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The internalization signal in the cytoplasmic tail of lysosomal acid phosphatase consists of the hexapeptide PGYRHV

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Lysosomal acid phosphatase (LAP) is rapidly internalized from the cell surface due to a tyrosine-containing internalization signal in its 19 amino acid cytoplasmic tail. Measuring the internalization of a series of LAP cytoplasmic tail truncation and substitution mutants revealed that the N-terminal 12 amino acids of the cytoplasmic tail are sufficient for rapid endocytosis and that the hexapeptide 411-PGYRHV-416 is the tyrosine-containing internalization signal. Truncation and substitution mutants of amino acid residues following Val416 can prevent internalization even though these residues do not belong to the internalization signal. It was shown recently that part of the LAP cytoplasmic tail peptide corresponding to 410-PPGY-413 forms a well-ordered β turn structure in solution. Two-dimensional NMR spectroscopy of two modified LAP tail peptides, in which the single tyrosine was substituted either by phenylalanine or by alanine, revealed that the tendency to form a β turn is reduced by 25% in the phenylalanine-containing peptide and by ~50% in the alanine-containing mutant peptide. Our results suggest, that in the short cytoplasmic tail of LAP tyrosine is required for stabilization of the tight turn and that the aromatic ring system of the tyrosine residue is a contact point to the putative cytoplasmic receptor.

Key words: 2D-NMR/endocytosis/internalization signal/lysosomal acid phosphatase

Introduction

Eukaryotic cells mediate endocytosis of cell surface proteins through specialized domains of the plasma membrane, the clathrin coated pits (Goldstein *et al.*, 1985; Hubbard 1989). Alternative internalization pathways independent of clathrin have been described (Sandvig and van Deurs, 1990; Keller *et al.*, 1992; Rothberg *et al.*, 1992). The LDL, transferrin, asialoglycoprotein, polymeric immunoglobulin and cation independent mannose 6-phosphate receptors are members of a group of cell surface receptors that are constitutively concentrated in clathrin coated pits and rapidly internalized through these structures, in the presence and absence of their ligands (Mostov *et al.*, 1986; Davis *et al.*, 1987; Jacopetta *et al.*, 1988; Lobel *et al.*, 1989; Fuhrer *et al.*, 1991). The coated pit localization and rapid endocytosis of this group of receptors is dependent upon an internalization signal in their cytoplasmic domains. Equivalent internalization signals have been detected in the cytoplasmic tails of the lysosomal membrane glycoprotein h-lamp-1 and human lysosomal acid phosphatase (LAP). These internalization signals do not share a linear consensus sequence but they all contain an essential tyrosine residue (Chen *et al.*, 1990; Collawn *et al.*, 1990; Peters *et al.*, 1990; Williams and Fukuda, 1990; Canfield *et al.*, 1991). In the LDL receptor and in LAP the essential tyrosine residues were recently shown to be part of a tight turn structure (Bansal and Gierasch, 1991; Eberle *et al.*, 1991).

LAP is synthesized and transported to the lysosomes as a membrane bound precursor. The large extracytoplasmic domain containing the catalytic site of the enzyme is separated from the 19 amino acid cytoplasmic domain by a single transmembrane domain (Pohlmann *et al.*, 1988). On its biosynthetic route newly synthesized LAP is transported from the endoplasmic reticulum to the Golgi apparatus from where it reaches the plasma membrane. Due to the tyrosine-containing internalization signal in its cytoplasmic tail it is rapidly internalized (Peters *et al.*, 1990) and recycles between plasma membrane and endosomes before it is delivered to lysosomes (Braun *et al.*, 1989). In the lysosomes the luminal domain is released from the membrane anchor by proteolytic cleavage to form mature LAP (Gottschalk *et al.*, 1989).

In this study we determined the sequence and structural requirements for rapid internalization of LAP. The N-terminal 12 amino acid residues of the cytoplasmic tail contain an active internalization signal represented by the hexapeptide 411-PGYRHV-416. This signal can be inactivated by truncation or substitution mutants of the seven C-terminal amino acid residues of the protein, which are not part of the internalization signal. Furthermore, it is shown by two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR) that the tight turn which has recently been determined for the tetrapeptide 410-PPGY-413 of the LAP cytoplasmic domain (Eberle *et al.*, 1991) is destabilized when the tyrosine residue is substituted by phenylalanine and the tendency to form this turn is reduced even more when the tyrosine is substituted by alanine.

Results

The internalization signal of LAP is located in the N-terminal 12 amino acid residues of the cytoplasmic tail

To determine the C-terminal extent of the Tyr413-containing internalization signal detected in the cytoplasmic tail of the LAP precursor (Peters *et al.*, 1990), a series of LAP cytoplasmic tail truncation mutants has been constructed by introduction of premature stop codons into the LAP cDNA (Figure 1). The LAP mutants were stably expressed in BHK-21 cells. The kinetics of internalization of cell surface

