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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

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Lysosomal acid phosphatase (LAP) is synthesized as a transmembrane protein with a short carboxy-terminal cytoplasmic tail of 19 amino acids, and processed to a soluble protein after transport to lysosomes. Deletion of the membrane spanning domain and the cytoplasmic tail converts LAP to a secretory protein, while deletion of the cytoplasmic tail as well as substitution of tyrosine 413 within the cytoplasmic tail against phenylalanine causes accumulation at the cell surface. A chimeric polypeptide, in which the cytoplasmic tail of LAP was fused to the ectoplasmic and transmembrane domain of hemagglutinin is rapidly internalized and tyrosine 413 of the LAP tail is essential for internalization of the fusion protein. A chimeric polypeptide, in which the membrane spanning domain and cytoplasmic tail of LAP are fused to the ectoplasmic domain of the M_r 46 kd mannose 6-phosphate receptor, is rapidly transported to lysosomes, whereas wild type receptor is not transported to lysosomes. We conclude that a tyrosine containing endocytosis signal in the cytoplasmic tail of LAP is necessary and sufficient for targeting to lysosomes.

Key words: endocytosis signal/internalization/lysosomes/targeting

Introduction

Several proteins of the lysosomal membrane have been identified in recent years (Lewis *et al.*, 1985; Chen *et al.* 1985; Lippincott-Schwartz and Fambrough, 1986; Barriocanal *et al.*, 1986). The contribution of these proteins to the specific functions of the lysosomal membrane, such as generation and maintenance of an acidic milieu, stability towards lysosomal hydrolases, and transport of low molecular weight components into and out of lysosomes remains to be established. Structural features common to this class of membrane proteins are a high number of *N*-linked sialylated oligosaccharides, a single membrane spanning domain and relatively short carboxy-terminal cytoplasmic tails of 10–11 residues (Fambrough *et al.*, 1988; Chen *et al.*, 1988; Viitala *et al.*, 1988; Howe *et al.*, 1988). In contrast to soluble proteins of the lysosomal matrix the lysosomal membrane proteins are routed from the Golgi complex to lysosomes independently of mannose 6-phosphate receptors. Minor fractions of some of the lysosomal membrane glycoproteins are located at the plasma membrane, where their appearance can be influenced by the state of differentiation of the cell (Viitala *et al.*, 1988;) or the lysosomal pH (Lippincott-Schwartz and Fambrough, 1987). The structural features that are involved in targeting of lysosomal membrane proteins are unknown. For a growing number of membrane glycoproteins it has been shown that sorting signals are localized within their cytoplasmic tails. The trafficking of receptors for LDL (Davis *et al.*, 1986, 1987) polymeric Ig (Mostov *et al.*, 1986), EGF (Prywes *et al.*, 1986), transferrin (Rothenberger *et al.*, 1987; Jacopetta *et al.*, 1988) and mannose 6-phosphate (Lobel *et al.*, 1989) depends on signals within their cytoplasmic tails. One of these trafficking signals mediates rapid endocytosis and contains an essential tyrosine residue. Exchange of a tyrosine residue within the cytoplasmic tail of the LDL receptor by a non-aromatic amino acid impairs its integration into coated pits and internalization (Davis *et al.*, 1987). Replacement of a cysteine by a tyrosine within the cytoplasmic tail converts the influenza virus hemagglutinin from a protein that is excluded from coated pits into one that is integrated into coated pits and recycled with kinetics comparable to that of endocytic receptors (Lazarovits and Roth, 1988).

Lysosomal acid phosphatase (LAP) resembles other lysosomal membrane proteins in some of its features such as dense glycosylation of the ectoplasmic domain, short cytoplasmic tail of 19 residues, mannose 6-phosphate receptor independent targeting, and partial localization at the cell surface (Pohlmann *et al.*, 1988; Waheed *et al.*, 1988; Gottschalk *et al.*, 1989a). The pathway to lysosomes includes passage of the trans Golgi, cell surface and endosomes. After synthesis at the endoplasmic reticulum the precursors of LAP are transported to the trans Golgi with a $t_{1/2}$ of 30 min, from where they reach the cell surface within less than 10 min. The cell surface associated precursors are subject to rapid internalization and recycling to the cell surface. On average precursors are retained for 5–6 h in this cell surface/endosome pool and recycle more than 15 times between the cell surface and endosomes before they are delivered to lysosomes (Braun *et al.*, 1989). After transfer to lysosomes the precursors are subject to limited proteolysis, which generates the soluble mature form of LAP (Gottschalk *et al.*, 1989b).

In the present study we have analyzed the role of the cytoplasmic tail of LAP for targeting to lysosomes. We show that deletion of the cytoplasmic tail results in accumulation of the truncated LAP precursors at the cell surface. The truncated LAP precursors are transferred by a slow process ($t_{1/2}$ ~1 day) from the cell surface to lysosomes, where they are processed to mature forms. Substitution of the single tyrosine in the cytoplasmic tail by phenylalanine arrests transport at the cell surface to a similar extent as deletion of the entire cytoplasmic tail. Furthermore, the cytoplasmic tail of LAP is sufficient to direct non-lysosomal membrane proteins to lysosomes.

Results

The transmembrane domain of LAP is required for intracellular retention and the cytoplasmic tail for efficient targeting to lysosomes

The membrane associated LAP precursor, which represents the transport form of LAP, and the soluble LAP, which represents the mature form of LAP located in lysosomes, can be differentiated by size and their behavior in detergent condensation. In expressing baby hamster kidney (BHK) cells the 63 kd precursor is largely recovered in the detergent phase, whereas the 52 kd mature form is exclusively found in the aqueous phase (Figure 1).

Translation termination codons were introduced into the LAP cDNA before (LAP-RP2) and after (LAP-RP3) the membrane spanning domain (see Figure 2). LAP-RP2 was secreted into the medium (Figure 1) and exhibited acid phosphatase activity (data not shown). The small fraction of LAP-RP2 found intracellularly was recovered in the aqueous phase. Thus, the luminal domain of LAP forms a catalytically active protein, but lacks the structural features necessary for intracellular retention and targeting to lysosomes. LAP-RP3 encoded polypeptides were retained intracellularly and were processed to a soluble form indistinguishable from the mature form derived from wild type LAP (Figure 1). Subcellular fractionation by Percoll density centrifugation showed that the mature form of LAP-RP3 is enriched in dense lysosomes (Figure 3, upper panel). Compared to wild type LAP the transport of LAP-RP3 to dense lysosomes was significantly slower. While wild type LAP is transported to dense lysosomes with a $t_{1/2}$ of 6–7 h (Gottschalk *et al.*, 1989b), LAP-RP3 is transported with a $t_{1/2}$ of ~24 h to dense lysosomes (Figure 3, lower left panel). A further difference between wild type and LAP-RP3 concerns the proteolytic processing in dense lysosomes. Wild type LAP precursors persist for 6–7 h in dense lysosomes before they are processed to the mature form (Gottschalk *et al.*, 1989b). LAP-RP3 precursors were hardly detectable in dense lysosomes, suggesting that they are processed immediately after delivery to lysosomes. The slow transport of LAP-RP3 changes the equilibrium distribution of LAP activity between dense lysosomes and light membranes. In BHK cells expressing wild type LAP, 40% of total LAP enzyme activity (precursor and mature form) are associated with dense lysosomes (Waheed *et al.*, 1988; Gottschalk *et al.*, 1989b), while this fraction is diminished to 28% in BHK cells expressing LAP-RP3 (Figure 3, lower right panel).

