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An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalization

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SUMMARY

Invariant chain (Ii) is a transmembrane protein that associates with the MHC class II molecules in the endoplasmic reticulum. Two regions of the 30 residue cytoplasmic tail of Ii contain sorting information able to direct Ii to the endocytic pathway. The full-length cytoplasmic tail of Ii and the two tail regions were fused to neuraminidase (NA) forming chimeric proteins (INA). Ii is known to form trimers and when INA was transfected into COS cells it assembled as a tetramer like NA. The INA molecules were targeted to the endosomal pathway and cotransfection with Ii showed that both molecules appeared in the same vesicles. By labelling the INA fusion proteins with iodinated antibody it was found that molecules with either endocytosis signal were expressed at the plasma membrane and internalized rapidly. Point mutations revealed that an LI motif within the first region of the cytoplasmic tail and an ML motif in the second region were essential for efficient internalization. The region containing the LI motif is required for Ii to induce large endosomes but a functional LI internalization motif was not fundamental for this property. The cytoplasmic tail of Ii is essential for efficient targeting of the class II molecules to endosomes and the dual LI and ML motif may thus be responsible for directing these molecules to the endosomal pathway, possibly via the plasma membrane.

Key words: invariant chain, MHC class II, endocytosis, internalization, membrane traffic

INTRODUCTION

The major histocompatibility complex (MHC) class II molecules are heterodimers (α and β chain) that associate in the endoplasmic reticulum (ER) with the invariant chain (Ii) (Sung and Jones, 1981; Kvist et al., 1982). Ii is a transmembrane protein that has 30 amino(N)-terminal residues exposed at the cytoplasmic side of the membrane, spans the membrane between residues 30 and 60, and has a large lumenal carboxy(C)-terminal domain (Claesson and Peterson, 1983; Strubin et al., 1984). In humans an alternative form of Ii results from translation initiation of an upstream start codon, which extends the cytoplasmic tail with 16 amino acids. These 16 N-terminal residues include a strong ER retention signal (Lotteau et al., 1990). Both forms may in addition contain an alternatively spliced exon, which, together with various types of post-translational modifications, can create numerous forms of the molecule (reviewed by Cresswell, 1992). The formation of αβIi complexes is believed to occur by the sequential addition of αβ heterodimers to a pre-existing core of trimeric Ii molecules (Marks et al., 1990; Lamb and Cresswell, 1992), the final complex being a stochiometric nine subunit complex (Roche et al., 1991). Following subunit assembly, the αβIi complex traverses the Golgi complex and approximately 2 hours after synthesis, Ii is proteolytically processed and dissociates from αβ in an endosomal compartment and αβ is transported to the plasma membrane, where it may interact with CD4-positive T-cells (reviewed by Germain and Margulies, 1993).

The endosomal sorting signal for newly synthesised αβIi complexes resides in the Ii cytoplasmic tail (Bakke and Dobberstein, 1990; Lotteau et al., 1990). Both deletion of the 11 N-terminal residues or residues 12-29 of the Ii tail resulted in Ii molecules that were still sorted to endosomes (Bakke and Dobberstein, 1990; Pieters et al., 1993), suggesting that the tail of Ii contains two independent sorting signals. The sorting signal could in principle be read at the level of the *trans*-Golgi network (TGN) resulting in transport directly to an endosomal compartment or Ii could follow a pathway to the plasma membrane before re-internalization. Some reports suggest that newly synthesised class II molecules reach the endosomes via a direct route (Neefjes et al., 1990; Peters et al., 1991), whereas we have found that a major fraction of both Ii and αβIi may be routed to endosomes via the plasma membrane (Roche et al., 1993).

Specific signals for endosomal or lysosomal targeting have been identified in the cytoplasmic tails of several transmembrane proteins. The signal in lysosomal membrane proteins such as h-Lamp 1 (Williams and Fukuda, 1990) and human lysosomal acid phosphatase (Peters et al., 1990; Lehmann et al., 1992; Eberle et al., 1991) involve a tyrosine, which, with a tight β-turn, is functionally comparable to the internalization signal found in cell surface receptors (Collawn et al., 1990; Bansal and Gierasch, 1991). However, a tyrosine signal does

not seem to be universal for sorting of lysosomal membrane proteins as the tyrosine-lacking tail of the LIMP II also contains a lysosomal sorting signal (Vega et al., 1991a,b). A novel di-leucine motif for lysosomal targeting has been identified in the cytoplasmic tails of the CD3-γ and -δ chains of the T-cell receptor complex (Letourneur and Klausner, 1992). They found a di-leucine and a tyrosine-based motif, which are individually sufficient to induce both endocytosis and delivery to lysosomes. One signal was enough for sorting via the plasma membrane whereas both signals were needed for a direct transport to lysosomes. Two similar signals for sorting to the endosomal pathway were also reported in the cytoplasmic tail of the mannose 6-phosphate receptor and the postulate was that a di-leucine motif mediates a direct transport of the cation-independent mannose 6-phosphate receptor to late endosomes, while the tyrosine motif is directing transport from the plasma membrane (Johnson and Kornfeld, 1992a).

The cytoplasmic tail of Ii is devoid of tyrosine, but contains sequences that resemble the LL signal described above. In this work we have fused the cytoplasmic portion of Ii to the transmembrane and extracellular region of neuraminidase, a type II plasma membrane protein (Van Rompuy et al., 1982). The chimeric protein was efficiently internalized after transport to the plasma membrane. Larger deletions confirmed that the cytoplasmic tail of Ii contains two autonomous internalization signals. By single point mutations we recognised a leucine-isoleucine motif in the most N-terminal region of the cytoplasmic tail and a methionine-leucine motif in the membrane proximal part of the tail that both independently in their context mediate endosomal sorting and efficient internalization.

MATERIALS AND METHODS

Expression vector and plasmid constructions

pSV51L is a late replacement vector with a short polylinker behind the SV40 promoter and this vector has been shown to give high transient expression of several proteins in simian cells (Huylebroeck et al., 1988; Bakke and Dobberstein, 1990). The cDNA for neuraminidase is from human influenza strain A7/Victoria3/75 (Van Rompuy et al., 1982). The INA construct was made by introducing by PCR a *Sac*II site at the beginning of the transmembrane region and a *Bam*HI site behind the coding region. The 1.3 kb *Sac*II-*Bam*HI fragment was then replacing a 0.9 kb fragment of pSVIi (Bakke and Dobberstein, 1990) also coding for the transmembrane and lumenal domain. Δ11INA was constructed by exchanging the same fragments in Δ11Ii (Bakke and Dobberstein, 1990) and Δ12-29INA constructed by this replacement in Δ12-29Ii (Pieters et al., 1993). LINA was constructed by inserting an Ii tail including the upstream methionine and the second methionine mutated to leucine (a gift from Dr E. O. Long, NIH, Rockville, MD; Strubin et al., 1986). Point mutations in the cytoplasmic tail of Ii were introduced by site directed mutagenesis in a single-stranded M13 mp19 vector by the method of Kunkel (1987). The cytoplasmic tail region used for mutagenesis was controlled by DNA sequencing and replaced with the same non-mutated region in the Ii or INA construct. The SSR cDNA (ER-p34) inserted into pSV51L (Prehn et al., 1990) was a gift from Dr S. Prehn, Max-Delbrück Institute, Berlin.

Antibodies

The rabbit polyclonal antibody WIC103 (R4/76) and the mouse monoclonal antibody NC71, which both recognise the lumenal domain of neuraminidase were a gift from Dr A. Douglas, Mill Hill, London. The mouse monoclonal antibody against the lumenal domain of the human lysosome-associated membrane protein LAMP2 (Carlsson et al., 1988) was donated by Dr S. Carlsson (Umea, Sweden). The rabbit anti SSR(ER-p34) antibody (Prehn et al., 1990) was a gift from Dr S. Prehn and T. Rappaport, Max-Delbrück Institute, Berlin). BU45 (The Binding Site, Birmingham, UK) recognises the lumenal domain of human Ii (Wraight et al., 1990). The cells were labelled for immunofluorescence with FITC- and Texas Red-conjugated goat anti-rabbit Ig and goat anti-mouse Ig antibodies (Dianova, Hamburg).

Cells and cell culture

The CV1 fibroblast cell line originates from African green monkey kidney cells (ATCC, number CCL70). COS cells are derived from CV1 cells transformed with an origin-defective mutant of SV40 coding for the wild-type T-antigen (Gluzman, 1981). The cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum (DMEM-FCS).

The expression vector pSV51 can equally well be used to transfect the simian COS cells and CV1 cells (see below). COS cells, expressing the large T-antigen, give a very high transfection efficiency (more than 50% of cells transfected), whereas the corresponding figure for CV1 cells is less than 10%. The morphology of CV1 cells is, however, more regular than COS cells and we chose to use CV1 cells for morphological studies and COS cells for uptake and metabolic labelling studies.