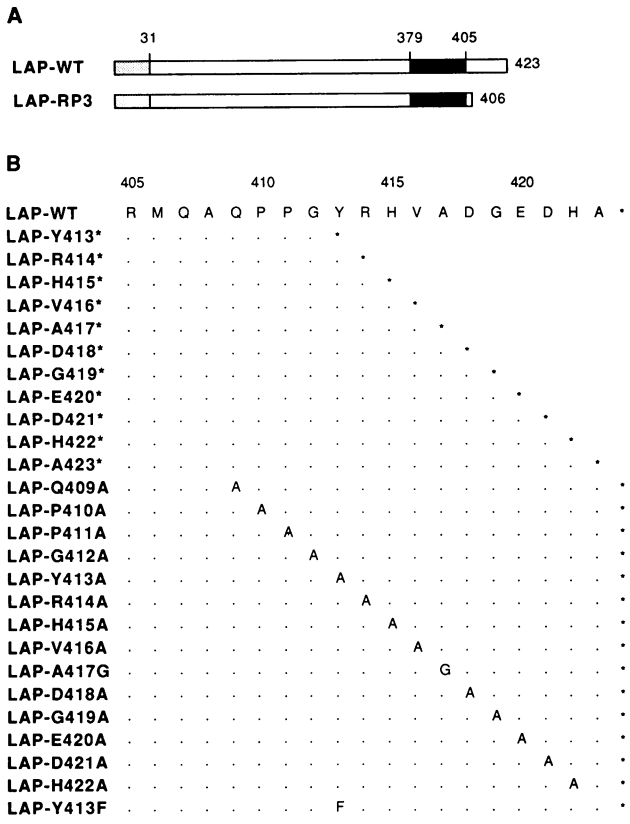

Fig. 1. Structure and amino acid sequences of LAP mutants. (**A**) Structure of wild-type LAP and deletion mutant LAP-RP3. The N-terminal signal sequence is indicated by a dotted box. The black box represents the transmembrane domain. The extracytoplasmic or luminal domain (amino acids 31–378) and the cytoplasmic domain (amino acids 405–423) are indicated by unfilled boxes. The numbers refer to the N-terminal amino acid after cleavage of the signal sequence, the first amino acid of the transmembrane domain, the cytoplasmic tail and the C-terminal amino acid (according to Peters *et al.*, 1990). (**B**) Amino acid sequence of deletion and substitution mutants in the cytoplasmic tail of LAP. Amino acid sequences of LAP wild-type and mutant cytoplasmic tails are given in one letter code (numbering according to Pohlmann *et al.*, 1988). Stop codons are indicated by asterisks. The mutant LAP-Y413F is identical to mutant LAP-CP4 in Peters *et al.* (1990).

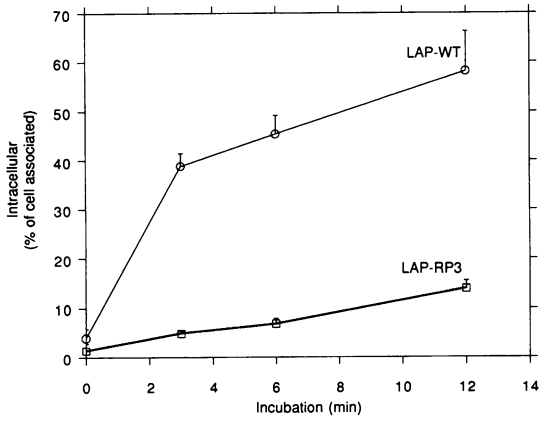

Fig. 2. Internalization kinetics of wild-type (LAP-WT) and tail-minus LAP (LAP-RP3). BHK-21 cells expressing wild-type (LAP-WT) or tail-minus LAP (LAP-RP3) were incubated with LAP antiserum for 2 h at 4°C. After removal of non-specifically bound antibodies LAP-specific antibodies were tagged with [^{125}I]protein A for 1 h at 4°C and cells were incubated for 0–12 min at 37°C. Cell surface associated and intracellular radioactivity were determined. Intracellular radioactivity is given as a percentage of total cell associated radioactivity. Error bars denote standard deviations.

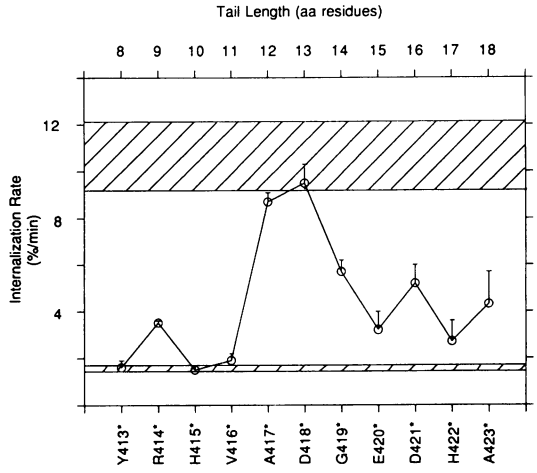

Fig. 3. Internalization of LAP truncation mutants. BHK-21 cells expressing LAP cytoplasmic tail truncation mutants (Figure 1) were incubated with LAP antiserum for 2 h at 4°C. After removal of non-specifically bound antibodies LAP-specific antibodies were tagged with [^{125}I]protein A for 1 h at 4°C and cells were incubated for 0 or 3 min at 37°C. Cell surface associated and intracellular radioactivity were determined and internalization rates, given as uptake of total cell associated radioactivity (in %) per minute, were calculated (see also Materials and methods). For comparison internalization rates of wild-type LAP (upper hatched area) and tail-minus LAP (LAP-RP3; lower hatched area) were also determined. Error bars denote standard deviations.

located LAP molecules was measured. Wild-type LAP (LAP-WT) and tail-minus LAP (LAP-RP3) expressing cells were incubated with an LAP-specific antiserum at 4°C. Bound antibodies were tagged by incubation with [^{125}I]protein A and cells incubated at 37°C for 0–12 min. Cell associated and intracellular radioactivity was determined. Internalization of wild-type LAP is rapid and levels off after the first 3 min, whereas the uptake of LAP-RP3 is >6-fold slower and linear for at least 12 min (Figure 2). Shorter incubations (not shown) revealed that the internalization of LAP-WT is almost linear during the first 3 min. Internalization rates (per min) were therefore determined by incubating cells that had been tagged at 4°C with LAP antiserum and [^{125}I]protein A, for 3 min at 37°C. The internalization of successive truncation mutants shows that a tail length of 12 residues is sufficient for rapid internalization (Figure 3). The mutants LAP-A417* and LAP-D418* with a tail length of 12 and 13 residues are internalized with 8.7 and 9.5 %/min, which approximates the internalization rate of wild-type LAP (10.7 ± 1.4 %/min, upper hatched area in Figure 3). Mutants with a tail length shorter than 12 residues are poorly internalized at 1.6 to 3.5 %/min, which is comparable with the internalization rate of the tail-minus mutant LAP-RP3 (1.6 ± 0.1 %/min, lower hatched area in Figure 3). These results indicate that all information required for rapid internalization is contained within the N-terminal 12 residues of the cytoplasmic tail. Interestingly, shortening of the C-terminus by one to five C-terminal amino acids yielding LAP mutants with a tail length of 14–18 residues also causes a substantial decrease in internalization rates (Figure 3).