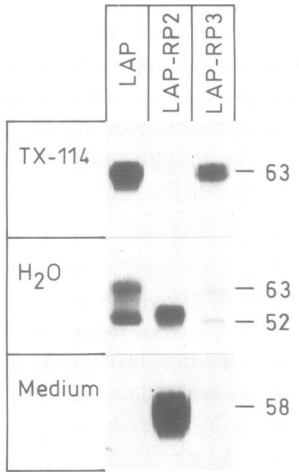

Fig. 1. Distribution of wild type and truncated forms of LAP in BHK cells. BHK cells expressing wild type LAP (LAP), LAP lacking the transmembrane domain and cytoplasmic tail (LAP-RP2) or LAP lacking the cytoplasmic tail (LAP-RP3) were metabolically labelled for 16 h with [^{35}S]methionine. The cell extract was phase separated with Triton X-114 into a detergent (TX-114) and an aqueous (H_2O) phase. The LAP was immunoprecipitated from the two phases and from the medium. The molecular weights of the membrane associated precursors (63 kd) and the mature forms (52 kd) of wild type LAP and of the secreted LAP-RP2 (58 kD) are indicated.

LAP-RP3 accumulates at the cell surface before transport to lysosomes

In BHK cells expressing wild type LAP between 15 and 20% of the LAP precursors are located at the cell surface. These precursors are in equilibrium with those in endosomes. In contrast to the intracellular precursors, the cell surface

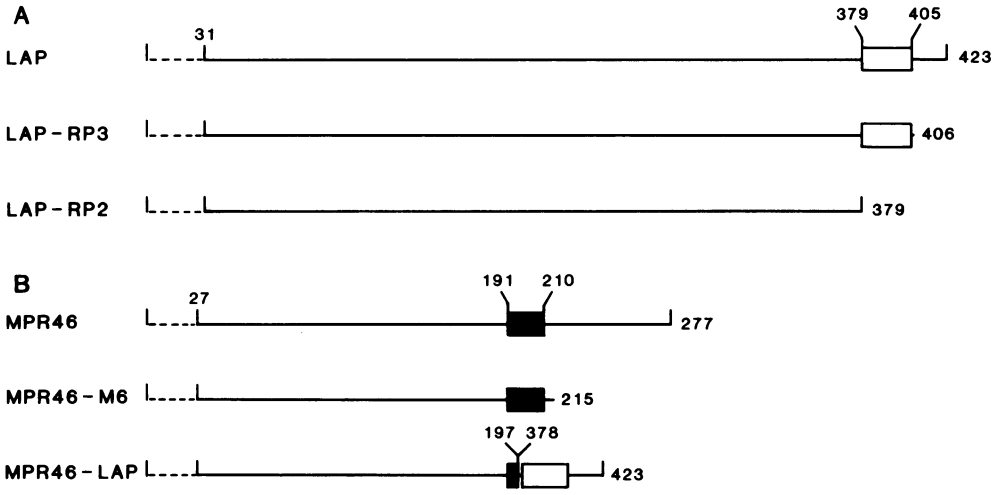

Fig. 2. Structure of the wild type and mutants of LAP, MPR46, and of the chimeric receptor-LAP polypeptides. N-terminal cleavable signal sequences are indicated by dashed lines. Boxes represent the transmembrane domain of LAP (open) and MPR46 (filled). The numbers refer to the first amino acid of the precursor with cleaved signal sequence, the membrane spanning domain, the cytoplasmic tail and to the C-terminal residue (for numbering of LAP and MPR46 see Pohlmann *et al.*, 1988 and 1987, respectively).

associated precursors are accessible at 4°C to antibodies, to neuraminidase or to lactoperoxidase catalyzed iodination (Braun *et al.*, 1989).

To determine the portion of cell surface-associated LAP, precursor cells were labeled for 2 h and chased for 2 h. Subsequently cells were incubated with neuraminidase at 4°C. The precursor LAP was immunoprecipitated from the detergent extract of cells and analyzed by SDS−PAGE or isoelectric focusing. Neuraminidase treatment decreases the size of the LAP precursors by about 3−4 kd and converts the acidic forms (pI 4.9−5.6) to more basic forms (pI 6.0−6.3) (Braun *et al.*, 1989). In BHK cells expressing wild type LAP, 15% of the precursors are accessible to neuraminidase as estimated by the shift of pI (Figure 4B). This fraction is too small to be detectable by SDS−PAGE (Figure 4A). In BHK cells expressing LAP-RP3 the pI of about 80% of the precursors was shifted to pI 6.0−6.3 (Figure 4B) and a reduction in size by 3−4 kd was apparent after separation by SDS−PAGE. This reduction in size was more obvious for the dimeric form of precursor LAP (Figure 4A). These results demonstrate that 2 h after synthesis about 80% of the LAP-RP3 precursors are located at the cell surface compared to 15% of wild type LAP.

To investigate the time span of accumulation of LAP-RP3 precursors at the cell surface, cells were labelled for 1 h, chased for 1−14 h and analyzed by SDS−PAGE (Figure 5). During the entire chase period essentially all LAP-RP3 precursors were accessible to neuraminidase (desialylation decreases the size of the molecules by about 4 kd, see Figure 4A) and hence are located at the cell surface. In the same type of experiment performed with wild type expressing cells only about 10−13% of total LAP precursors were located at the cell surface. Furthermore, in contrast to wild type LAP (due to the slow transport of LAP-RP3 precursors to lysosomes) the total LAP signal hardly declined over the chase period of 14 h [compare with Braun *et al.* (1989) for wild type LAP data].

The arrest of the LAP-RP3 precursors at the cell surface was also demonstrated with the immunofluorescence approach of Lazarovits and Roth (1988). LAP antibodies were bound at 0°C to the cell surface of BHK cells and examined for their localization directly after binding and after

