in high amounts at the plasma membrane, and intracellularly its localization is confined to

**Fig. 9.** (A) Outline of Ii, neuraminidase (NA) and the Ii-NA hybrid molecules INA. The transmembrane regions are indicated by gray boxes. Glycosylation sites for N-linked carbohydrate side chains are indicated by asterisks. Sequences of the Ii, NA and INA cytoplasmic tails are shown in the lower half. (B) Pulse-chase analysis of newly synthesized invariant chain (Ii), neuraminidase (NA) and the Ii-NA chimaeric protein INA in COS cells. COS cells were transfected with Ii, NA or INA. After 20 hours, newly synthesized proteins were labelled with Tran-<sup>35</sup>S-label for 20 minutes and chased for the times indicated at the bottom of panel D. Cells were lysed and proteins immunoprecipitated using anti-Ii mAb VIC Y1 (panel A), or anti-neuraminidase mAb NC75 (panels B and C). One half of the immunoprecipitated material was treated with endoglycosidase H before SDS-PAGE and autoradiography. Asterisks, complex-type; and o, high-mannose carbohydrate forms. For quantification, the gels were exposed to a phosphorimager screen and the percentage of endo H-resistant proteins determined (panel D).

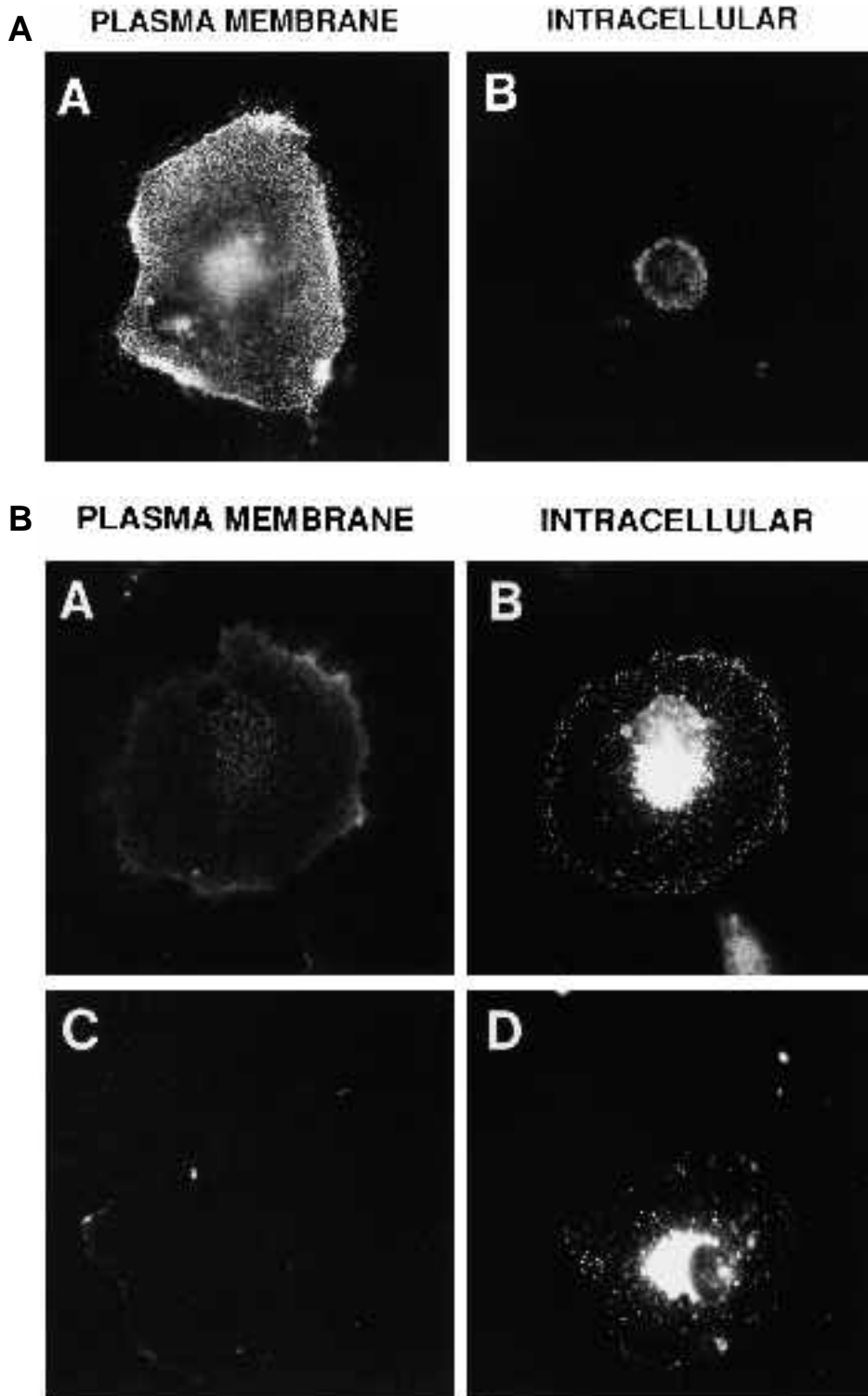
the Golgi region. No accumulation in intracellular vesicles or vacuolar structures could be detected. Internalization of anti-IiC antibodies was monitored after binding at 4°C to the surface of cells expressing either 12-29Ii or 12-29Ii<sup>LI-AA</sup>. As shown in Fig. 8, panel C, the internalization of 12-29Ii<sup>LI-AA</sup> dropped to the same levels as for the 1-20Ii mutant molecules. In conclusion, these results show that the leucine-isoleucine pair at position 7-8 of the Ii cytoplasmic tail is essential for internalization from the cell surface and for targeting of Ii molecules to endocytic vacuoles.