The use of divalent immunoglobulin and [^{125}I]protein A for tagging LAP molecules at the cell surface may induce

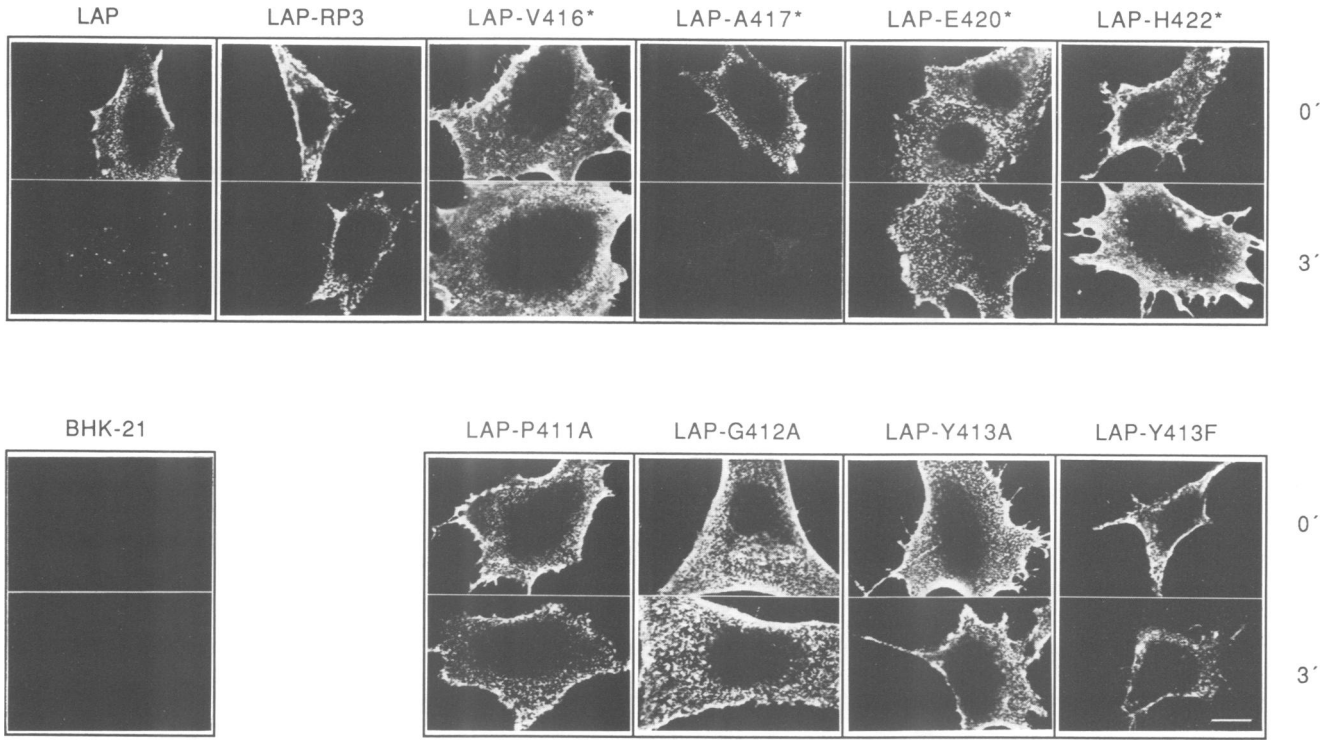

Fig. 4. Internalization of LAP cytoplasmic tail mutants detected in an immunofluorescence based internalization assay. BHK-21 cells expressing wild-type LAP or the LAP mutants indicated were incubated with an anti-LAP antiserum for 1 h at 4°C. Cells were washed and fixed immediately (0′) or incubated at 37°C for 3 min (3′) and subsequently fixed under conditions preserving the integrity of the plasma membrane. Rhodamine conjugated immunoglobulin was used as second antibody. Analysis was performed with a confocal laser scanning microscope. Bar: 10 μm.

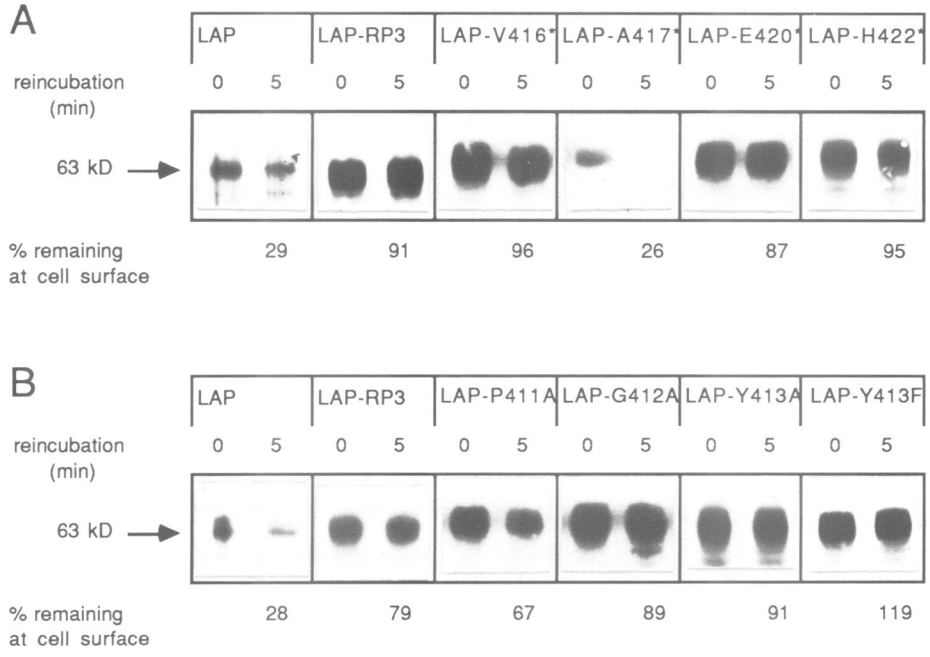

Fig. 5. Internalization of biotinylated LAP cytoplasmic tail mutants. BHK-21 cells expressing wild-type LAP or the LAP mutants indicated were surface biotinylated with sulfo-NHS-biotin at 4°C. Plasma membrane located LAP (or LAP mutants) were immunoprecipitated either directly or after reincubation at 37°C for 5 min. The immunoprecipitates were subjected to SDS−PAGE, transferred to nitrocellulose and biotinylated LAP was detected with streptavidin−peroxidase and an enhanced chemiluminescence system (see Materials and methods). The position of the 63 kDa membrane bound LAP form is indicated by an arrow. Signals were quantified by densitometry, the amount of LAP (or LAP mutants) remaining at the cell surface after reincubation at 37°C for 5 min was determined [given as percentage of control (no reincubation) below the respective lanes].