Transient expression in CV1 cells

This procedure is similar to the one used by Huylebroeck et al. (1988): 70% confluent cells were split 1:5 into 35 mm wells one day before transfection. Plasmid DNA (2 μg) was dissolved in 0.25 ml DMEM with 25 mM HEPES and mixed with 0.25 ml DMEM-HEPES containing 1 mg/ml DEAE-dextran. The DNA mixture was left at room temperature for 30 minutes. The cells were washed twice in DMEM-HEPES before the DNA (0.5 ml per 35 mm well) was added and then incubated at room temperature. After 45 minutes they were washed twice in DMEM-HEPES before incubation in DMEM-FCS with 0.1 mM chloroquine (Sigma) for 3-4 hours (37°C, 5% CO_2). The cells were then exposed to 0.1 mM sodium butyrate (Merck) in DMEM-FCS for 20-24 hours and then further incubated in DMEM-FCS overnight before they were used for experiments.

Transient expression in COS cells

The cells were seeded into 35 mm wells the day before transfection as described above for the CV1 cells. Plasmid DNA (0.5 μg) was dissolved in 0.1 ml DMEM with 10% NuSerum (Collaborative Research). The solution was mixed with 1 ml DMEM-NuSerum containing 400 μg/ml DEAE-dextran and 0.1 mM chloroquine. The cells were washed twice in PBS (pH 7.4) before the DNA was added (1 ml per 35 mm well). After 3-4 hours at 37°C, 6% CO_2 the cells were given a DMSO shock (10% DMSO, 2-3 minutes) and grown for a further 2 days in DMEM-FCS for expression of the proteins.

Antibody uptake and labelling of cells for immunofluorescence microscopy

Transiently transfected CV1 cells were cultivated on glass coverslips and fixed in methanol for 3 minutes at −20°C two days after transfection with the various proteins. The cells were labelled with the appropriate antibodies for 30 minutes at room temperature and the secondary fluorochrome-tagged antibodies for another 30 minutes before mounting in Mowiol.

Cells transfected with INA constructs cells were labelled on ice for 30 minutes with NC71 before incubation at 37°C for various times. After fixation in methanol the internalized antibody-antigen complexes were visualized by a FITC-conjugated goat anti-mouse antibody. To detect the total distribution of the expressed protein in these cells, the cells were incubated with the polyclonal anti-NA serum WIC103 and a Texas Red-conjugated goat anti-rabbit antibody.

To avoid fixation artefacts due to the use of a specific fixation in all experiments we also fixed cells on parallel coverslips in paraformaldehyde and permeabilised with Triton as described earlier (Bakke and Dobberstein, 1990). This procedure showed the same staining pattern as that seen after methanol fixation. BU45 was used to monitor internalization of Ii constructs as outlined earlier (Roche et al., 1993).

Purification and ^{125}I-labelling of antibodies

Mouse ascites NC71 (IgG_1) was precipitated with 0.18 g/ml Na_2SO4 and purified on a Protein A-Sepharose (Pharmacia) column at pH 8.0 before iodination by IODO-GEN™ as described by the manufacturer (Pierce). The amount of acid-soluble and -precipitable material was determined by TCA precipitation and counting in a Cobra Auto-Gamma γ-counter. The amount of soluble radioactivity in the samples was usually as low as 1-2%.

Plasma membrane expression of proteins

Transiently transfected COS cells in 35 mm wells were incubated in 0.5 ml DMEM containing ^{125}I-labelled NC-71 (1 mg/ml) for 2 hours on ice and washed 6 times in ice-cold PBS with 2% FCS before lysis in 1 ml 1 M NaOH. The samples were counted in the γ-counter to measure the amount of protein expressed on the surface. To relate the surface expression to total amount of expressed protein parallel wells were labelled with [^{35}S]cysteine for 2 hours and immunoprecipitated as above using NC-71 and WIC103. After SDS-PAGE and fluorography the relative amounts of protein in the bands were measured in a gel scanner.

Internalization of ^{125}I-labelled antibodies

Transiently transfected COS cells in 35 mm wells were incubated with ^{125}I-labelled NC71 in DMEM-FCS (~1 μg/ml) on ice for 2 hours. The cells were then washed six times in ice-cold PBS with 2% FCS. The chase was performed in DMEM-FCS at 37°C for different periods of time. Cells were then cooled on ice and treated twice with 0.5 M acetic acid in 0.15 M NaCl (pH 2.5) for 7 minutes. This step removed 95-98% of the surface-bound antibody. The cells were removed from the wells by lysis in 1 M NaOH. The acid wash and the lysed samples were counted in the γ-counter. Each time point was performed in duplicate. Internalized antibody was calculated as the antibody resistant to the low pH wash relative to the antibody bound before the chase period. Typically, when cells were sham-transfected with the vector without insert and incubated with ^{125}I-NC71 on ice and washed, the cell bound activity was less than 1% of the activity bound to cells transfected with INA-variants.

Metabolic labelling of COS cells and immunoprecipitation

COS cells in 35 mm wells were labelled two days after transfection. They were starved in cysteine/methionine-free DMEM for 30 minutes before labelling in 0.5 ml of the same medium containing 80-100 μCi/ml [^{35}S]cysteine/[^{35}S]methionine for 20 minutes (pulse chase) or continuously for 3 hours. For pulse-chase experiments the cells were washed extensively and chased in DMEM at 37°C. After labelling and chase the cells were placed on ice, washed twice in ice-cold PBS (pH 7.4) and lysed with 1 ml ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40) containing a cocktail of protease inhibitors (4 μg/ml PMSF, 2 μg/ml antipain, 2 μg/ml leupeptin and 1 μg/ml pepstatin A) for 20 minutes. Samples were centrifuged (10,000 ***g***) for 10 minutes at 4°C to remove cell nuclei. The supernatants were either used directly for immunoprecipitation or frozen at −80°C.

One μl antibody serum or ascites was added to 1 ml lysate, and the antibodies were allowed to bind for at least 2 hours at 4°C. 35 μl of a 1:1 slurry of Protein A-Sepharose (Pharmacia) equilibrated in buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40) was added and incubation at 4°C continued for 1-2 hours. The beads were then washed twice in buffer A, twice in buffer A containing 500 mM NaCl, and once in 10 mM Tris-HCl (pH 7.5). For EndoH treatment half of the precipitated material was treated with 1 mU EndoH in 50 μl 0.15 M sodium citrate containing the cocktail of protease inhibitors for 12 hours at 37°C. Proteins bound to the beads were analysed by SDS-PAGE on a 12% polyacrylamide gel. Fluorography of the gels was performed using Entensify as directed by the manufacturer (New England Nuclear). Immunoprecipitation of the crosslinked samples followed the same protocol except that instead of buffer A a special lysis buffer (see below) was used to wash the beads and DTT was not included in the sample buffer.

Chemical crosslinking

Transiently transfected and metabolically labelled COS cells were washed twice in PBS and extracted at 1×10^6 cells/ml in a lysis buffer (1% polyoxyethylene 9 lauryl ether (Sigma), 0.13 M NaCl, 0.02 M bicine (Sigma), pH 8.2) for 20 minutes at 4°C. Then 50 mM 3,3 dithio-bis succinimide propionate ester (DSP) (Sigma) in DMSO was added to the extracts to a final concentration of 0.5 mM. After incubating for 30 minutes at 4°C, 10 mM glycine and protease inhibitors (see above) were added. These samples were immunoprecipitated as described above using the WIC 103 antibody.

RESULTS

Expression and multimerisation of invariant chain-neuraminidase fusion proteins

Previous studies have shown that Ii contains sorting signals in the cytoplasmic tail necessary for targeting to and accumulation in endosomal vesicles (Bakke and Dobberstein, 1990; Lotteau et al., 1990). In a recent study we reported that there seem to be two signals for endosomal routing within the cytoplasmic tail of Ii, one within the first 11 amino acids and another within the remaining 19 residues, which both are able to direct Ii to endosomes (Pieters et al., 1993). In addition the full-length tail of Ii could mediate sorting of the plasma membrane protein neuraminidase to endosomes. To show the existence of two autonomous and independent endosomal sorting signals in the Ii-tail, we have constructed fusion proteins where the short cytoplasmic tail of neuraminidase (NA) has been replaced by regions of the cytoplasmic tail of Ii (see Fig. 1).