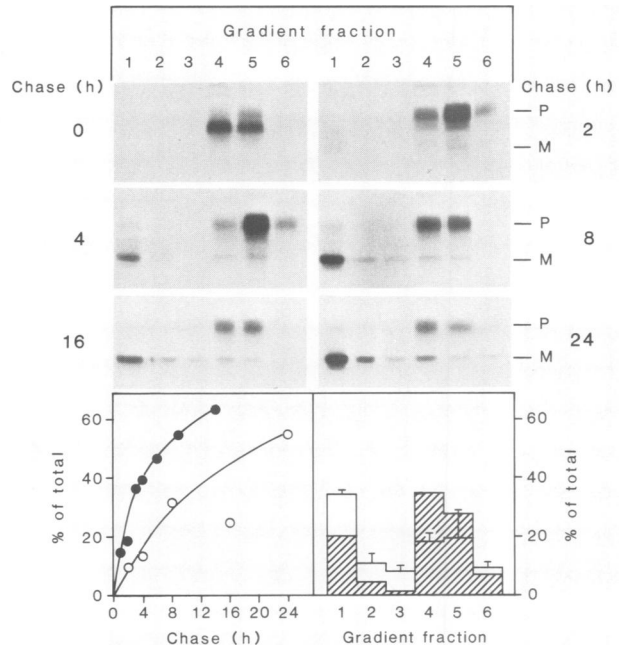

Fig. 3. Transport of LAP-RP3 mutant protein. **Upper panel**: LAP-RP3 expressing BHK cells were labeled for 1 h and chased for 0, 2, 4, 8, 16 or 24 h as indicated. The post-nuclear supernatants were subjected to Percoll density centrifugation. LAP-RP3 was immunoprecipitated from the gradient fractions and seperated by SDS−PAGE. P, LAP-RP3 precursor, membrane bound; M, LAP-RP3, mature, soluble form. **Lower left panel**: LAP polypeptides in dense lysosomes (gradient fractions 1 and 2) and light membranes (gradient fractions 4 and 5) were quantified by densitometry, and LAP in dense lysosomes expressed as percentage of total LAP (--o--). For comparison the data on transport of wild type LAP to lysosomes taken from Gottschalk *et al.* (1989b) are shown (--●--). *Lower right panel*: distribution of β-hexosaminidase (clear area) and acid phosphatase (hatched area) activities in gradient fractions 1−6 (from bottom to top). Bars indicate the variation of β-hexosaminidase activity in four individual gradients.

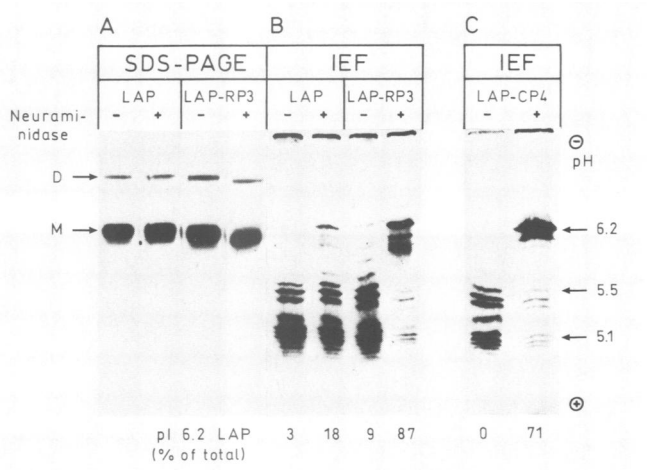

Fig. 4. Wild type LAP, LAP-RP3 and LAP-CP4 precursors accessible to neuraminidase at 4°C. Cells labeled and chased for 2 h at 37°C, respectively were incubated for 1 h at 4°C with or without neuraminidase. After phase separation of the cell extracts with Triton X-114, the precursor was immunoprecipitated from the detergent phase. Aliquots of the immunoprecipitates were subjected to SDS−PAGE (**A**) or isoelectric focussing (**B**, **C**). The numbers below the lanes represent the percentage of precursor shifted by neuraminidase to pH 6.0−6.3. M and D denote positions of LAP monomers and LAP dimers resistant to boiling in SDS and dithiothreitol, respectively.

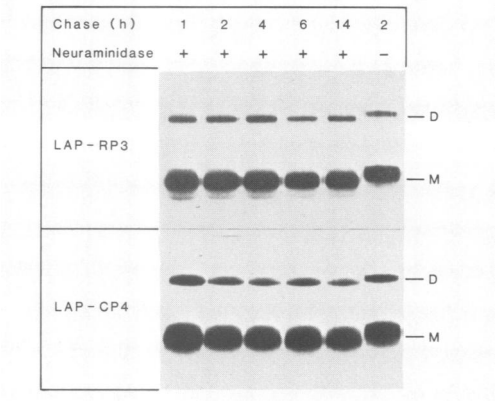

Fig. 5. Cell surface as the major localization of LAP-RP3 and LAP-CP4 precursors during a prolonged chase. BHK cells expressing LAP-RP3 or LAP-CP4 labeled for 1 h were chased for 1−14 h and then incubated at 4°C with neuraminidase as indicated above the lanes. After phase separation with Triton X-114 the precursors were immunoprecipitated and analyzed by SDS−PAGE. The positions of monomeric (M) and dimeric (D) forms are indicated.

reculturing of the cells for 60 min at 37°C. The punctuated pattern of antibodies bound at 4°C (Figure 6A,B) was replaced by a diffuse staining when the cells were fixed prior to incubation with LAP antibodies (not shown). It reflects therefore an antibody induced rather than a natural clustering of cell-surface associated LAP. After reculturing the cells at 37°C the antibodies bound to wild type LAP were rapidly internalized and accumulated in vacuoles (Figure 6A, bottom left), which distribute as the lysosomal marker arylsulfatase B (not shown) and hardly any antibodies were detectable at the cell surface (Figure 6B, bottom left). The traces of antibodies still present at the cell surface are attributed to the recycling of LAP (Braun *et al.*, 1989). In cells expressing LAP-RP3 the antibodies remained associated with the cell surface even after an incubation for 1 h at 37°C (Figure 6A,B, middle).

These results indicate that newly synthesized LAP-RP3 accumulates at the cell surface. From the cell surface LAP-RP3 precursors are transferred to dense lysosomes with a $t_{1/2}$ of ~1 day. Two independent observations suggest that