a crosslinking of LAP precursors due to multiple binding sites in the immunoglobulin as well as in protein A (Surolia *et al.*, 1982). Crosslinking in turn could alter the internalization rate of LAP. We therefore followed the internalization of LAP by two alternative assays, the first of which avoids the use of protein A, while the second is

independent of immunoglobulins as well. The former assay has been described before (Lazarovits and Roth, 1988; Peters *et al.*, 1990). LAP antibodies were bound to the surface of LAP expressing cells and the location of the antibodies was determined by immunofluorescence either immediately or after incubation of the cells at 37°C for 3 min. The results given in Figure 4 show that the majority of wild-type LAP molecules tagged with antibody at the cell surface have disappeared from the plasma membrane after incubation for 3 min at 37°C (intact plasma membrane in the absence of Triton X-100, Figure 4) and can be detected in vesicular structures below the membrane (membranes permeabilized with Triton X-100, data not shown). In contrast, the majority of the tail-minus mutant LAP-RP3 remains at the surface after incubation at 37°C for 3 min. The results of this immunofluorescence assay performed on a selected set of tail substitution and truncation mutants exactly parallel the internalization of LAP tail mutants in the antibody/[^{125}I]protein A internalization assay (Figures 2 and 3).

To exclude an influence of the divalent immunoglobulins on the internalization rates of LAP mutants, an internalization assay based on selective biotinylation and immunoprecipitation of cell surface associated LAP was performed. Wild-type LAP, the LAP mutant lacking the cytoplasmic domain (LAP-RP3) or LAP truncation and substitution mutants were covalently labeled with sulfo-NHS-biotin on the surface of expressing BHK cells at 4°C. Cells were recultured at 37°C for 5 min while control plates were kept at 4°C. Subsequently, cell surface located LAP molecules were selectively immunoprecipitated and subjected to SDS–PAGE. Proteins were transferred to a nitrocellulose membrane, LAP molecules labeled with an LAP-specific antiserum followed by detection with an enhanced chemiluminescence system and quantification by densitometry. The results depicted in Figure 5A and B show that the surface LAP signal in wild-type LAP expressing cells is reduced to 29% (Figure 5A) or 28% (Figure 5B) and in LAP-RP3 expressing cells to 91% (Figure 5A) or 79% (Figure 5B) of the control after warming to 37°C for 5 min. This indicates that >70% of cell surface associated wild-type LAP are internalized within 5 min, while this fraction is 15% for LAP-RP3 (average of the two experiments shown in Figure 5A and B). Furthermore, the amount of LAP molecules detected at the cell surface is more than four times higher in LAP-RP3 expressing cells than in wild-type LAP expressing cells (compare signals in 0 min lanes of LAP and LAP-RP3 in Figure 5) due to the accumulation of LAP molecules lacking the tyrosine-containing internalization signal at the cell surface (Peters *et al.*, 1990); wild-type LAP and LAP-RP3 expressing BHK cells have the same level of LAP expression. The percentage of LAP molecules remaining at the cell surface after reincubation for 5 min at 37°C of cells expressing the LAP deletion mutants LAP-V416* (96%), LAP-A417* (26%), LAP-E420* (87%) and LAP-H422* (95%), respectively (Figure 5A), fully agrees with the results of the immunoglobulin/[^{125}I]protein A internalization assay (Figures 2 and 3).

A series of alanine substitution mutants identifies the hexapeptide PGYRHV as the LAP internalization signal

To determine the amino acid residues contributing to the Tyr413-containing internalization signal in the cytoplasmic tail of LAP, the codons for Gln409–His422 were individually changed to codons for alanine by *in vitro* mutagenesis (the codon for Ala417 was changed to a Gly codon) (Figure 1). The resulting mutants were stably expressed in BHK-21 cells and the internalization rate of each mutant was determined by the antibody internalization/[^{125}I]protein A assay (Figure 6). Substitution of the six amino acid residues between Pro411 and Val416 including the tyrosine residue 413, which was previously identified to be critical for endocytosis (Peters *et al.*, 1990), results in a diminished internalization rate of 1.7–5.5 %/min as compared with 10.7 %/min for wild-type LAP. Even the conservative substitution of the essential Tyr residue 413 for Phe causes a reduction of the internalization rate to 3.1 ± 0.2 %/min. The Tyr413 to Phe substitution mutant (LAP-Y413F) is identical to LAP-CP4 in Peters *et al.* (1990). Furthermore, substitution of Asp418, Gly419 and Glu420, which are distal of the C-terminal boundary of the internalization signal (Val416), causes a reduction of internalization rates to ~4 %/min (Figure 6). Four of these substitution mutants were also analyzed with the immunofluorescence based internalization assay as well as with the biotinylation assay. The results of these assays confirmed the poor internalization of LAP-P411A, LAP-G412A, LAP-Y413A and LAP-Y413F (Figures 4 and 5). Together with the data of the LAP cytoplasmic tail truncation mutants the results for the substitution mutants identify the hexapeptide 411-PGYRHV-416 as the internalization signal in the cytoplasmic tail of LAP (Figure 8). This internalization signal can be functionally inactivated by substitution or truncation of amino acids that are located in the C-terminal part of the tail (418-DGEDHA-423).

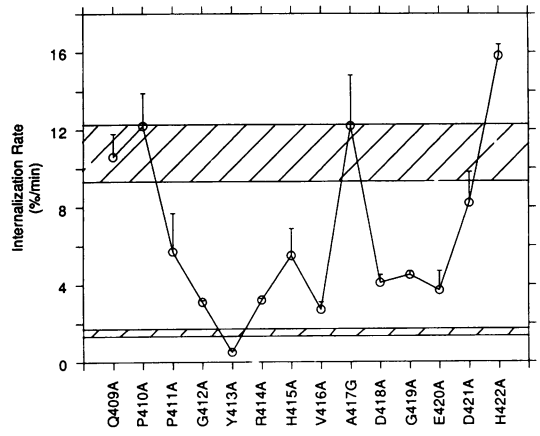

Fig. 6. Internalization of LAP substitution mutants. Internalization of LAP cytoplasmic tail substitution mutants (Figure 1) in expressing BHK-21 cells was determined as described in the legend to Figure 3 (see also Materials and methods).