COS cells giving a high frequency of transfection were transfected with INA, Δ11INA and Δ12-29INA and biosynthetically labelled with [^{35}S]cysteine for 20 minutes before chase periods of 1, 2.5 or 5 hours. Proteins from cell lysates were then immunoprecipitated with antibodies recognising neuraminidase. A fraction was digested with endoH to remove high mannose sugars before SDS-PAGE and visualization by fluorography. As shown in Fig. 2 the proteins were relatively stable and densitometric measurements showed that the half lives for all three constructs were more than 5 hours. Furthermore, initial transport kinetics is relatively slow for these molecules as approximately 2 hours were needed for 50% of the carbohydrate moieties to be converted to complex type sugars, a process that takes place in the *trans*-Golgi.

Chemical crosslinking has indicated that Ii in complex with the class II molecules forms trimers (Roche et al., 1991; Lamb and Cresswell, 1992) and influenza neuraminidase is reported to assemble as a tetramer (Varghese et al., 1983). To study the multimerisation state of INA chemical crosslinking DSP was applied after transfection of COS cells. NA without crosslinking appeared as monomers and dimers and a third band probably representing tetramers, judging by their molecular

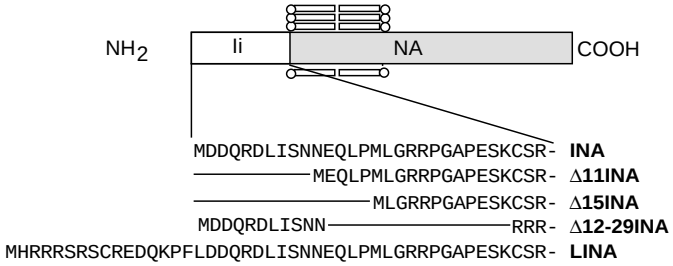

Fig. 1. Amino acid sequences of the cytoplasmic N-terminal domains of INA deletion mutants. Deletions are indicated by horizontal lines. The transmembrane and lumenal part of the molecule is shown in grey.

mass (Fig. 3). Crosslinked NA and INA migrated as tetramers. The reporter molecule INA thus resembles Ii by making multimers although they differ in their state of multimerisation.

Localization of invariant chain-neuraminidase fusion proteins

The localization of the fusion protein in transfected CV1 cells was visualized by immunofluorescence microscopy and compared to intracellular localization of LAMP2, a protein found mostly in late endosomes and lysosomes (Chen et al., 1985). As shown in Fig. 4, INA, Δ11INA and Δ12-29 INA were detected on the plasma membrane and in intracellular compartments and overlapped with LAMP2, indicating that these proteins are sorted to late endosomes and possibly lysosomes. Using the same expression vector Ii has been found to induce large vesicular endosomal structures (Romagnoli et al., 1993; Pieters et al., 1993; Fig. 11, this paper), but such a dramatic effect was not observed for the INA fusion proteins. Δ15Ii accumulate at the plasma membrane (Bakke and Dobberstein, 1990) and Δ15INA with an identical cytoplasmic tail showed a similar behaviour (Fig. 4G). To investigate further the nature of the vesicles with INA molecules a 30 minute pulse of Texas Red-conjugated ovalbumin was internalized and followed by various chase periods. After fixation the endogenous INA proteins were labelled with FITC-conjugated antibodies. Double immunofluorescence microscopy revealed that the vesicles with INA proteins colocalized with the internalized OVA both immediately after uptake and after a one

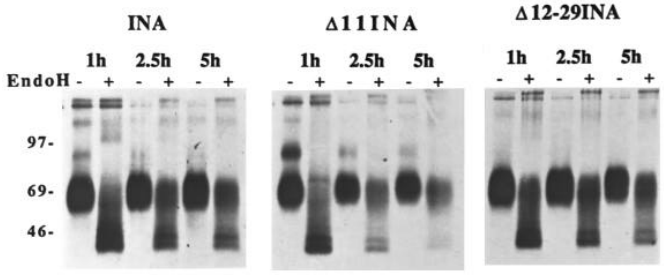

Fig. 2. Pulse-chase analysis of INA molecules in COS cells. Transiently transfected COS cells were pulse-labelled with [^{35}S]cysteine for 20 minutes and chased in DMEM at 37°C for 1, 2.5 and 5 hours. The proteins were immunoprecipitated using WIC103. One half of the immunoprecipitated material was treated with endoglycosidase H before SDS-PAGE (12%) and fluorography. Size standards (in kDa) are shown on the left.

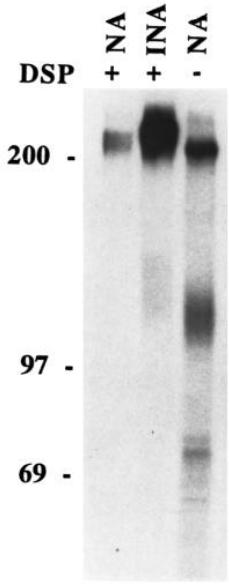

Fig. 3. DSP crosslinking of NA and INA. Transiently transfected COS cells were labelled with [^{35}S]cysteine for one hour. The two first lanes represent samples crosslinked with DSP (+) whereas the sample in the third lane was not crosslinked (–). The samples were immunoprecipitated using WIC103 and run on a 6-12% SDS-PAGE gel under nonreducing conditions. Size standards (in kDa) are shown on the left.

hour chase (data not shown). This, together with the LAMP colocalization and the antibody uptake shown later (Fig. 7) indicates that INA, Δ11INA and Δ12-29 INA are all sorted to similar endocytic compartments.

In the above studies all the INA constructs were visible at the cell surface. To quantitate the relative amount of protein on the plasma membrane, a ^{125}I-labelled monoclonal antibody towards NA (NC71) was incubated with the cells on ice and the bound fraction was calculated. Δ15INA had, as expected from the immunofluorescence, the highest cell surface expression level (set to 1.0) whereas the levels for Δ11INA, Δ12-29INA and INA were significantly lower (0.26, 0.10 and 0.11; see Fig. 5).

The ER retention signal in Ii may also retain NA

Upstream of the main form of human Ii cDNA there is an alternative start codon that extends the cytoplasmic tail with 16 amino acids. This results in a 35 kDa form of Ii, which is expressed at a lower efficiency than Ii (Strubin et al., 1986). When the main start codon was mutated the 35 kDa Ii molecule was found to be retained in the ER in HeLa cells (Lotteau et al., 1990) and in CV1 cells (M. Gedde-Dahl and O. Bakke, unpublished). This extended cytoplasmic tail was fused with neuraminidase and the resulting protein (LINA, Fig. 1) showed a similar reticular expression pattern to that of the rough ER molecule SSR (p34-ER) (Fig. 4H and I). Thus, the ER retention signal in the extended tail of Ii may also retain NA in this part of the biosynthetic pathway.

Plasma membrane internalization of INA molecules

Membrane proteins may enter the endocytic pathway either by direct sorting from the Golgi (e.g. the TGN) or via internal-