#### Hybrid Ii-neuraminidase (INA)

To investigate whether the Ii cytoplasmic tail is sufficient for targeting to endosomes, it was fused to the transmembrane and luminal portion of the influenza neuraminidase molecule (NA) (van Rompuy et al., 1982). Neuraminidase is a type II membrane protein expressed at the surface of cells infected with influenza virus (Sivasubramanian and Nayak, 1987). As outlined in Fig. 9A, cDNAs coding for neuraminidase and for a hybrid Ii-neuraminidase (INA) molecule, were cloned into the eukaryotic expression vector pSV51L.

The synthesis and acquisition of complex-type carbohydrate side chains of Ii, NA and INA was analyzed in transfected COS cells. Newly synthesized proteins were labelled 24 hours after transfection for 20 minutes using Tran-<sup>35</sup>S-label and chased for 1, 2, 3 and 4 hours. Cells were lysed and proteins were immunoprecipitated with the monoclonal anti-Ii antibody VIC-Y1 or anti-neuraminidase antibody NC75. To determine complex-type carbohydrate addition half of the immunoprecipitated material was digested with endoglycosidase H. The immunoprecipitated material was subjected to SDS-PAGE and autoradiography. The results are presented in Fig. 9B, panels A-C. The percentage of endoglycosidase H-resistant molecules at each time point is shown in Fig. 9B, panel D.

As judged by the resistance to endo H digestion, about 20% of Ii molecules had acquired complex-type carbohydrate after 4 hours chase (Fig. 9B, panels A and D). In contrast, nearly 80% of NA molecules were converted to the complex type already after 3 hours chase (Fig. 9B, panels B and D). For INA about 60% of the molecules were converted to the complex type within 4 hours (Fig. 9B, panels



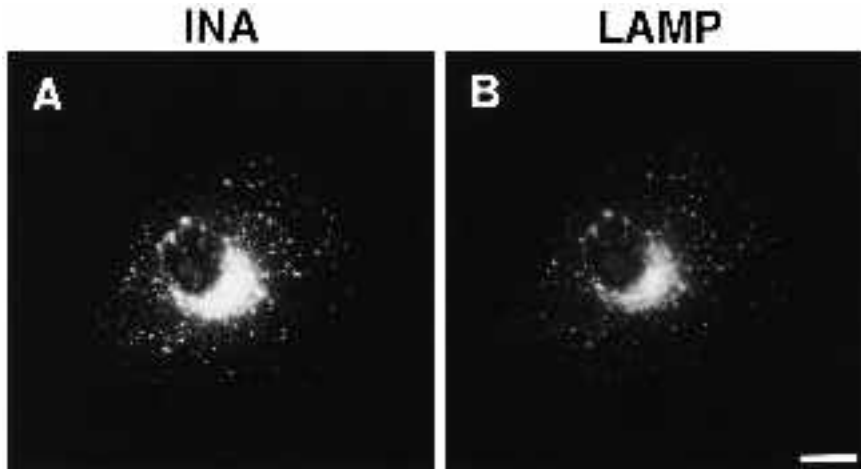
**Fig. 10.** Localization of neuraminidase (NA) and the invariant chain-neuraminidase fusion protein (INA) in CV1 cells. Cells were grown on coverslips and transfected with plasmids encoding NA or INA. (A) Cells expressing NA were labelled on ice with a mixture of polyclonal anti-NA antisera followed by Texas Red-conjugated second antibody (plasma membrane, panel A). After paraformaldehyde fixation and permeabilization with Triton X-100 the cells were labelled using monoclonal anti-NA antibody NC75, followed by FITC-conjugated second antibody (intracellular, panel B). (B) Cells expressing INA were labelled on ice with a mixture of polyclonal anti-neuraminidase antisera followed by Texas Red-conjugated second antibody (plasma membrane, panel A and C). After paraformaldehyde fixation and permeabilization with Triton X-100 the cells were labelled using monoclonal anti-Ii (VIC-Y1, panel B) or monoclonal anti-NA antibody (NC75, panel D) followed by FITC-conjugated second antibody.