The tendency to form a type I β turn in the LAP cytoplasmic tail at 410-PPGY-413 is reduced when Tyr413 is replaced by phenylalanine or alanine

A type I β turn structure has recently been determined for the 5-PPGY-8 sequence in an 18 amino acid peptide representing the cytoplasmic tail of lysosomal acid phosphatase by 2D-NMR (Eberle *et al.*, 1991; the position of the turn corresponds to amino acids 410-PPGY-413 in the LAP precursor). The sequence of the wild-type LAP tail peptide (LAP-WT) is given in Table IA and the tetrapeptide forming the tight turn is boxed. The preferred β turn

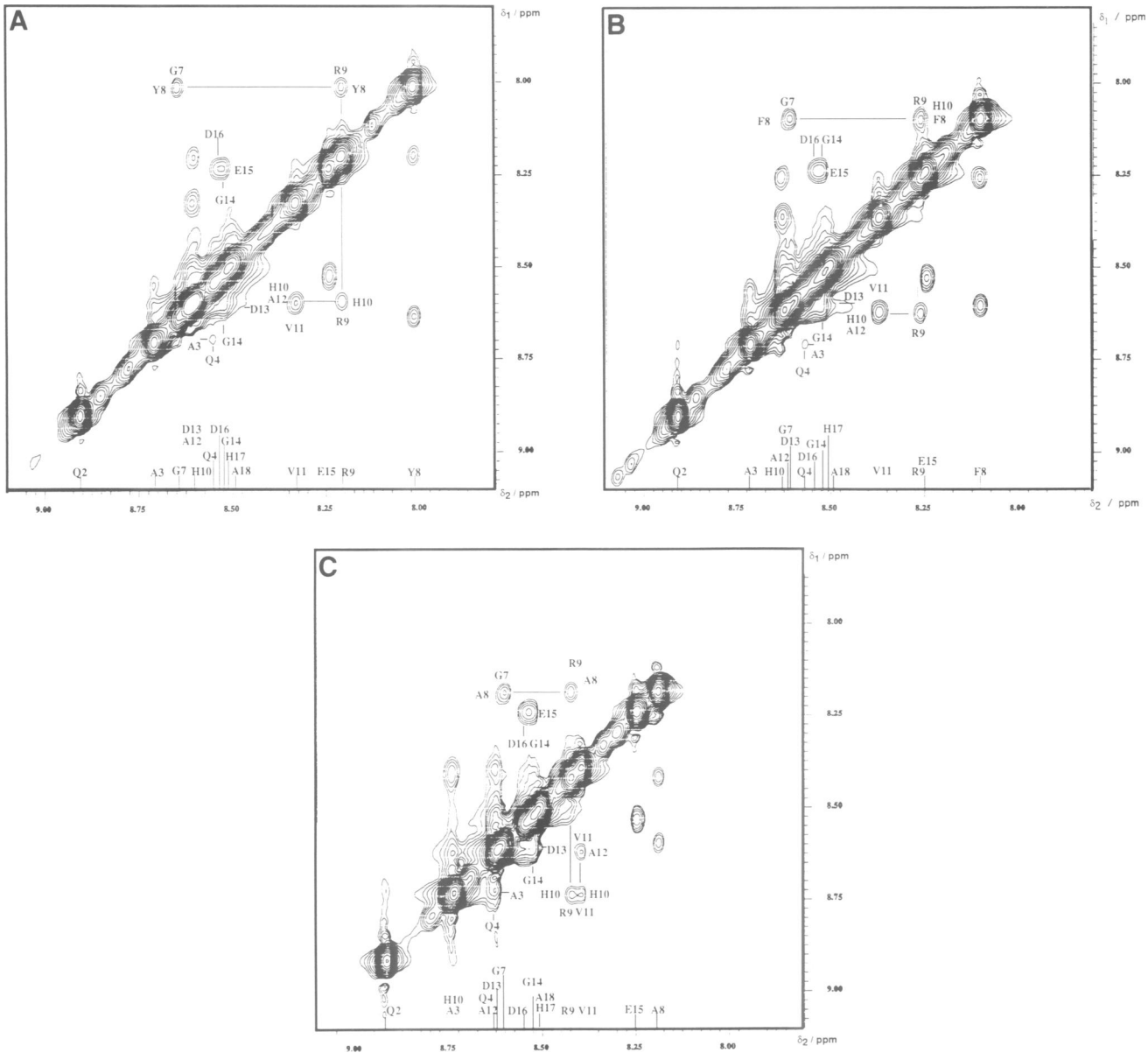

Fig. 7. Regions of the amide proton part of 2D-NOESY spectra (t_{mix} = 200 ms). (**A**) 18 amino acid wild-type LAP tail peptide (LAP-WT); (**B**) 18 amino acid mutant Tyr8→Phe LAP tail peptide (LAP-F8); (**C**) 18 amino acid mutant Tyr8→Ala LAP tail peptide (LAP-A8). The resonances of all three peptides were assigned completely by analysis of homonuclear proton 2D double spectra. The chains of sequential NH−NH NOEs—Ala3−Pro5, Pro6−Ala12 and Asp13−Asp16—are indicated. In LAP-F8 the NOE between Gln2 and Ala3 is also visible, whereas this NOE is beyond the lowest level drawn in LAP-WT and LAP-A8. In the case of prolines the Cδ protons are equivalent to amide protons (not shown). The NH−NH Ala12−Asp13 NOEs were not observed due to spectral overlap. In all three peptides Hα−NH(i+2) NOEs from Pro6−Tyr/Phe/Ala8, Gly7−Arg9 and Asp13−Glu15 were observed and in LAP-WT and LAP-F8 Arg9−Val11, additionally.

conformation of 5-PPGY-8 is in fast exchange with random structure. At a given time >50% of the total peptide exhibits the turn and the other molecules show a random coil structure (Eberle *et al.*, 1991). A reinvestigation of LAP-WT at a different pH and with different data processing revealed chains of sequential NH−NH nuclear Overhauser effects (NOEs) and some Hα(i)−NH(i+2) NOEs (Figure 7A). Since no Hα−NH(i+3/i+4) or Hα(i)−Hβ(i+3) NOEs characteristic of a stable helix were observed, the sequential and Hα(i)−NH(i+2) NOEs indicate nascent helices N- and C-terminal of the 5-PPGY-8 turn (Figure 7A; Wüthrich, 1986; Dyson *et al.*, 1988).