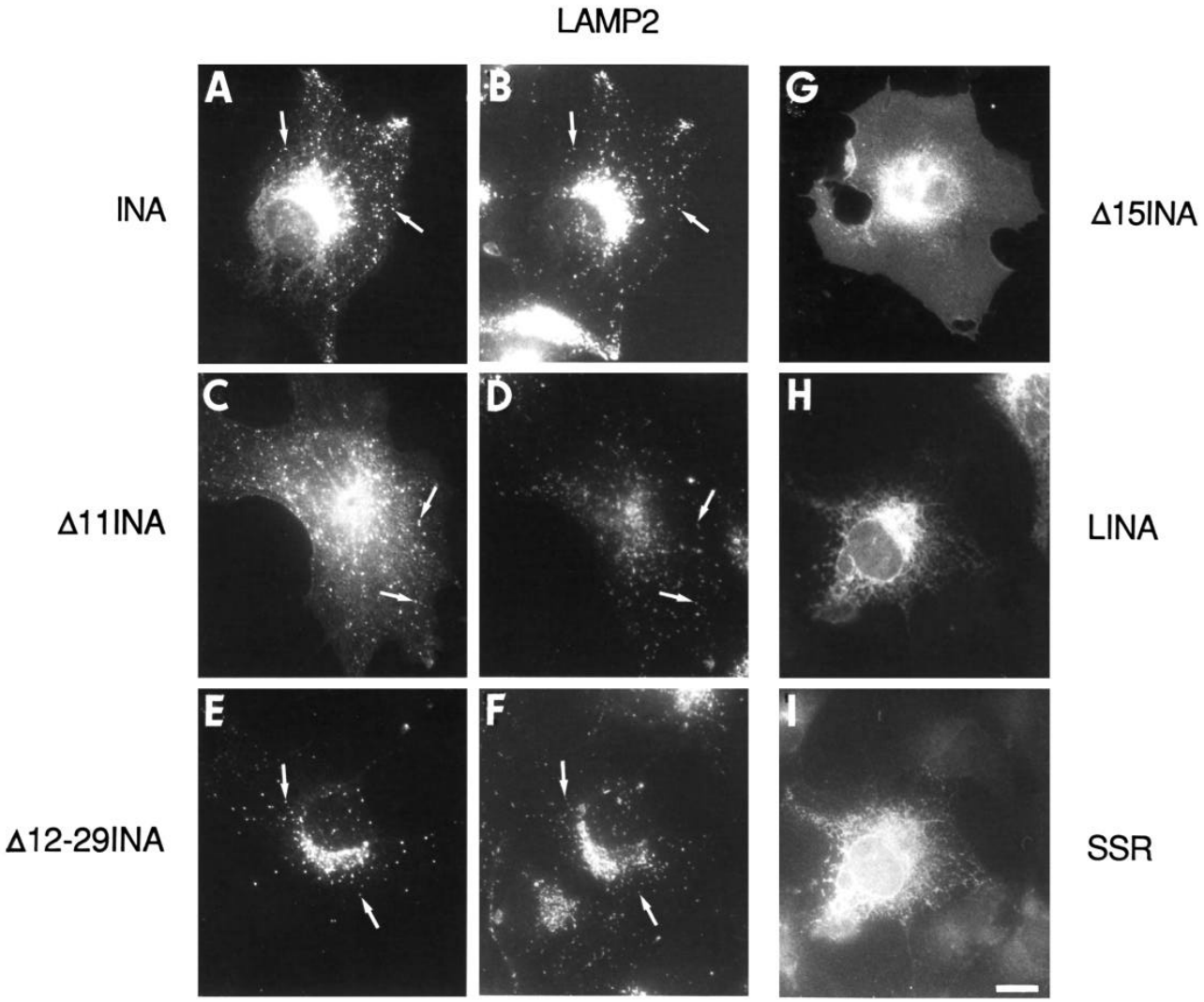

Fig. 4. Localization of INA and INA deletion mutants in CV1 cells and colocalization with LAMP and SSR. CV1 cells transiently transfected with INA (A and B), Δ11INA (C and D) and Δ12-29INA (E and F) were double-labelled with the polyclonal anti-NA serum WIC103 and the monoclonal anti-LAMP antibody LAMP2. INA variants were visualized by a Texas Red-conjugated goat anti-rabbit second antibody (A,C and E) and LAMP was visualized by a FITC-conjugated goat anti-mouse second antibody (B,D and F). Colocalization is marked by white arrows. (G) Expression of Δ15INA. Cells cotransfected with LINA (NA with the long tail of Ii) and the rough ER protein SSR were labelled with NC71 and a polyclonal antibody against SSR (H and I). Bar, 20 μm.

ization from the cell surface. As shown in Figs 4 and 5, INA, Δ11INA and Δ12-29INA are all found at the plasma membrane besides the endocytic compartments. The variation in plasma membrane expression seen in Fig. 5 could be caused by differences in the sorting efficiency from TGN to endosomes, variable internalization rates from the plasma membrane or by different molecular stability. To monitor internalization from the surface, transfected COS cells were incubated with ^{125}I-labelled antibodies on ice and chased at 37°C. As shown in Fig. 6, INA and Δ12-29INA were very rapidly internalized, approximately 25% within the first minute. Δ11INA was internalized more slowly (6% the first minute) whereas Δ15INA (and Δ20INA) was not internalized above background internalization level for NA. From this we conclude that the Ii tail contains two efficient independent internalization signals. To exclude the possibility that the rapid internalization rates were induced by antibody binding, BFA was used to stop protein transport to the plasma membrane (Roche et al., 1993). ^{125}I-NC71 binding was then used to quantify remaining surface protein, and this showed similar internalization rates to those obtained by the above method (data not shown). The variation in internalization rates also corresponds qualitatively with the relative level of plasma membrane expression (Fig. 5) and might thus explain the observed differences.

To monitor the internalization by microscopy, CV1 cells transfected with INA, Δ11INA, Δ12-29INA and Δ15INA, were labelled with NC71 on ice for 30 minutes and reheated to 37°C. Cells on coverslips were fixed after various times and internalized antibody as well as total protein was labelled with fluorochrome-tagged antibodies. After 5 minutes the internalized antibody was seen in vesicles for the first three constructs and some of the vesicles costained with total protein (data not

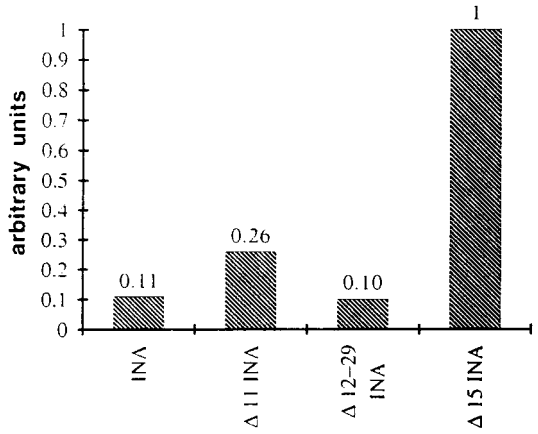

Fig. 5. Cell surface expression of INA and INA deletion mutants in COS cells. Transfected COS cells were labelled with ^{125}I-NC71 for 2 hours on ice. The cells were washed and the bound antibody measured in a γ-counter. Parallel dishes were labelled with [^{35}S]methionine for 3 hours and the total protein was immunoprecipitated using NC71 and WIC103. After SDS-PAGE and fluorography the bands were quantified by densitometric analysis and the values were used to correct for protein expression levels of the various proteins.

shown). The overlap between internalized antibody and total protein increased with time until the 30 minute time point which is shown in Fig. 7. Thereafter the antibody was found in fewer vesicles and was no longer detected after 2-3 hours most likely due to degradation. For Δ15INA only minor amounts of NC71 was internalized after 30 minutes. From the biochemical and morphological data we conclude that INA with the first, second or both internalization signals may be routed to their destination in endosomes via the plasma membrane, although we cannot exclude the possibility that direct transport also occurs from the TGN to endosomes.

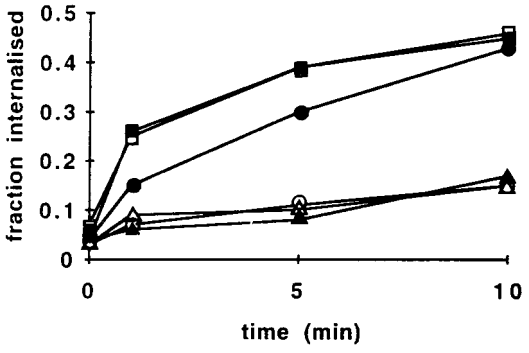

Fig. 6. Internalization of ^{125}I-labelled NC71 in COS cells expressing different INA constructs. Transfected COS cells were incubated with ^{125}I-NC71 for 2 hours on ice and chased in medium at 37°C for different time periods. The radioactivity in the low pH wash and the acid resistant fractions removed from the wells by NaOH were measured in a γ-counter. The internalized fractions were calculated as the fraction of the total amount of cell-bound activity resistant to the low pH wash. □, INA; ■, Δ12-29INA; ●, Δ11INA; ○, Δ15INA; ▲, NA; △, Δ20INA. Each construct was tested at least twice, giving similar results.

Influence of point mutations: both LI and ML motifs mediate rapid internalization

It has been shown that a LL or IL motif is able to mediate lysosomal sorting of the γ and δ chain of the CD3 complex (Letourneur and Klausner, 1992). A similar motif was also detected in the mannose 6-phosphate receptors (Johnson and Kornfeld, 1992a,b). The first 11 N-terminal amino acids of Ii contain a leucine-isoleucine (LI) motif and the leucine was mutated to alanine in Δ12-29 INA (Fig. 8). This point mutation abolished localization in vesicles, and lead to a strong plasma membrane expression (data not shown). Internalization studies using ^{125}I labelled NC71 also showed that the internalization rate was dramatically reduced (Fig. 9A).

Between residues 12 and 30 there are no LL/LI/IL motifs in the human invariant chain. In the mouse and rat sequence, however, there is an IL motif at position 16-17, which corresponds to a ML motif in the human homologue (Bakke and Dobberstein, 1990). Point mutations of either one of these residues to alanine changed Δ11INA to a molecule that was located primarily at the plasma membrane and reduced the internalization rate to background level (Fig. 9B). This shows that the motif for efficient internalization may also be ML in the proper context. There is also a third leucine in the Ii tail (leu 14) surrounded by glutamine and proline, but mutation of this residue to alanine did not alter the rapid NC71 uptake of Δ11INA (data not shown). Phosphorylation may play a role for the internalization of receptors (reviewed by Trowbridge, 1991). Within the 11 first residues of the Ii tail there is only one candidate for phosphorylation, a serine at position 9. Point mutation of this serine to alanine (Δ12-29INA ser 9 to ala) did not, however, alter the intracellular distribution of the Δ12-29 INA molecule or the internalization rate (Fig. 9A) indicating that phosphorylation of the cytoplasmic tail is not involved in this process.