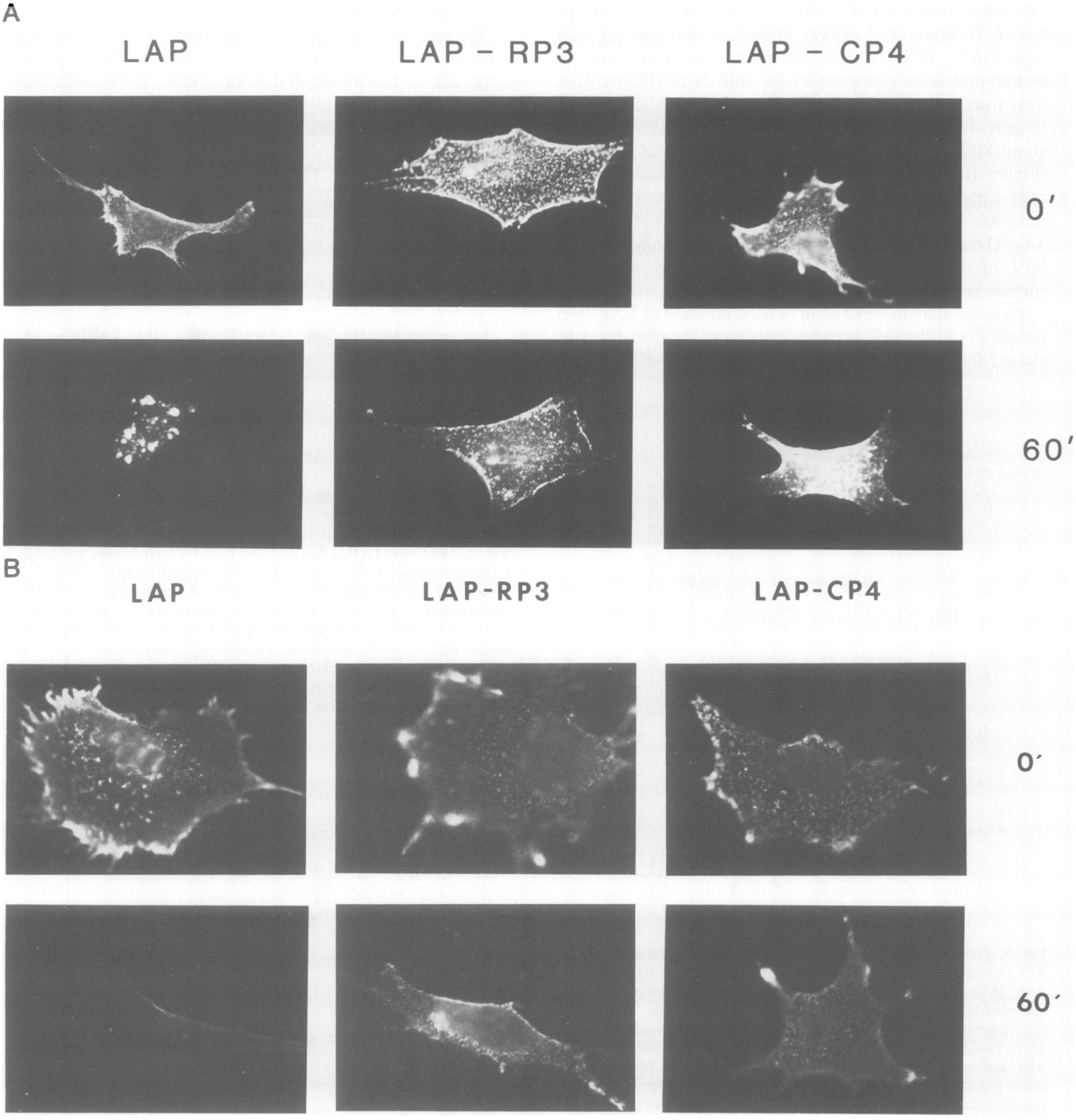

Fig. 6. Internalization of wildtype and mutant LAP. LAP, LAP-RP3 and LAP-CP4 expressing BHK cells were incubated with an anti-LAP antiserum for 1 h at 0°C. **(A)** Cells were washed and fixed immediately (0′) or incubated at 37°C for 60 min and then fixed with paraformaldehyde (60′). Cells were permeabilized with detergent and rhodamine conjugated immunoglobulin was used as second antibody. **(B)** For selective detection of cell surface associated antibodies cells were incubated with the second antibody (0′) or recultured at 37°C for 60 min and then incubated with the second antibody (60′) before fixation. Detergent permeabilization was omitted. The microscope was focused on the cell periphery of cells with surface staining and on vesicles when intracellular staining was observed.

LAP-RP3 precursors do not accumulate intracellularly during transport from the cell surface to lysosomes. Firstly, LAP-RP3 precursors are accessible to neuraminidase during prolonged chase (Figure 5), and secondly, LAP-RP3 precursors are not found in dense lysosomes (Figure 3).

Tyrosine 413 in the cytoplasmic tail is essential for rapid internalization

Tyrosine residues in the cytoplasmic tails of the LDL-receptor, a mutant hemagglutinin and the M_r 300 kd mannose 6-phosphate receptor are essential for rapid internalization of these polypeptides (Davis *et al.*, 1986, 1987; Lazarovits and Roth 1988; Lobel *et al.*, 1989). The cytoplasmic tail of LAP contains a single tyrosine in position 413. In LAP-CP4 this tyrosine was changed to phenylalanine (Figure 7). In LAP-CP4-expressing BHK cells, labeled for 2 h and chased for 2 h, 70% of the LAP-CP4 precursors were accessible to neuraminidase at 4°C (Figure 4C). During a chase for 1–14 h no intracellular LAP-CP4 precursors were detectable (Figure 5, lower panel). In the antibody internalization assay antibodies bound at 0°C to cells expressing LAP-CP4 remained cell-surface localized after reculturing for 1 h at 37°C as shown by the staining pattern of permeabilized (Figure 6A, bottom right) and non-permeabilized (Figure 6B, bottom right) cells. These results indicate that tyrosine 413 is an essential component of the signal in the cytoplasmic tail which is required for rapid internalization.

The cytoplasmic tail of LAP confers rapid internalization and lysosomal targeting to non-lysosomal proteins

The experiments outlined above had substantiated that the cytoplasmic domain is essential for rapid internalization of cell-surface located LAP precursors and thereby for its efficient transport to lysosomes, and that tyrosine 413 is an essential part of this endocytosis signal. To test whether this signal is not only essential but also sufficient for rapid internalization and lysosomal targeting a chimeric polypeptide consisting of the ectoplasmic and transmembrane domain of the plasma membrane sorted glycoprotein hemagglutinin (HA) and the cytoplasmic tail of LAP was constructed (HA–LAP, Figure 7B). In contrast to wild type HA the chimera HA–LAP is rapidly internalized since anti-HA antibodies bound to the cell surface at 0°C remained at the plasma membrane of wild type HA expressing cells whereas they were transferred to vesicular structures in HA–LAP expressing cells upon reculturing for 1 h at 37°C (Figure 8A, left and middle). Substitution of the single tyrosine residue in the cytoplasmic domain of the HA–LAP chimera (HA–CP4, Figure 7B) by phenylalanine reverted the rapid internalization almost completely (Figure 8A, right). The internalization (HA–LAP) or non-internalization (HA–CP4) of HA-associated antibodies during the reculturing period was mirrored by the disappearance or persistence of immunofluorescent staining at the cell surface, respectively, if the cells were not permeabilized prior to staining (Figure 8B).

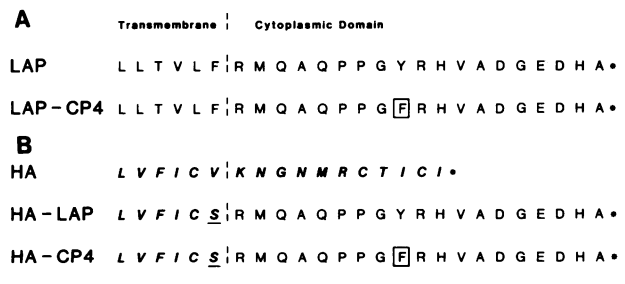

Fig. 7. Amino acid sequences of LAP mutants and HA–LAP chimeras. **(A)** In the mutant LAP-CP4 tyrosine 413 in the cytoplasmic domain of wild type (LAP) was changed to phenylalanine (boxed). **(B)** The ectoplasmic and transmembrane domains of influenza virus hemagglutinin (HA) were fused to the cytoplasmic domain of lysosomal acid phosphatase (HA–LAP). The C-terminal valine of the hemagglutinin (HA) transmembrane domain was changed to serine (underlined) due to the introduction of an *Xho*I restriction site (see Materials and methods). For construction of HA–CP4 the tyrosine 413 of the HA–LAP cytoplasmic domain was changed to phenylalanine (boxed). Sequences are shown in one letter code.