Mutant LAP tail peptides in which Tyr8 is replaced by Phe (LAP-F8) or Ala (LAP-A8; Table IA) exhibit the same pattern of NOEs in 2D-NOESY spectra indicating that the mutant peptides contain the same structural elements as LAP-WT (Figure 7A−C). Assuming the same basic conformation in wild-type and both mutant LAP tail peptides, the intensities of the NOEs correlate with the fraction of the peptide populations containing an ordered tight turn structure versus random coil structure and hence give a quantitative estimate of the stability of the β turn involving 5-P-P-G-Y/F/A-8. The intensity of the sequential NH−NH NOEs linking Gly7 and Phe8 in LAP-F8 is reduced to 75% and the NOE linking Gly7 and Ala8 in LAP-A8 to 53% of the intensity measured for the NOE linking Gly7 and Tyr8 in LAP-WT (Table IB).

In LAP-WT the two β protons of Tyr8 exhibit different chemical shifts at low temperature. This difference decreases with rising temperature and vanishes above 310 K. For the β protons of Phe8 in LAP-F8 the chemical shift is degenerate

even at 277 K (data not shown). A difference in chemical shifts can be expected for a side chain which is fixed at low temperature (LAP-WT). A loss of the difference in the chemical shifts at 310 K indicates that the side chain with the aromatic ring rotates freely at 310 K. The fact that a difference in chemical shifts for the β protons of LAP-F8 was not even detected at 277 K, suggests that the aromatic ring in this mutant peptide is free to rotate even at low temperature, although it cannot be excluded that the difference in chemical shifts is lost coincidentally.

The difference in chemical shifts of the α protons of Gly7 is smaller in LAP-F8 than in LAP-WT (0.03 versus 0.05 p.p.m.) and is even zero in LAP-A8. This result independently indicates that a lower fraction of the LAP-F8 and LAP-A8 peptides adopts an ordered structure as compared with LAP-WT.

$^3J_{NHH\alpha}$ coupling constants observed for a helical conformation of the dihedral angle are ~4 Hz and those for random structures are 7–8 Hz. A fast interconverting mixed population leads to averaging of these values weighted by the populations (Jardetzky and Roberts, 1981). This coupling constant is shifted from 5.5 Hz for Tyr8 in LAP-WT to 6.2 Hz for Phe8 in the mutant peptide LAP-F8 indicating a higher percentage of random structure in the mutant peptide. In contrast, the value detected for Arg9 shifts from 7.5 Hz for LAP-WT to 6.3 Hz for LAP-F8. The $^3J_{NHH\alpha}$ coupling constant for the dihedral angle of Ala8 in LAP-A8 is 5.3 Hz, i.e. even below the value for the corresponding position in LAP-WT. This might be due to the different side chains—tyrosine and alanine—in LAP-WT and LAP-A8 at position 8 rather than reflecting the local structure. Taken together these results show that the tendency to form a β turn is reduced by 25% in LAP-F8 and by ~50% in LAP-A8 in comparison with LAP-WT.

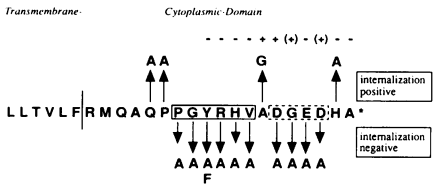

Fig. 8. The LAP internalization signal. The amino acid sequence of part of the transmembrane domain and the cytoplasmic tail of LAP are shown in one letter code. Internalization positive substitution mutants are indicated by an upward arrow and internalization negative substitution mutants by a short (residual internalization activity) or longer (no internalization activity) downward arrow. Internalization negative deletion mutants are indicated by '−' and internalization positive deletion mutants by '+' or for reduced activity '(+)'. The internalization signal of LAP is boxed with a solid line and the 'modulating element' (see Discussion) is boxed with a broken line.

Discussion

Amino acid residues contributing to the internalization signal in the cytoplasmic domain of LAP

The 19 amino acid cytoplasmic domain of the LAP precursor contains a signal for rapid internalization from the cell surface via clathrin coated pits (Peters *et al.*, 1990; Hille *et al.*, 1992). The single tyrosine residue in the cytoplasmic tail was shown to be an essential constituent of this internalization signal. Determination of internalization rates of cytoplasmic tail truncation and substitution mutants presented in this study show that the N-terminal 12 amino acid residues of the tail—ending with Val416—are sufficient for rapid endocytosis of LAP and that the internalization signal consists of the hexapeptide 411-PGYRHV-416.

The internalization of membrane bound LAP precursor mutants from the plasma membrane has been determined by binding an LAP-specific antiserum to the surface of expressing BHK-cells at 4°C. Subsequently, specifically bound LAP antibodies were tagged with radioactively labeled protein A. Since protein A as well as the bivalent immunoglobulins used in this assay contain more than one binding site for LAP or immunoglobulin, respectively, it is conceivable that this experimental procedure causes clustering of LAP molecules at the plasma membrane. This phenomenon could have an influence on the internalization rate of the tagged LAP molecules. To exclude such an artifact, the internalization of a selected set of LAP truncation and substitution mutants was measured in two alternative assays.

First, wild-type LAP and nine LAP cytoplasmic tail

Table I. Wild-type and mutant LAP cytoplasmic tail peptides (A) and correlation of their turn stabilities and internalization indices (B)

A

	1				5					10				15				
LAP-WT	M	Q	A	Q	P	P	G	Y	R	H	V	A	D	G	E	D	H	A
LAP-F8	.	.	.	.	.	.	.	F	.	.	.	.	.	.	.	.	.	.
LAP-A8	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.

B

Peptide	Internalization index (%/min)[a]	$^3J_{NHH\alpha}$ coupling constant (Hz)[b] Pos. 8	Pos. 9	NOE intensities (NH7–NH8)[c]
LAP-WT	10.7 (1.00)	5.5	7.5	466 (1.00)
LAP-F8	3.1 (0.29)	6.2	6.3	352 (0.75)
LAP-A8	0.5 (0.05)	5.3	7.1	246 (0.53)

[a]Internalization indices are given in %/min, compare Figure 6.
[b] $^3J_{NHH\alpha}$ coupling constants of residues Tyr, Phe, Ala8 and Arg9 are given.
[c]NOE intensities of a structurally important sequential NOE are given. The numbers are calibrated relative to the intensities of diagonal signals; relative internalization indices and NOE intensities are given in parenthesis.