In the above studies we have transplanted the two regions of the cytoplasmic tail to neuraminidase and performed mutations. The machinery that reads the sorting signals might, however, depend on the tertiary structure of the full-length tail and we also performed point mutations on this construct as outlined in Fig. 8. Immunofluorescence microscopy (micrograph not shown) and internalization measurements (Fig. 9B) showed as expected that mutation of both signals was needed to block internalization. When the first signal was deleted by point mutations (leu7 to ala) the internalization rate was approx. 10%/minute (Fig. 9B), which is more rapid than for Δ11INA where the first region is deleted.

Both INA and Ii mutants are sorted to the same compartments

In this study we have concentrated on the sorting of INA fusion proteins. Full-length Ii, Δ11Ii (Bakke and Dobberstein, 1990) and Δ12-29Ii (Pieters et al., 1993) are also sorted to endosomes in CV1 cells and we wanted to investigate the localization of the fusion proteins. CV1 cells were co-transfected with various INA and Ii constructs and the two molecules were detected by double immunofluorescence. As shown in Fig. 10A Ii was found both in small endosomes and in the large vesicular structures and INA was present in the same structures (Fig. 10B). When Δ11Ii was transfected with Δ11INA there was also a high degree of colocalization (Fig. 10C and D). Δ12-29 Ii shows a stronger surface expression than Δ12-29INA, with

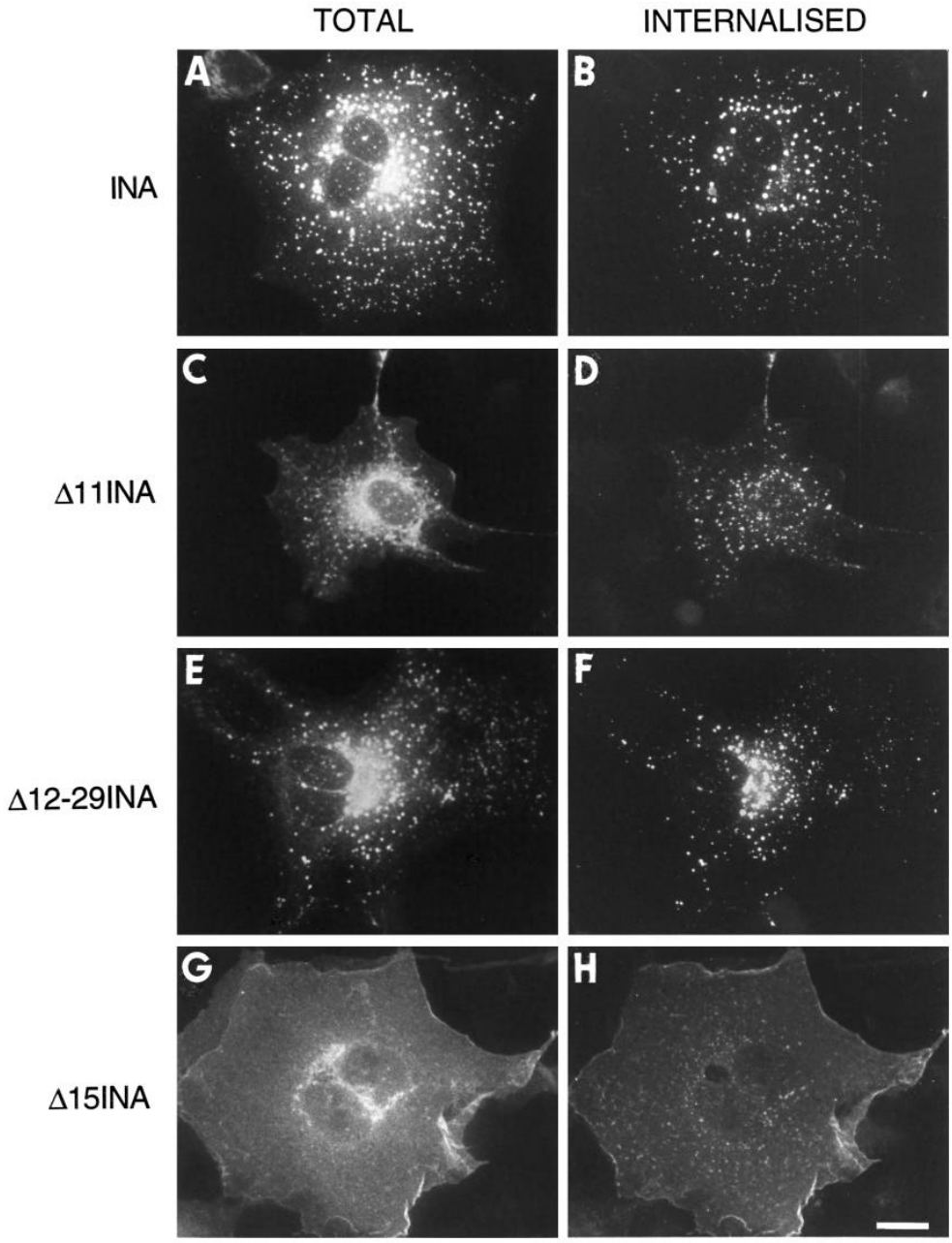

Fig. 7. Localization of internalized antibody and total protein in CV1 cells transfected with different INA mutants. CV1 cells transfected with INA, Δ11INA, Δ12-29INA and Δ15INA were labelled with NC71 on ice for 30 minutes. After internalization at 37°C for 30 minutes, the cells were fixed in methanol. Internalized NC71 was visualized by a FITC-conjugated second antibody. Localization of total protein was detected by the polyclonal anti-NA serum WIC103 and a Texas Red-conjugated goat anti-rabbit antibody. (A,C,E,G) Total distribution of protein detected by WIC103, (B,D,F,H) internalized NC71. Bar, 20 μm.

only a minor part in large endosomal structures (Fig. 10E and F). The large vesicles were also in this case found to contain Δ12-29INA (Fig. 10F). In addition to the colocalization of molecules with identical tails shown in Fig. 10 each of the three INA constructs (INA, Δ11INA, Δ12-29INA) colocalized equally well with the other Ii constructs (Ii, Δ11Ii or Δ12-29Ii) (micrographs not shown).

Although the two molecules might possibly influence each other when expressed in the same cells, the above data strongly suggest that both Ii and INA constructs with various cytoplasmic tails are sorted in a similar manner. This impression was further reinforced when we performed uptake studies using BU45 and transfected with Ii, Δ11Ii and Δ12-29Ii. This showed that BU45 was rapidly internalized to the same endosomes that contained endogenous protein (data not shown). Furthermore, the same mutations that abolished the rapid internalization rates of INA also dramatically reduced internalization of the corresponding Ii mutant protein and resulted, for the double mutant, in a high level of plasma membrane expression (Fig. 11D). From this we conclude that the sorting of Ii to endosomes via the plasma membrane depend on the same two internalization signals defined for INA.

Effect of point mutations of the LI/ML signal on the large vesicles induced by the invariant chain

As demonstrated in Fig. 10A, Ii expressed at a high level induced large vesicular structures. These structures were not seen for the Δ11Ii mutant (Romagnoli et al., 1993; Pieters et al., 1993; Fig. 10C, this paper) suggesting that this region of the molecule is essential for the formation of these structures. This region contains the LI endocytosis signal and it was suggested that this signal is essential for the induction of the

large vesicles (Pieters et al., 1993). However, as shown in Fig. 11, mutations of the LI signal alone did not alter the formation of the vesicles. Likewise, a mutation of the second signal (leu 17 to ala) did not influence large vesicle formation whereas a double mutation resulted in plasma membrane expression. In conclusion, although the formation of the large vesicular structures seem to require the first 11 residues of the Ii tail the LI motif within this sequence is not essential for the formation of these structures.

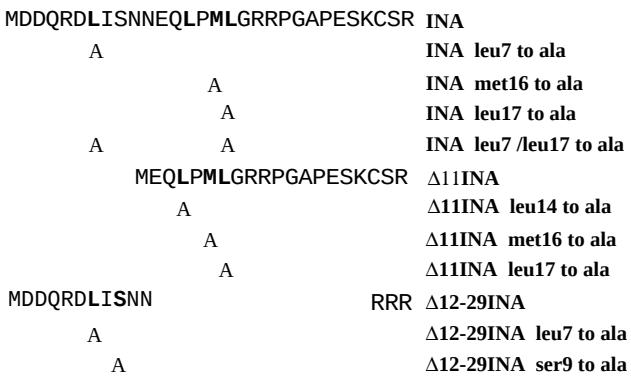

Fig. 8. Point mutations in INA deletion mutants. Point mutations are indicated by vertical dotted lines. Deletions are indicated by sparsely dotted horizontal lines.