As a second indicator molecule we utilized the M_r 46 kd mannose 6-phosphate receptor (MPR46). A chimeric protein consisting of the ectoplasmic domain and 6 amino acids of the transmembrane domain of MPR46 and the complete transmembrane and cytoplasmic domains of LAP was constructed (MPR46–LAP; see Figure 2B and Materials and methods). Wild type MPR46 recycles between Golgi apparatus and endosomes and between plasma membrane and endosomes; it does not enter lysosomes in detectable amounts (Stein *et al.*, 1987a; Bleekemolen *et al.*, 1989). An endocytosis signal necessary for rapid internalization is residing in the cytoplasmic tail of the receptor (B.Weber and R.Pohlmann, unpublished results). This is also shown in the antibody internalization assay depicted in Figure 9; receptor antibodies bound to the cell surface of BHK cells expressing wild type MPR46 are endocytosed into small vesicular structures most likely representing endosomes within 1 h of reculturing at 37°C. In contrast receptor antibodies bound to truncated MPR46 lacking all but 6 amino acids of the cytoplasmic domain (MPR46-M6; Figure 2B) remain at the plasma membrane (Figure 9A and B, left and middle). Receptor antibodies bound to the cell surface of BHK cells expressing the MPR46–LAP chimera were readily internalized into larger lysosome-like vesicles (Figure 9A, right) and cleared from the plasma membrane after 1 h of reculturing at 37°C (Figure 9B, right). Since the transmembrane domain of LAP is not sufficient for rapid internalization of LAP, rapid endocytosis of the MPR46–LAP chimera is most likely due to the cytoplasmic tail of LAP.

The half life of MPR46–LAP polypeptides is approximately 2 h, as determined by metabolic labeling of expressing BHK cells for 1 h, and chasing for appropriate times, followed by immunoprecipitation and quantification of MPR46–LAP polypeptides (data not shown). Despite this short half life MPR46–LAP fusion protein must be considered folded correctly since it binds to phosphomannan in a mannose 6-phosphate dependent manner (B.Weber and R.Pohlmann, unpublished) and undergoes carbohydrate processing as wild type MPR46. Furthermore comparable amounts of wild type MPR46 (17%) and MPR46-LAP chimera (18%) are expressed at the cell surface as detected by [^{125}I] antibody binding. 45% of cell surface bound [^{125}I] MPR46 antibodies were internalized within 3 min upon warming to 37°C in cells expressing MPR46–LAP, as compared with 51% in wild type MPR46-expressing cells. If the MPR46–LAP chimera recycles between endosomes and cell surface as do wild type MPR46 and LAP it probably exits from the recycling pool much faster and is delivered

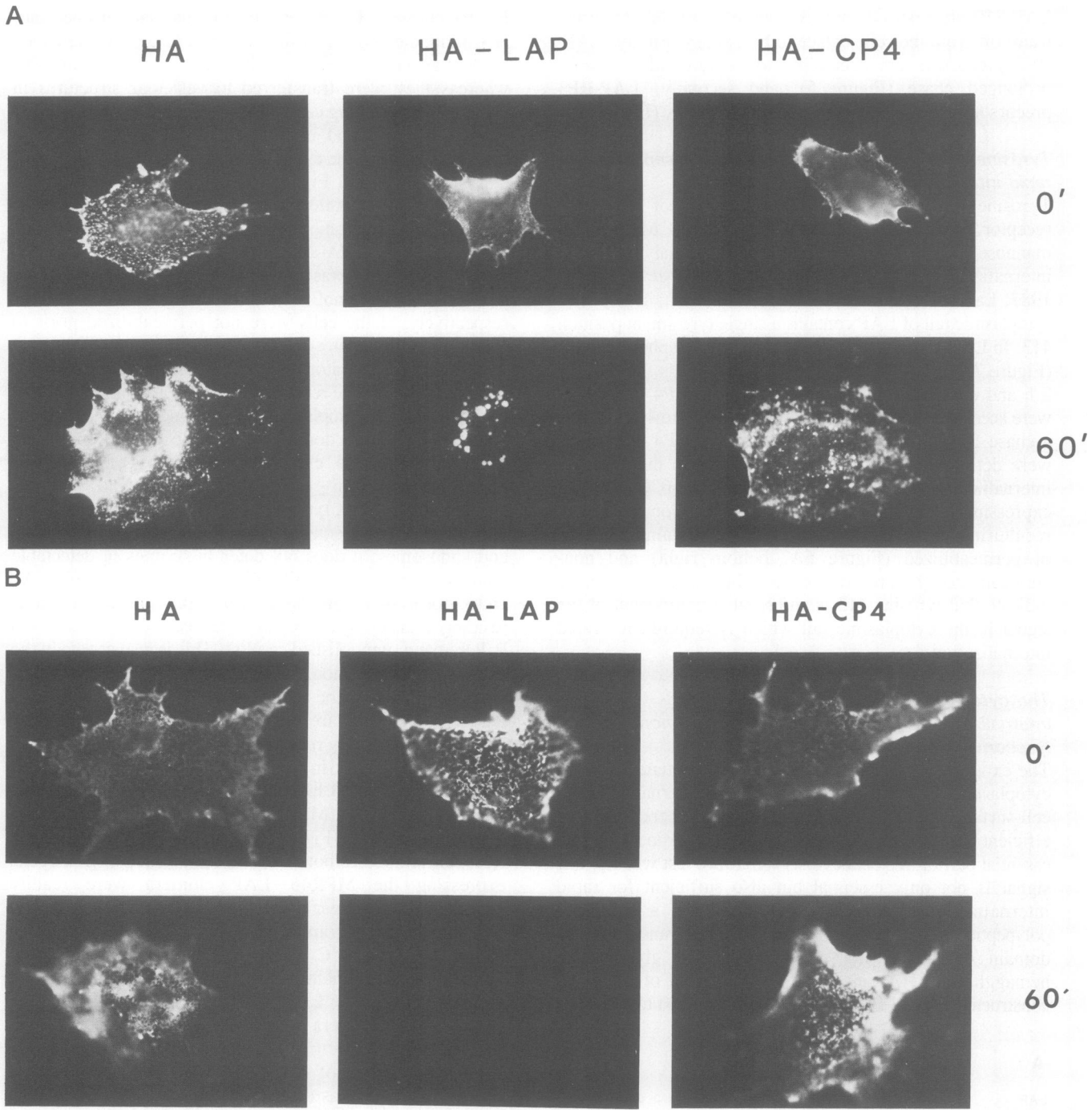

Fig. 8. Internalization of hemagglutinin (HA) and hemagglutinin-LAP fusion proteins (HA−LAP, HA−CP4). HA, HA−LAP and HA−CP4 expressing BHK cells were incubated with an anti-HA antiserum for 1 h at 0°C. Subsequently cells were processed for indirect immuofluorescence as described in Figure 6 legend. **(A)** with detergent permeabilization, **(B)** without detergent permeabilization.

to lysosomes. In contrast to the ectoplasmic domain of LAP, that of MPR46 is likely to be sensitive to lysosomal proteinases since MPR46 does not enter this compartment. To test this hypothesis lysosomal proteolysis was inhibited by preincubation of MPR46−LAP and wild type MPR46 expressing cells with lysosomal proteinase inhibitors leupeptin or/and pepstatin. Metabolic labeling for 2 h and subsequent chase for 6 h, followed by immunoprecipitation of MPR46-related polypeptides, showed that the chimera was stabilized in the presence of these inhibitors whereas no detectable effect on the yield of wild type MPR46 was observed (Figure 10).