mutants were subjected to an endocytosis assay based on the internalization of immunoglobulins specifically bound to LAP precursors at 4°C upon reculturing of the cells at 37°C. Subsequently, the location of the immunoglobulins was detected by indirect immunofluorescence. This assay was first introduced by Lazarovits and Roth (1988) and has been applied since to several other membrane proteins (Peters *et al.*, 1990; Williams and Fukuda, 1990; Keller *et al.*, 1992). The results obtained for the nine selected LAP cytoplasmic tail truncation and substitution mutants (Figure 4) exactly reflect those in the immunoglobulin/[^{125}I]protein A based internalization assay (Figures 3 and 6), excluding a major effect of a putative clustering of LAP precursors at the plasma membrane caused by protein A. To exclude an influence of putative clustering caused by the divalent immunoglobulin on internalization, cell surface-located proteins were covalently derivatized with sulfo-NHS-biotin at 4°C as described by Lisanti *et al.* (1988). The disappearance of biotin-labeled proteins from the cell surface after reculturing the cells at 37°C can be used as a quantitative measure of internalization of these molecules during the reculturing period. A loss of biotinylated LAP molecules from the cell surface for example by proteolytic cleavage or other kinds of destruction can be excluded since the LAP tail-minus mutant LAP-RP3, which is known to stay at the plasma membrane for a longer time (Peters *et al.*, 1990) hardly disappears from the cell surface during the reculturing period of 5 min (Figure 5). The internalization rates determined with this surface biotinylation/internalization assay for the nine selected LAP mutants (Figure 5) again exactly reflect the results of the immunoglobulin/[^{125}I]protein A internalization assay (Figures 3 and 6) and exclude an influence of a possible clustering of surface LAP molecules caused by divalent immunoglobulins on internalization. Taken together the findings in the immunofluorescence and surface biotinylation internalization assays clearly validate the results of the immunoglobulin/[^{125}I]protein A internalization assay.

Similarities between the internalization signals of three constitutively endocytosed surface receptors and the internalization signal of LAP

In the cytoplasmic domains of three constitutively internalized endocytic receptors, the LDL (Chen *et al.*, 1990), transferrin (TF; Collawn *et al.*, 1990) and cation independent mannose 6-phosphate receptors (MPR300; Canfield *et al.*, 1991; Jadot *et al.*, 1992), internalization signals have been determined by extensive analysis of cytoplasmic domain truncation and substitution mutants (Table II). Like the LAP internalization signal determined in this study the signals consist of a short contiguous stretch of four or six amino acids. These internalization signals all contain an essential tyrosine and three out of four signals have a bulky hydrophobic amino acid (valine or phenylalanine) located three residues C-terminal of the essential tyrosine (Table II). It has been shown recently for the MPR300 signal, that exact spacing between the tyrosine and the valine is essential and that the valine can be replaced by other bulky hydrophobic amino acids without loss of function (Jadot *et al.*, 1992).

Replacement of the internalization signal of the TF receptor by the hexapeptide internalization signals of the MPR300 and LDL receptor results in internalization rates

Table II. Internalization signals in the cytoplasmic domains of endocytic receptors and lysosomal acid phosphatase

LDL-R	I	N	F	D	N	P	V	**Y**	Q	K
MPR300	V	S	Y	K	**Y**	S	K	V	N	K
TF-R	E	P	L	S	**Y**	T	R	F	S	L
LAP	Q	P	P	G	**Y**	R	H	V	A	D

MPR300, bovine cation independent mannose 6-phosphate receptor; LDL-R, light density lipoprotein receptor; TF-R, transferrin receptor; LAP, lysosomal acid phosphatase; the amino acid sequences are given in single letter code; internalization signals are boxed; essential tyrosine residues are in bold.

of the chimeric proteins of 97 and 56% of wild-type TF receptor, respectively (Collawn *et al.*, 1991). Substitution of the YSKV tetrapeptide of MPR300 by putative four amino acid internalization motifs from several recycling receptors also gave functional internalization signals (Jadot *et al.*, 1992). These results show that internalization signals are interchangeable between receptors. The efficiency of the internalization signals may differ intrinsically or may be modulated by their environment. Transplantation of the PPGY tetrapeptide forming a tight turn in the LAP cytoplasmic tail (Eberle *et al.*, 1991) into the appropriate position of the TF receptor resulted in an inactive chimera, whereas transplantation of the tetrapeptide YRHV from the LAP internalization signal (Table II) into the TF receptor and MPR300 was sufficient to promote rapid internalization of the resulting fusion proteins (J.F.Collawn, A.Lai and I.S.Trowbridge, personal communication; Jadot *et al.*, 1992) indicating that the two N-terminal amino acids of the LAP internalization signal preceding the transplanted YRHV can be replaced functionally by the environment provided by the TF receptor or MPR300 (see Table II).

It has been shown in a 2D-NMR study that the PPGY tetrapeptide in the 18 amino acid cytoplasmic tail peptide of LAP adopts a tight turn structure in solution with tyrosine in position $i+3$ of the turn (Eberle *et al.*, 1991). A similar tight turn structure has been observed for the NPVY tetrapeptide of the LDL receptor internalization signal (Bansal and Gierasch, 1991). For the YTRF tetrapeptide of the TF receptor a tight turn with tyrosine in position i and phenylalanine in position $i+3$ was proposed (Collawn *et al.*, 1990). These results suggest that tyrosine-containing internalization signals are correlated with a tight turn conformational motif. The HA-II adaptor complex located in clathrin coated pits at the plasma membrane is considered to be the cytoplasmic receptor interacting with tyrosine-containing internalization signals (Glickman *et al.*, 1989). The precise function of the tyrosine side chain in the interaction between signal and receptor is not known at present. In three endocytic receptors, LDL-R, TF-R and MPR300, which have cytoplasmic domains larger than the cytoplasmic tail of LAP the tyrosine residue in the internalization signal can be replaced by the aromatic amino acid residues phenylalanine and tryptophan without impairment of endocytosis (Davis *et al.*, 1987; McGraw and Maxfield, 1990; Canfield *et al.*, 1991). Interestingly, substitution of the essential tyrosine in the internalization signal of MPR300 by tryptophan and phenylalanine in the context of a truncated MPR300 cytoplasmic tail of 29 amino acids resulted in severe reduction of internalization (Jadot

et al., 1992). This indicates that in a favorable structural context tyrosine can be replaced by an aromatic residue. The hydroxyl group of the tyrosine appears therefore not to be essential for recognition of the internalization signal by the putative cytoplasmic receptor. In short cytoplasmic tails, such as those of LAP and the truncated MPR300, where the tyrosine cannot be replaced by phenylalanine or tryptophan, the tyrosine hydroxyl group may be required to stabilize the internalization signal in a steric conformation favourable for recognition by a putative cytoplasmic receptor (see below). In the larger cytoplasmic tails an appropriate folding of the polypeptide chain may be sufficient to stabilize the conformation necessary for interaction with the cytoplasmic receptors. On the other hand it cannot be excluded that the larger cytoplasmic tails contain additional contact sites for interaction with the cytoplasmic receptors, which stabilize the binding of these mutants to the receptor and thereby maintain the near wild-type level of internalization.