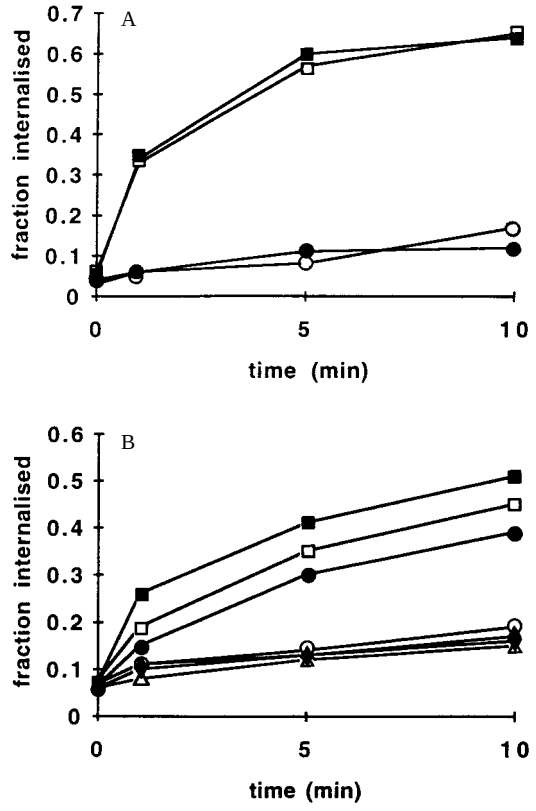

Fig. 9. Internalization of ^{125}I-NC71 in COS cells transfected with mutants of INA, Δ12-29INA and Δ11INA. (A) Internalization of Δ12-29INA and mutants. □, Δ12–29INA; ■, Δ12–29INA *ser9 to ala*; ●, Δ12–29INA *leu7 to ala*; ○, NA. (B) Internalization of Δ11 INA mutants and INA mutants. ■, INA; □, INA *leu7 to ala*; ●, Δ11INA; ○, Δ11INA *leu17 to ala*; ▲, Δ11INA *met16 to ala*; ▼, INA *leu7 to ala / leu17 to ala*; △, Δ20INA. Internalization of ^{125}I-NC71 and calculation of the internalized fractions were performed as described in the legend to Fig. 6 and in Materials and Methods.

DISCUSSION

It has been shown that signals within the cytoplasmic tail of Ii are responsible for transport of Ii alone and in complex with MHC class II to the endocytic pathway (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Roche et al., 1992; Simonsen et al., 1993). The N-terminal 18 residues of the cytoplasmic tail of Ii are identical in human, rat and mouse apart from 2 amino acids (Bakke and Dobberstein, 1990) and this region also contains the essential information for sorting of Ii to endosomes. The N-terminal tail of Ii seems to contain at least two regions that individually may both sort Ii to endosomes, one signal between residue 1-11 and another from 12-29 (Pieters et al., 1993). In this work we have shown that both regions may independently sort a reporter molecule (neuraminidase) to the endocytic pathway. Neuraminidase is a more stable molecule than Ii and colocalization with both internalized markers and the lysosomal protein LAMP2 indicates that Δ11INA and Δ12-29INA are present in early and late endosomes and possibly lysosomes.

The various fusion proteins were detected at the plasma membrane. The level of plasma membrane expression correlated well with internalization rate. For example Δ15INA showed a high level of surface expression and the bound antibodies were not internalized. INA and Δ12-29INA, which showed the lowest level of surface expression were extremely rapidly internalized; 25% per minute. This internalization rate is similar to that of Ii in B cells and fibroblasts (Roche et al., 1993). The rapid internalization contrasts with the slow biosynthetic transport from the ER, which requires 2 hours to reach the regions of Golgi where complex-type sugars are added. Slow transport from the ER to the Golgi is also reported for Ii (Bakke and Dobberstein, 1990) and as both molecules form multimers the delay could be caused by the machinery that aids folding, multimerisation and proofreading in the biosynthetic pathway (Copeland et al., 1986).

Our results demonstrate clearly that the tail of Ii contains two autonomous efficient internalization signals. The Ii tail does not contain the conventional tyrosine motif that mediates efficient internalization of many membrane proteins (for a review see Trowbridge, 1991), but variations of the di-leucine motif are found to mediate sorting to endosomes/lysosomes (Letourneur and Klausner, 1992; Johnson and Kornfeld, 1992a,b). Mutating either the LI motif or the ML motif within Δ12-29INA or Δ11INA prevented internalization and resulted in a strong surface expression of the mutated protein. As each of the two signals promote internalization, mutation of both signals was required to prevent internalization of INA with the full-length cytoplasmic tail.

Removal of 15 residues from the cytoplasmic tail of Ii (Bakke and Dobberstein, 1990) or INA (Δ15INA) resulted in molecules that were not sorted to endosomes but accumulated at the plasma membrane. In these constructs only the LI signal was deleted and our results indicate that the ML signal does not function at the very amino-terminal end of the molecule. Letourneur and Klausner (1992), however, report that the di-

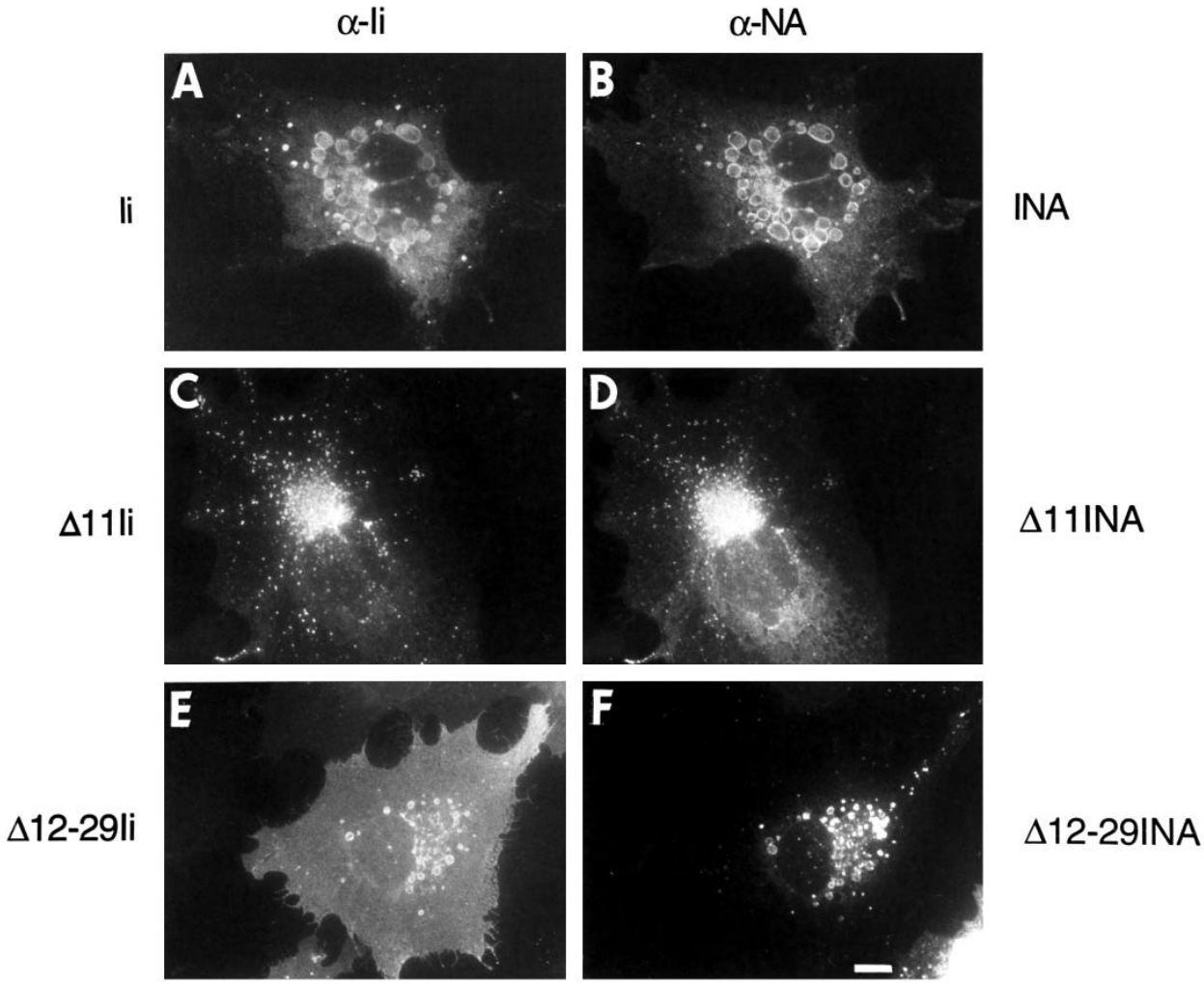

Fig. 10. Immunofluorescence analysis of CV1 cells co-transfected with Ii and INA deletion mutants. CV1 cells were co-transfected with Ii + INA, Δ11Ii + Δ11INA and Δ12–29Ii + Δ12–29INA. The cells were fixed in methanol and stained for Ii variants with the monoclonal anti-Ii antibody BU45 and a FITC-conjugated second antibody (A,C,E) and for INA variants with the polyclonal anti-NA serum WIC103 and a Texas Red-conjugated second antibody (B,D,F). Bar, 20 μm.