A Percoll density gradient centrifugation of post nuclear supernatant from BHK cells metabolically pulse−chase labeled for 2 h each under the proteolytic protection of leupeptin and pepstatin, produced direct evidence of delivery of the MPR46−LAP chimera to dense lysosomes (Figure 11, bottom panel). More than 50% of immunoprecipitable MPR46−LAP were associated with heavy membranes whereas wild type MPR46 and truncated receptors lacking the cytoplasmic domain (MPR46-M6) were absent from dense lysosomes and associated with fractions of low density (Figure 11, top and middle panel), which are enriched in markers for the endoplasmic reticulum, Golgi, plasma

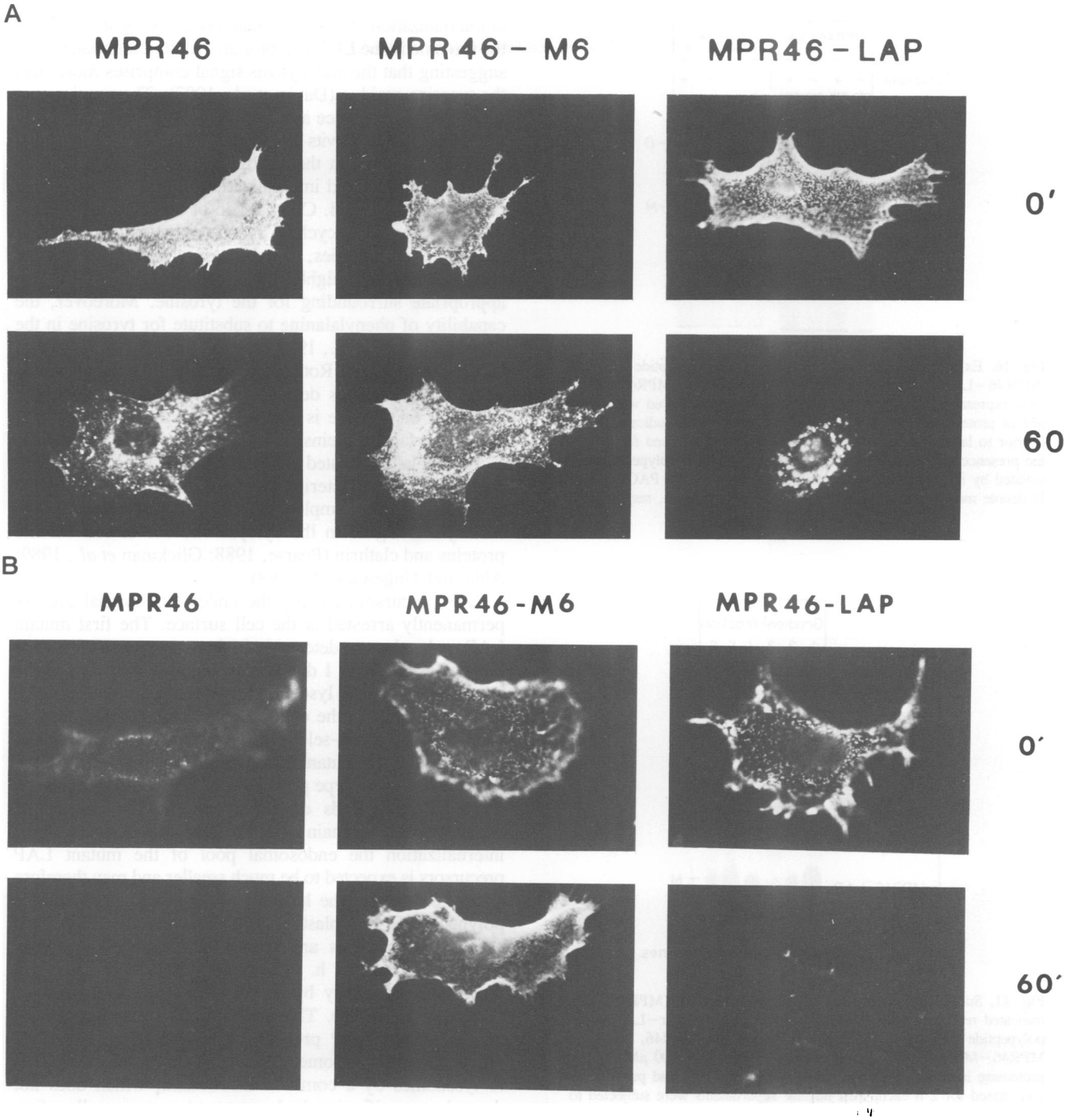

Fig. 9. Internalization of MPR46, MPR46−M6 and MPR46−LAP. MPR46, MPR46−M6 and MPR46−LAP expressing BHK cells were incubated with an anti-MPR46 antiserum for 1 h at 0°C. Subsequently cells were processed for indirect immunofluorescence as described in Figure 6 legend. **(A)** with detergent permeabilization, **(B)** without detergent permeabilization.

membrane and endosomes (Lemansky *et al.*, 1984). These observations show that the MPR46−LAP chimeras are delivered to lysosomes, while the wild type MPR46, as well as receptors lacking a cytoplasmic domain, are excluded from lysosomes.

Discussion

Transport of LAP precursor lacking the cytoplasmic tail

A tyrosine-containing endocytosis signal in the cytoplasmic tail is required for targeting of LAP to lysosomes. LAP precursors are associated for about 5−6 h with a cell surface/endosome pool before they are delivered to lysosomes. Within these 5−6 h they recycle more than 15 times between the cell surface and endosomes. At steady state about 4 times more LAP precursors are present in endosomes than at the cell surface (Braun *et al.*, 1989). The present study shows that the recycling between the cell surface and endosomes is interrupted if the cytoplasmic tail of LAP is deleted, or tyrosine 413 in the cytoplasmic tail is changed to phenylalanine. The mutant LAP precursors accumulate at the cell surface. This indicates that the cytoplasmic tail contains a signal required for internalization

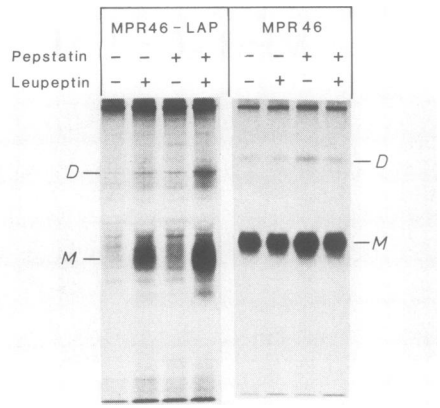

Fig. 10. Expression of chimeric receptor−LAP polypeptide (MPR46−LAP) in comparison to wild type receptor (MPR46). BHK cells expressing MPR46 or MPR46−LAP were incubated with 100 μM of proteinase inhibitors pepstatin and leupeptin as indicated for 16 h prior to labeling. Cells were labeled for 2 h and chased for 6 h in the presence of the same inhibitors. Receptor related polypeptides were isolated by immunoprecipitation and analyzed by SDS−PAGE. M and D denote monomeric and dimeric receptor polypeptides, respectively.