C and D). As addition of complex-type carbohydrate occurs in the Golgi we conclude that transport of both NA and INA from the ER to Golgi compartments was considerably faster than that of Ii.

#### Cellular location of neuraminidase and INA

The intracellular location of NA and INA was analyzed by indirect immunofluorescence. Cells were grown on cover-

slips and transfected with recombinant plasmids encoding NA or INA. After 20 hours the cells were incubated on ice with rabbit anti-NA antiserum followed by Texas Red-conjugated second antibody to label plasma membrane molecules. After fixation of the cells in paraformaldehyde and permeabilization with Triton X-100, intracellular molecules were labelled using anti-NA or anti-Ii monoclonal antibodies and FITC-conjugated second antibody. The results are



**Fig. 11.** Double labelling of transfected CV1 cells for INA and LAMP. Cells expressing INA were grown on coverslips, fixed and permeabilized. Cells were incubated with anti-Ii mAb VIC Y1 and rabbit anti-LAMP antiserum followed by FITC-conjugated goat anti-mouse and Texas Red-conjugated goat anti-rabbit second antibody. Bar, 20  $\mu$ m.

depicted in Fig. 10A and B. NA is expressed strongly at the plasma membrane, and intracellularly is found in the Golgi area (Fig. 10A, panels A and B). INA, in contrast, shows a weak plasma membrane expression (Fig. 10B, panels A and C), and a strong intracellular expression in a punctate pattern throughout the cytoplasm (Fig. 10B, panels B and D).

Note that expression of INA did not result in formation of large vacuolar structures (Fig. 1), even when INA transfected cells were analyzed 30 hours after transfection (results not shown).

The INA-positive structures contained lysosomal marker proteins. Fig. 11 shows that INA colocalized with lysosome-associated membrane protein (LAMP). We therefore conclude that INA is located in endosomal structures that are related to lysosomes.

## DISCUSSION

### Ii accumulates in large endosomal structures

Ii, when expressed in large amounts in CV1 cells, accumulated in large vacuolar structures. These Ii-positive vacuoles were not formed when cells were transfected with the vector alone; formation of the vacuoles was induced by the high expression of Ii. The Ii-positive vacuoles contained marker proteins for the *trans*-Golgi network, endosomes and lysosomes. In addition, they could be reached by internalized transferrin and by Texas Red-coupled ovalbumin. We conclude that Ii is targeted to and retained in the endocytic pathway, thereby creating large vacuolar structures.

Formation of Ii-positive large vacuoles required the presence of the N-terminal 11 amino acid residues. Deletion mutants lacking 11 amino acid residues from the N-terminus were only found in small vesicles even 30 hours after transfection. In cells expressing Ii mutant proteins that contained only the first 11 amino acid residues of the cytoplasmic tail (1-29Ii) these mutant proteins accumulated in large vacuoles. Within the 11 N-terminal amino acid residues of Ii a leucine-isoleucine pair is found, which could be part of an endocytosis signal. Such a motif has been found in the cytoplasmic tail of the T-cell antigen receptor and chains to mediate endocytosis (Letourneur and

Klausner, 1992), and in the cytoplasmic tail of the CI-MPR to mediate sorting to endosomes in the TGN. When this leucine-isoleucine pair was changed to alanine-alanine, accumulation in vacuoles was completely abolished.

Also in HeLa cells large Ii-positive vacuolar structures have been observed after transient expression of Ii and were proposed to be autophagosomes (Lotteau et al., 1990). Autophagic vacuoles have been described to originate from specialized regions in the endoplasmic reticulum, and to mature into degradative vacuoles containing lysosomal marker proteins (Dunn, 1990a,b). Autophagic markers could be colocalized with endosomal markers, CI-MPR and lysosomal markers (Tooze et al., 1990; Rabinowitz et al., 1992). We cannot exclude the possibility that the Ii-positive large vacuoles are autophagosomes, as was suggested earlier (Lotteau et al., 1990).

Ii or Ii mutant proteins present at the plasma membrane were found to contain, besides complex-type, high-mannose carbohydrate moieties. Complex-type sugar addition may be incomplete; it may however be possible that part of the Ii molecules are not transported through the Golgi but follow an alternative route to endosomes (Lotteau et al., 1990).

The chimaeric protein INA, which is almost completely converted to the complex-type carbohydrate form was never found to accumulate in large vacuoles. This indicates that sequences located either within the transmembrane region and/or the luminal region may contribute to Ii accumulation in vacuoles.