leucine signal is functional at the carboxy-terminal end of the molecule. This may suggest a requirement for residues at the amino side of the signal. In this study we changed the leucine 14 to alanine in Δ11INA without any effect on internalization, but more elaborate studies are needed to elucidate a potential larger recognition motif. The Ii cytoplasmic tail is found to be phosphorylated (Spiro and Quaranta, 1989) and phosphorylation might play a role for internalization as suggested for membrane receptors (Trowbridge, 1991). However, alteration of the only phosphorylation candidate within the eleven residue cytoplasmic tail of Δ12-29INA did not influence internalization, so excluding an absolute requirement for phosphorylation in this process. This is in agreement with the results for the Man-6P/IGF-II receptor where mutation of the phosphorylation sites had no detectable effect on the sorting of cathepsin D (Johnson and Kornfeld, 1992a).

The internalization rate of INA (25%/minute) is similar to the rate found for Ii in a human B cell line and stably transfected fibroblasts (Roche et al., 1993). Furthermore Δ12-29INA with the LI signal alone was just as efficiently internalized as INA, whereas constructs containing only the ML-based motif was less efficient. However, as the context and the localization of the two signals within the tail is different we may not draw a general conclusion about the efficiency of the two motifs.

In this study we have concentrated on the Ii cytoplasmic tail transplanted to NA, and the characteristics of this fusion molecule are in many respects similar to the native Ii molecule. Crosslinking studies show that INA like NA alone form multimers that migrate as tetramers on SDS gels. This multimerisation is most likely determined by the tetrameric neuraminidase as Ii is a trimer (Roche et al., 1991; Lamb and Cresswell, 1992; Gedde-Dahl and Bakke, unpublished). Although the multimerisation state is different, cotransfection studies showed that both signals within the Ii tail sorted NA to the same endocytic compartments as Ii and mutated Ii molecules. As stated above the rate of internalization for Ii and INA is similar and the cytoplasmic tail mutations that repressed internalization of INA also inhibited internalization of Ii and resulted in plasma membrane accumulation. Finally, NA fused with the extended Ii tail (LINA) was arrested in the ER like the native Ii molecule with this extension. Both the sorting and

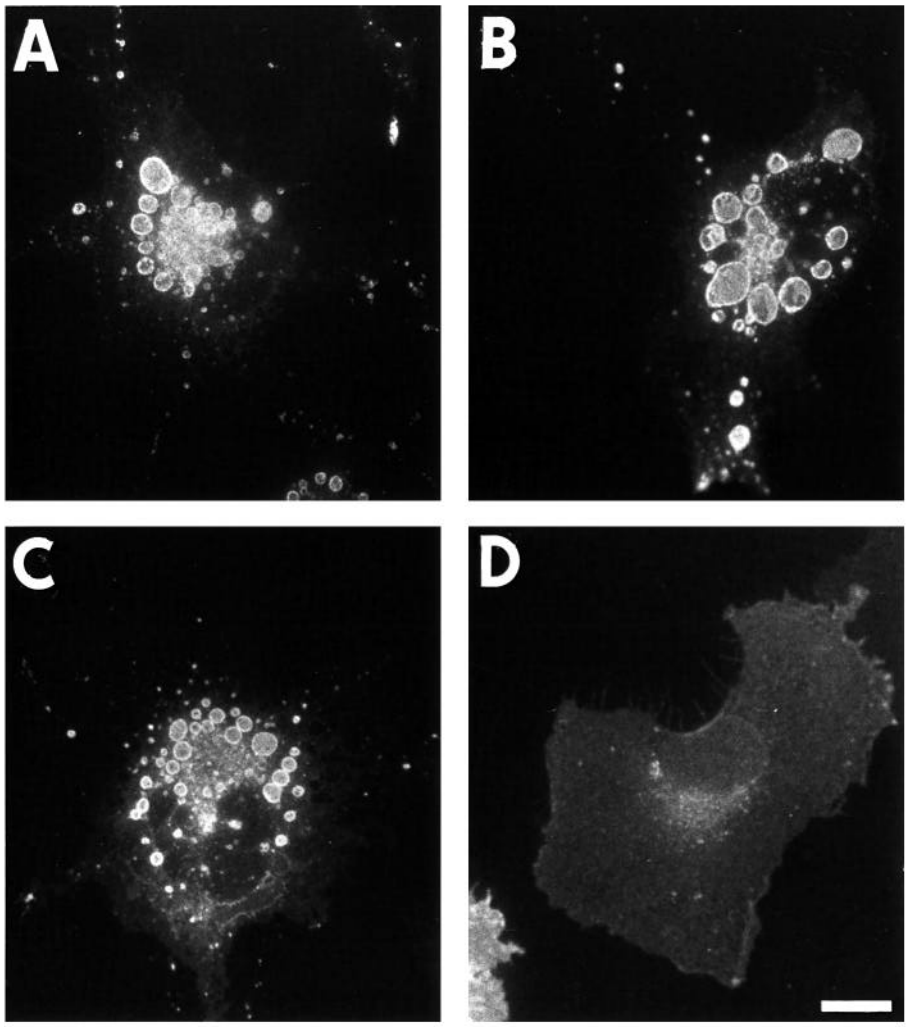

Fig. 11. Localization of Ii mutants with signals mutated individually in CV1 cells. CV1 cells transfected with Ii (A), Ii *leu7 to ala* (B), Ii *leu17 to ala* (C) and Ii *leu7 to ala / leu17 to ala* (D) were fixed in methanol and stained with the monoclonal antibody BU45 and an FITC-conjugated goat anti-mouse second antibody. Bar, 20 μm.

retention signals of Ii are thus localized to sequences in the cytoplasmic tail.

Despite the similarities of the Ii protein and the INA fusion protein there seems to be a major difference; Ii, but not INA, induces the large endosomal vesicles (Romagnoli et al., 1993; Pieters et al., 1993). These vacuoles might depend on features within the amino-terminal 11 residues of the cytoplasmic tail and other features in the remaining Ii molecule as neither Δ11Ii nor the INA molecules could create such large vesicular structures. Our results demonstrate that the LI signal within these 11 residues is not essential as long as the other ML-based sorting signal is intact. The sequence requirements for endosomal retention within these 11 amino acids still remain to be elucidated.

Whether the αβIi complex is delivered to endosomal compartments via internalization from the plasma membrane (Roche et al., 1993) or following a direct pathway from the Golgi apparatus (reviewed by Neefjes and Ploegh, 1992) is controversial and both routes may be functional. Cell surface appearance of Ii has been reported previously (Claesson-Welsh et al., 1986; Reske et al., 1987; Wraight et al., 1990; Koch and Stockinger, 1991) and the significance of this may be that the plasma membrane is an intermediate in the transport pathway to endosomes for both the complex and Ii alone. Our data show that the INA molecule is routed via the plasma membrane and thereafter internalized and will accordingly support such a transport route although we cannot exclude the possibility that endosomal sorting also takes place in the TGN. The sorting signals might thus be read both at the level of the TGN and at the plasma membrane. At variable cellular states the same molecule could be sorted either directly from the TGN to the endocytic pathway or to endosomes via the plasma membrane. Such a split sorting dependent on differentiation state is suggested for the transport of LAMP1 to lysosomes in the studies of Carlsson and Fukuda (1992).

The LL/LI signal is present in numerous molecules like the tyrosine kinase receptors (Hanks et al., 1988) in addition to the two M6PR (Johnson and Kornfeld, 1992a,b) and the T-cell receptor (Letourneur and Klausner, 1992), where they were first elucidated. As with the γ-chain of the T-cell receptor the motif is also present in molecules that are not normally sorted to the endocytic pathway. In a study by Nilsson et al. (1991) the cytoplasmic Ii tail was exchanged with that of galactosyltransferase, which contains a di-leucine, and the resulting molecule was in fact sorted to endosomes. One putative function of the di-leucine signal might be to sort molecules to the endocytic pathway for degradation if they are missorted to the plasma membrane and possibly TGN. Why two internalization signals are present within the cytoplasmic tail of Ii, both of which seem to mediate efficient internalization is not

known. Also, the detailed context requirements for the LL/LI/IL/ML-based sorting signals have not been explored. It will thus be a challenge to elucidate these and search for interacting molecules to understand the function of such seemingly simple signals.