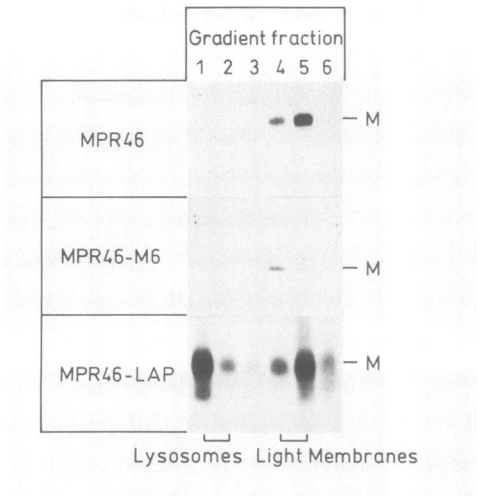

Fig. 11. Subcellular distribution of wild type receptor (MPR46), truncated receptor (MPR46−M6) and chimeric receptor−LAP polypeptide MPR46−LAP. BHK cells expressing MPR46, MPR46−M6 or MPR46−LAP were incubated with 100 μM of proteinase inhibitors pepstatin and leupeptin for 16 h and pulse labeled and chased for 2 h each. Post nuclear supernatants were subjected to subcellular fractionation by Percoll gradient centrifugation. Receptor related polypeptides were isolated by immunoprecipitation and analyzed by SDS−PAGE. Dense lysosomes migrate in fractions 1 and 2 and light membranes in fractions 4 and 5 as indicated. M, monomeric receptor polypeptides.

of LAP and that tyrosine 413 is an essential part of this signal.

The importance of tyrosine-containing signals within the cytoplasmic tail for rapid internalization of cell surface associated proteins has been reported for the LDL-receptor (Davis *et al.*, 1986, 1987), an artificially created mutant of the influenza virus hemagglutinin (Lazarovits and Roth, 1988) and the 300 kd mannose 6-phosphate/IGF II receptor (Lobel *et al.*, 1989). In the LDL receptor phenylalanine and tryptophan can substitute for the tyrosine, while a change to charged or uncharged aliphatic residues results in a loss of internalization. Nonsense mutations following immediately the tyrosine in the LDL receptor also disrupt internalization, suggesting that the endocytosis signal comprises more than the tyrosine residue (Davis *et al.*, 1987). The requirement for a suitable sequence around the tyrosine is supported by the studies of Lazarovits and Roth (1988). They showed that changing residues in the short cytoplasmic tail of HA to tyrosine caused rapid internalization of HA only in one of three positions tested. Comparison of the sequence around the tyrosines in recycling receptors show no apparent sequence homologies, suggesting that a variety of combinations of neighboring residues can provide the appropriate surrounding for the tyrosine. Moreover, the capability of phenylalanine to substitute for tyrosine in the LDL tail (Davis *et al.*, 1987) but not in the tail of the mutant HA (Lazarovits and Roth, 1988) and of LAP suggests that neighboring residues determine whether phenylalanine is sufficient or tyrosine is required.

Cell surface proteins, which are rapidly internalized, cluster in clathrin-coated pits before endocytosis via clathrin-coated vesicles. Clustering in coated pits is mediated by the protein adaptor complex HAII, which recognizes the endocytosis signal in the cytoplasmic tail of cell surface proteins and clathrin (Pearse, 1988; Glickman *et al.*, 1989; Ahle and Ungewickell, 1989).

LAP precursors lacking the endocytosis signal are not permanently arrested at the cell surface. The first mutant LAP molecules are detectable in dense lysosomes 2 h after biosynthesis. About 1 day is required to deliver half of the precursors to dense lysosomes. We suppose that the LAP precursors lacking the endocytosis signal are delivered to lysosomes by a non-selective, stochastic process. It is not clear whether the mutant LAP precursors recycle to the cell surface as do wild type precursors. This would be expected if recycling depends on signals in the luminal and/or transmembrane domain of LAP. Due to the slow rate of internalization the endosomal pool of the mutant LAP precursors is expected to be much smaller and may therefore escape detection. The half life of an average cell surface polypeptide in fibroblasts is 20 h (Draye *et al.*, 1988). Cell surface polypeptides are usually degraded in lysosomes within less than 1 h. Therefore, their half lives are determined primarily by the transport rate to lysosomes (Draye *et al.*, 1988). The similarity of the transport rates of the mutant LAP precursor and average cell surface polypeptides to lysosomes suggests that they are transported to lysosomes by a common mechanism, which does not depend on specific signals. In contrast to average cell surface polypeptides, the mutant LAP precursors are not degraded in lysosomes but processed to soluble forms, which are indistinguishable from the mature form of wild type LAP.

The cytoplasmic tail inhibits proteolytic processing of LAP

The proteolytic processing of the LAP precursor after delivery to lysosomes is initiated by a cleavage within the cytoplasmic tail. This reaction is catalyzed by a thiol proteinase and occurs with a half time of 6−7 h. Only the product of the thiol proteinase serves as a substrate for an intralysosomal aspartyl proteinase, which rapidly generates the soluble mature form of LAP (Gottschalk *et al.*, 1989b). The rapid processing of truncated LAP after delivery to lysosomes supports the view that the presence of the

cytoplasmic tail delays generation of soluble LAP and that inhibition of premature proteolytic processing may be one of the tail functions.

The cytoplasmic tail of LAP is sufficient to target non-lysosomal proteins to lysosomes

The cytoplasmic tail of LAP was sufficient to induce rapid internalization of HA and MPR46. Internalization of the HA chimera was dependent on tyrosine 413 in the cytoplasmic LAP tail. When lysosomal proteolysis was inhibited, transport of the MPR46−LAP chimera to lysosomes could be demonstrated. The MPR46-LAP chimera contained the transmembrane domain and cytoplasmic tail of LAP. Since truncated forms of MPR46 and LAP containing their own transmembrane domains, but lacking their cytoplasmic tails, are not targeted to lysosomes, transport of the MPR46−LAP chimera to lysosomes can be ascribed to signals in the cytoplasmic tail provided by LAP. The fusion between MPR46 and LAP was not precisely at the border between the ectoplasmic domain of MPR46 and the transmembrane domain of LAP (the chimera contained the 6 initial residues of the transmembrane domain of MPR46). We therefore had to rule out the possibility that due to aberrant folding of the ectoplasmic domain the chimera was transported to lysosomes by default. The chimera binds mannose 6-phosphate, it forms dimers and its *N*-linked oligosaccharides are processed to complex type structures. Generation of a ligand binding site, dimerization and oligosaccharide processing involve a series of post-translational modifications of the ectoplasmic domain (Hille *et al.*, 1990). In addition, the chimera was transported to the cell surface, where it mediated the endocytosis of receptor antibodies. These data indicate that the ectoplasmic domain of the chimera is folded correctly.

The chimeric receptor had a half life of ≥ 2 h, indicating that it is transported to lysosomes much faster than wild type LAP. Transport of LAP to lysosomes is delayed by efficient recycling to the cell surface. The inability of the transmembrane domain and the cytoplasmic tail of LAP to induce a similar recycling of the chimeric receptor suggests that the structural requirements for recycling of LAP are provided by the ectoplasmic domain of LAP.