### The cytoplasmic tail of Ii contains two endosomal targeting signals

Analysis of Ii mutants containing deletions in the membrane proximal region revealed two endosomal targeting signals within the cytoplasmic tail of Ii. Ii mutants containing either amino acid residues 1-11, or 12-29 of the cytoplasmic tail were found in endosomes, albeit to a different extent. This explains why mutations in one endosomal targeting sequence (as e.g. in 12-15Ii) did not affect the final location of Ii, as the second endosomal targeting sequence was still operative.

While the Ii mutant containing residues 1-11 was found in large vacuolar structures and in high amounts at the

plasma membrane, the mutant containing residues 12-29 was found in low amounts at the plasma membrane and intracellularly in small vesicular structures. Within the first 11 amino acid residues of the Ii cytoplasmic tail, a leucine-isoleucine pair is present at positions 7 and 8. Pairs of leucine-leucine and leucine-isoleucine have been shown to function as endosomal/lysosomal targeting signals in the cytoplasmic tail of the T-cell antigen receptor and chains (Letourneur and Klausner, 1992) and the cytoplasmic tail of the CI-MPR (Johnson and Kornfeld, 1992). We demonstrated that the mutation of this leucine-isoleucine pair to alanine-alanine completely abolished endosomal targeting. Thus, the Ii cytoplasmic tail contains two independent endosomal targeting signals, one of which is located within residues 12-29, the other consisting of a leucine-isoleucine motif.

Ii molecules containing both or one of the two endosomal targeting sequences were rapidly internalized from the cell surface. Thus, at least part of the Ii molecules reached the endosomes after transport to and internalization from the plasma membrane. It remains to be established, however, whether or not internalization from the plasma membrane is the major route to endosomes. The low steady-state levels of Ii molecules at the cell surface suggests that the cell surface is not a resident intermediate for transport to endosomes. It is conceivable that an equilibrium may exist between the endosomal pool of Ii molecules and the plasma membrane pool and that molecules that arrive at the plasma membrane will be internalized when they contain either one or both of the endosomal targeting sequences. This would however mean that the same sequences that function as intracellular sorting signals also function as internalization signals. In fact, this has been shown to be the case for the leucine-leucine motif: when present in the Tac antigen, it functions as an internalization signal (Letourneur and Klausner, 1992), and when present in the cytoplasmic tail of the CI-MPR, it functions in sorting from the TGN to endocytic compartments (Johnson and Kornfeld, 1992). Tyrosine-containing signals in the cytoplasmic tail of the CI-MPR and a tyrosine-glycine-containing signal in the tail of Igp A also serve alternative routes; plasma membrane internalization and intracellular targeting to endosomes (Johnson and Kornfeld, 1992; Harter and Mellman, 1992).

The identification of the leucine-isoleucine motif as an endosomal targeting signal in the cytoplasmic tail of Ii furthermore explains the results obtained by Nillson et al., (1991). These investigators found that the Ii cytoplasmic tail could be exchanged with that of galactosyltransferase without altering the endosomal location of this chimaeric molecule. In fact, a leucine-leucine pair is present at positions 6 and 7 of the galactosyltransferase cytoplasmic tail (Masri et al., 1988), which might be responsible, in the absence of the galactosyltransferase transmembrane region, for targeting to endosomes.

### The Ii cytoplasmic tail is sufficient for targeting a passenger molecule to endosomes

The Ii cytoplasmic tail was found to be sufficient to target a fusion protein with neuraminidase (NA) as passenger (INA) to endocytic compartments. Only low amounts of INA were found at the plasma membrane. The endocytic

INA-positive structures were identified as lysosome-related compartments as they were labelled with antibodies to lysosome-associated membrane protein (LAMP). NA, in contrast, was mainly located at the plasma membrane and intracellularly confined to the Golgi region.

A number of differences existed however, between localization and transport of Ii and INA. First, while Ii induced and was found in large vacuolar endosomes, INA expression did not induce these structures. Second, in contrast to Ii, carbohydrate moieties on INA molecules were almost completely modified to the complex type, indicating that these molecules are readily transported through the Golgi complex. Third, INA molecules were far more stable than Ii wild type or Ii mutant molecules.

Thus, the Ii cytoplasmic tail does not contain all the information for retention in the same endosomal location of Ii. As INA only contains the cytoplasmic tail of Ii, the transmembrane and/or luminal domain of Ii must also contribute to the correct endosomal location of Ii. The difference in localization between INA and Ii may be due to the presence of a retention sequence in the Ii transmembrane and/or luminal domain.

Taken together, the results we present here show that Ii contains two independent targeting signals for the endocytic pathway in its cytoplasmic tail. One of the proposed functions of Ii is to target MHC class II molecules to endocytic compartments where they can combine with antigenic peptides. The complexity of signals that regulate Ii transport to and retention in the endosomal system may be required to ensure the versatility of class II molecules, maximizing their exposure to exogenous antigens, as was suggested by Brodsky (1992).

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