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REFERENCES

Bakke, O. and Dobberstein, B. (1990). MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell* **63**, 707-716.

Bansal, A. and Gierasch, L. M. (1991). The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell* **67**, 1195-1201.

Carlsson, S. R., Roth, J., Piller, F. and Fukuda, M. (1988). Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. Major sialoglycoproteins carrying polylactosaminoglycan. *J. Biol. Chem.* **263**, 18911-18919.

Carlsson, S. R. and Fukuda, M. (1992). The lysosomal membrane glycoprotein lamp-1 is transported to lysosomes by two alternative pathways. *Arch. Biochem. Biophys.* **296**, 630-639.

Chen, J. W., Murphy, T. L., Willingham, M. C., Pastan, I. and August, J. T. (1985). Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* **101**, 85-95.

Claesson, L. and Peterson, P. A. (1983). Association of human gamma chain with class II transplantation antigens during intracellular transport. *Biochemistry* **22**, 3206-3213.

Claesson-Welsh, L., Scheynius, A., Tjernlund, U. and Peterson, P. A. (1986). Cell surface expression of invariant gamma-chain of class II histocompatibility antigens in human skin. *J. Immunol.* **136**, 484-490.

Collawn, J. F., Stangel, M., Kuhn, L. A., Esekogwu, V., Jing, S. Q., Trowbridge, I. S. and Tainer, J. A. (1990). Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell* **63**, 1061-1072.

Copeland, C. S., Doms, R. W., Bolzau, E. M., Webster, R. G. and Helenius, A. (1986). Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* **103**, 1179-1191.

Cresswell, P. (1992). Chemistry and functional role of the invariant chain. *Curr. Opin. Immunol.* **4**, 87-92.

Eberle, W., Sander, C., Klaus, W., Schmidt, B., von Figura, K. and Peters, C. (1991). The essential tyrosine of the internalization signal in lysosomal acid phosphatase is part of a beta turn. *Cell* **67**, 1203-1209.

Germain, R. N. and Margulies, D. H. (1993). The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* **11**, 403-450.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-182.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Huylebroeck, D., Maertens, G., Verhoeyen, M., Lopez, C., Raeymakers, A., Jou, W. M. and Fiers, W. (1988). High-level transient expression of influenza virus proteins from a series of SV40 late and early replacement vectors. *Gene* **66**, 163-181.

Johnson, K. F. and Kornfeld, S. (1992a). The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol.* **119**, 249-257.

Johnson, K. F. and Kornfeld, S. (1992b). A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. *J. Biol. Chem.* **267**, 17110-17115.

Koch, N. and Stockinger, B. (1991). Molecules that modify antigen recognition. *Curr. Opin. Immunol.* **3**, 10-15.

Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* **154**, 367-382.

Kvist, S., Wiman, K., Claesson, L., Peterson, P. A. and Dobberstein, B. (1982). Membrane insertion and oligomeric assembly of HLA-DR histocompatibility antigens. *Cell* **29**, 61-69.

Lamb, C. A. and Cresswell, P. (1992). Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *J. Immunol.* **148**, 3478-3482.

Lehmann, L. E., Eberle, W., Krull, S., Prill, V., Schmidt, B., Sander, C., von Figura, K. and Peters, C. (1992). The internalization signal in the cytoplasmic tail of lysosomal acid phosphatase consists of the hexapeptide PGYRHV. *EMBO J.* **11**, 4391-4399.

Letourneur, F. and Klausner, R. D. (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell* **69**, 1143-1157.

Lotteau, V., Teyton, L., Peleraux, A., Nilsson, T., Karlsson, L., Schmid, S. L., Quaranta, V. and Peterson, P. A. (1990). Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348**, 600-605.

Marks, M. S., Blum, J. S. and Cresswell, P. (1990). Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J. Cell Biol.* **111**, 839-855.

Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J. and Ploegh, H. L. (1990). The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* **61**, 171-183.

Neefjes, J. J. and Ploegh, H. L. (1992). Intracellular transport of MHC class II molecules. *Immunol. Today* **13**, 179-184.

Nilsson, T., Lucocq, J. M., Mackay, D. and Warren, G. (1991). The membrane spanning domain of beta-1, 4-galactosyltransferase specifies trans Golgi localization. *EMBO J.* **10**, 3567-3575.

Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. and von Figura, K. (1990). Targeting of a lysosomal membrane protein: a tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO J.* **9**, 3497-3506.

Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. and Geuze, H. J. (1991). Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments (see comments). *Nature* **349**, 669-676.

Pieters, J., Bakke, O. and Dobberstein, B. (1993). The MHC class II associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J. Cell Sci.* **106**, 831-846.

Prehn, S., Herz, J., Hartmann, E., Kurzchalia, T. V., Frank, R., Roemisch, K., Dobberstein, B. and Rapoport, T. A. (1990). Structure and biosynthesis of the signal-sequence receptor. *Eur. J. Biochem.* **188**, 439-445.

Reske, K., Mohle, U., Sun, D. and Wekerle, H. (1987). Synthesis and cell surface display of class II determinants by long-term propagated rat T line cells. *Eur. J. Immunol.* **17**, 909-914.

Roche, P. A., Marks, M. S. and Cresswell, P. (1991). Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* **354**, 392-394.

Roche, P. A., Teletski, C. L., Karp, D. R., Pinet, V., Bakke, O. and Long, E. O. (1992). Stable surface expression of invariant chain prevents peptide presentation by HLA-DR. *EMBO J.* **11**, 2841-2847.

Roche, P. A., Teletski, C. L., Stang, E., Bakke, O. and Long, E. O. (1993). Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. *Proc. Nat. Acad. Sci. USA* **90**, 8581-8585.

Romagnoli, P., Layet, C., Yewdell, J., Bakke, O. and Germain, R. N. (1993). Relationship between invariant chain expression and major histocompatibility complex class II transport into early and late endocytic compartments. *J. Exp. Med.* **177**, 583-596.

Simonsen, A., Momburg, F., Drexler, J., Hämmerling, J. H. and Bakke, O. (1993). Intracellular distribution of the MHC class II molecules and the associated invariant chain (Ii) in different cell lines. *Int. Immunol.* **5**, 903-917.

Spiro, R. C. and Quaranta, V. (1989). The invariant chain is a phosphorylated subunit of class II molecules. *J. Immunol.* **143**, 2589-2594.

Strubin, M., Mach, B. and Long, E. O. (1984). The complete sequence of the mRNA for the HLA-DR-associated invariant chain reveals a polypeptide with an unusual transmembrane polarity. *EMBO J.* **3**, 869-872.

Strubin, M., Long, E. O. and Mach, B. (1986). Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. *Cell* **47**, 619-625.

Sung, E. and Jones, P. P. (1981). The invariant chain of murine Ia antigens: its glycosylation, abundance and subcellular localization. *Mol. Immunol.* **18**, 899-913.

Trowbridge, I. S. (1991). Endocytosis and signals for internalization

(published erratum appears in *Curr. Opin. Cell Biol.* (1991) **3**, 1062). *Curr. Opin. Cell Biol.* **3**, 634-641.

Van Rompuy, L., Jou, W. M., Huylebroeck, D. and Fiers, W. (1982). Complete nucleotide sequence of a human influenza neuraminidase gene of subtype N2 (A/Victoria/3/75). *J. Mol. Biol.* **161**, 1-11.

Varghese, J. N., Laver, W. G. and Colman, P. M. (1983). Structure of the influenza virus glycoprotein antigen neuraminidase at 2. 9 Å resolution. *Nature* **303**, 35-40.

Vega, M. A., Rodriguez, F., Segui, B., Cales, C., Alcalde, J. and Sandoval, I. V. (1991a). Targeting of lysosomal integral membrane protein LIMP II. The tyrosine-lacking carboxyl cytoplasmic tail of LIMP II is sufficient for direct targeting to lysosomes. *J. Biol. Chem.* **266**, 16269-16272.

Vega, M. A., Segui-Real, B., Garcia, J. A., Cales, C., Rodriguez, F., Vanderkerckhove, J. and Sandoval, I. V. (1991b). Cloning, sequencing, and expression of a cDNA encoding rat LIMP II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36. *J. Biol. Chem.* **266**, 16818-16824.

Williams, M. A. and Fukuda, M. (1990). Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* **111**, 955-966.

Wraight, C. J., van Endert, P., Moller, P., Lipp, J., Ling, N. R., MacLennan, I. C., Koch, N. and Moldenhauer, G. (1990). Human major histocompatibility complex class II invariant chain is expressed on the cell surface. *J. Biol. Chem.* **265**, 5787-5792.

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