Targeting of other lysosomal membrane proteins

For several proteins of the lysosomal membrane the primary structure is known (Howe *et al.*, 1988; Viitala *et al.*, 1988; Fukuda *et al.*, 1989; Noguchi *et al.*, 1989). They all have a short carboxy-terminal cytoplasmic tail, which contains a single tyrosine residue. In a related study Mathews and Fambrough (1988) observed that the transmembrane and cytoplasmic domains of the lysosomal membrane protein LEP-100 are sufficient to target the ectoplasmic domain of the VSVG-protein, a plasma membrane protein, to lysosomes, while the opposite chimera containing the ectoplasmic domain of LEP-100 and the transmembrane domain and cytoplasmic tail of the VSVG-protein accumulates at the cell surface. They concluded that the transmembrane and cytoplasmic domains of LEP-100 contain the signals required for targeting to lysosomes.

It is therefore conceivable that passage of the cell surface is obligatory for many lysosomal glycoproteins and that a tyrosine containing endocytosis signal is essential for their efficient routing from the cell surface to lysosomes.

Materials and methods

Recombinant DNAs

The coding region of influenza virus HA from fowl plague virus, strain A/FPV/Rostock/34(H7N1) contained in plasmid pUcHA651 (Kuroda *et al.*, 1986) was subcloned into M13mp11 and kindly provided by E. Kretschmer and H.-D.Klenk, Marburg, FRG. The cDNAs for LAP (Pohlmann *et al.*, 1988) and MPR46 (Pohlmann *et al.*, 1987) were subcloned into M13mp18. For *in vitro* mutagenesis the procedure of Nakamaye and Eckstein (1986) was used. The codons for the LAP residues 380 and 407 were changed to amber codons (LAP-RP2 and LAP-RP3). The codon of MPR46 residue 216 was changed to an opal codon (MPR46-M6), the codon of LAP residue tyrosine 413 was changed to the phenylalanine codon TTC (Figure 7A). For construction of HA−LAP chimeras (HA−LAP and HA−CP4) a *Xho*I restriction site was introduced into the LAP cDNA at nucleotide 1209 and a *Xho*I restriction site into hemagglutinin DNA at nucleotide 1674. The HA DNA encoding the N-terminal part of the protein and the LAP cDNA encoding the C-terminal part of the protein were fused at these artificial *Xho*I restriction sites. This manipulation changed valine 552, the last amino acid residue of the HA transmembrane domain, to serine (underlined in Figure 7B). The codon for tyrosine 413 of HA−LAP was changed to the phenylalanine codon TTC yielding the chimera HA−CP4 (Figure 7B). For construction of the MPR46−LAP chimera a *Pst*I site was introduced at position 587 of the MPR46 cDNA. The MPR46 cDNA encoding the N-terminal part of the receptor was fused at this artificial *Pst*I site to the unique *Pst*I site of the LAP cDNA fragment encoding the C-terminal part of the protein. The construct encodes for the N-terminal 197 amino acids of MPR46 (with exchange of phenylalanine 196 to serine), followed by the transmembrane and cytoplasmic domain of LAP (see Figure 2).

The cDNAs encoding LAP mutants were subcloned into the *Eco*RI site of the expression vector pBEH (Artelt *et al.*, 1988). The *Bgl*II−*Bgl*II DNA fragment encoding HA was subcloned into the *Bam*HI site of plasmid pBEH. DNAs encoding the chimeras HA−LAP and HA−CP4 were subcloned as *Bgl*II−*Hin*dIII DNA fragments into *Bam*HI and *Hin*dIII restriction sites of the same expression vector. The MPR46 mutants and the MPR46−LAP chimera were subcloned as *Bgl*II−*Eco*RI inserts into *Bgl*II/*Eco*RI cut pBHE-P29Δ5′. In the pBHE-P29Δ5′ the 5′-untranslated sequence of the MPR46 cDNA is shortened to 7 bp to improve expression (Wendland *et al.*, 1989). All mutations were confirmed by sequencing of single and double stranded DNA.

Transfection of BHK cells

Baby hamster kidney cells (clone BHK-21), 5×10^5 cells/6 cm dish, were transfected with 10−20 μg of plasmid DNA, 0.5−1 μg pSV2pac (Vara *et al.*, 1986) using the calcium phosphate technique (Wigler *et al.*, 1977). No glycerol shock was applied. Two days after transfection the medium was supplemented with 2 μg/ml puromycin and 6 days later with 5 μg/ml puromycin. Alternatively 0.5−1 μg pSV2neo (Southern and Berg, 1982) were included in calcium phosphate precipitates, additionally, medium was supplemented with 2 μg/ml puromycin two days after transfection and with 30 μg/ml puromycin and 500 μg/ml G418 six days later. Single colonies were picked and either analyzed for L-tartrate inhibitable LAP activity (Waheed *et al.*, 1985) or synthesis of MPR46 or hemagglutinin related proteins.

Metabolic labeling and preparation of cell extracts

BHK cells in 35 or 60 mm dishes were labeled with 0.74−11.1 MBq [^{35}S]methionine ($\geq$24.6 TBq/mmol) as described (von Figura *et al.*, 1983; Lemansky *et al.*, 1985). During chase, the medium was supplemented with 0.25 mg/ml methionine. The cells were harvested by scraping and extracts of cells and media were prepared as described (Lemansky *et al.*, 1985). In some experiments soluble and membrane-associated cellular proteins were separated by Triton X-114 condensation (Stein *et al.*, 1987b).

Subcellular fractionation in Percoll gradients, immunoprecipitation, neuraminidase treatment and electrophoresis were performed essentially as described (Braun *et al.*, 1989; Wendland *et al.*, 1989).

Determination of cell surface expression and internalization of MPR46−LAP chimera and wild type MPR46

The monoclonal antibody 21D3 (A. Hille, unpublished) against the luminal domain of human MPR46 was iodinated as described (Parker and Strominger, 1983). Surface associated and total cellular wild type MPR46 and MPR46−LAP chimera were determined essentially as described (Wendland *et al.*, 1990).

For determination of antibody internalization, confluent 35 mm dishes of BHK cells expressing MPR46−LAP chimera or wild type MPR46 were incubated for 3×5 min at 4°C with medium (MEM containing 20 mM HEPES, pH 7.2 and 7.5% FCS), followed by an incubation for 2 h at 4°C

with [^{125}I] 21D3 antibody (360 000 c.p.m. in 0.6 ml medium). Subsequently cells were washed 3 times with medium at 4°C and either recultured for 3 min at 37°C or kept at 4°C. After incubation in medium at 4°C for another 5 min cells were washed 3 times with 0.6 ml 25 mM glycine/HCl, pH 2.5, 150 mM NaCl at 4°C for 8 min each. The cells were harvested by scraping in 2×0.5 ml 1 M NaOH. Radioactivity released by acidic washes (cell surface bound antibodies) and cell bound radioactivity (internalized antibodies) was determined.

Endocytosis assay

BHK cells expressing LAP, MPR46 or HA-related polypeptides were washed 3× with ice cold PBS and incubated for 1 h at 4°C with antisera diluted 1:150–1:300 in PBS. Subsequently cells were washed 3× with medium supplemented with 7.5% fetal calf serum to remove unbound antibodies and either fixed directly or incubated for 60 min at 37°C, 5% CO_2 and then fixed. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100. The cells were then incubated with second antibodies (goat anti-rabbit immunoglobulin or rabbit anti-goat immunoglobulin) conjugated to rhodamine. Alternatively the cells—either with or without reculturing at 37°C for 60 min— were incubated with the second antibody at 4°C and then fixed. This permitted the selective detection of cell surface associated antibodies.

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