The 2D-NMR data presented in this study show that the tight turn in the wild-type LAP peptide (LAP-WT) is more stable than in the phenylalanine or alanine-containing mutant peptides (LAP-F8 and LAP-A8). The observed higher stability of LAP-WT as compared with LAP-F8 is likely to be caused by electrostatic interaction of the tyrosine hydroxyl group with the charged side chains of Gln4, Arg9 or His10 preventing the Tyr8 side chain from rotating. A similar reduction in turn stability was observed in a tyrosine to phenylalanine mutant nonapeptide of the LDL receptor cytoplasmic tail (Bansal and Gierasch, 1991). These observations suggest that the tyrosine hydroxyl group stabilizes the tight turn motif also *in vivo* in the context of the membrane glycoproteins and hence support the hypothesis that the tyrosine residue is essential for stabilization of the tight turn motif in short cytoplasmic tails rather than for direct interaction between signal and receptor. A threshold value for the signal−receptor interaction may explain why a 20−30% loss of stability in the tight turn as detected in the LAP phenylalanine mutant peptide (LAP-F8) is sufficient to diminish the internalization rate of the LAP-Y413F mutant to 29% of wild-type level (Table IB).

The minimum requirement for this class of internalization signals therefore seems to be an aromatic amino acid residue presented in the context of a tight turn and a bulky hydrophobic side chain three residues after the aromatic amino acid. In a short cytoplasmic tail the aromatic residue apparently has to be a tyrosine, the hydroxyl group of which stabilizes the turn, whereas in a larger cytoplasmic domain any aromatic residue is sufficient, possibly because the turn is stabilized by appropriate folding of the polypeptide chain. In this model the contact points of the internalization signal for interaction with the receptor are built by the aromatic ring system and the bulky hydrophobic side chain.

Influence of amino acid residues C-terminal of Val416 on the activity of the internalization signal

Truncation or substitution mutants of the C-terminal six amino acids of the cytoplasmic domain of LAP were found to inhibit the function of the PGYRHV internalization signal. This unexpected finding may be explained by the results of the modified 2D-NMR analysis of the wild-type LAP tail peptide presented here. The nascent helix observed C-terminal of the tight turn (Figure 7A) may be disturbed when point or truncation mutations are introduced into the C-terminal part of the cytoplasmic tail. An incomplete structural formation of the C-terminal six amino acids could block the interaction between internalization signal and cytoplasmic receptor. Therefore, the nascent helix C-terminal of the internalization signal may be considered as a modulating element, which is inert in its wild-type steric conformation but which inhibits signal−receptor interaction as soon as its native conformation is disturbed by truncation or substitution mutations.

It will be possible to address the question of interaction between internalization signals and their cytoplasmic receptors as soon as these receptors have been characterized in more detail and binding sites for the internalization signals have been mapped on the adaptor complexes.

Materials and methods

Construction of LAP mutants and LAP-expressing plasmids

LAP truncation mutants (LAP-Y413*−LAP-A423*, Figure 1) and LAP substitution mutants (LAP-Q409A−LAP-H422A, and LAP-Y413F, Figure 1) were generated by oligonucleotide-directed mutagenesis of the wild-type human LAP cDNA (Pohlmann *et al.*, 1988) using the method of Nakamaye and Eckstein (1986). For the truncation mutants, the codons for the LAP residues 407 (LAP-RP3) and 413−423 (LAP-Y413*−LAP-A423*) were changed to stop codons. In the case of the substitution mutants, the codon of LAP residue Tyr413 was changed to the phenylalanine codon TTC, the codon for Ala417 to the glycine codon GGA, the codons for residues Gly419, Glu420 and Gln409 were changed to the alanine codon GCG, the codons of residues Gly412, Tyr413, Arg414, His415, Val416, Asp421 and His422 were changed to the alanine codon GCC, and the codons of residues Asp418, Pro410 and Pro411 were changed to the alanine codon GCT. The cDNAs encoding LAP mutants were subcloned into the *Eco*RI site of the expression vector pBEH (Artelt *et al.*, 1988) and all mutants were confirmed by sequencing single and double stranded DNA (Sanger *et al.*, 1977).

Stable expression in BHK-21 cells

Baby hamster kidney cells (clone BHK-21) (3×10^5) seeded in a 20 cm^2 tissue culture flask were transfected as described (Peters *et al.*, 1990). After selection in medium supplemented with 5 μg/ml puromycin single colonies were picked and assayed for L-tartrate inhibitable LAP activity (Waheed and van Etten, 1985).

Antibody/[^{125}I]protein A internalization assay

BHK cells on subconfluent 3 cm dishes were washed twice with Hanks' solution [137 mM NaCl, 0.5 mM KCl, 0.3 mM $Na_2HPO_4\times2\ H_2O$, 0.4 mM KH_2PO_4, 0.5 mM glucose (H_2O) and 0.4 mM $NaHCO_3$] at 4°C. LAP antiserum diluted 1:100 in minimal essential medium (MEM) containing 0.1% BSA and 20 mM HEPES pH 7.4 was bound to the cell surfaces for 2 h at 4°C. After washing five times with Hanks' solution at 4°C, cells were incubated at 4°C with [^{125}I]protein A (specific activity: 1.2×10^7 c.p.m./μg; 7×10^5 c.p.m. in 1 ml of medium containing 0.1% BSA and 20 mM HEPES, pH 7.4) for 1 h. Non-specifically bound radioactivity was removed by washing five times with Hanks' at 4°C. To assay the rate of endocytosis cells were incubated in MEM supplemented with 0.1% BSA and 20 mM HEPES pH 7.4 at 37°C for 0−12 min. To determine the cell associated radioactivity cells were incubated for 3×8 min with 0.2 M acetic acid, pH 2.5, containing 0.5 M NaCl, the solution was collected and radioactivity determined. Cells were harvested in 1 ml 1 N NaOH and radioactivity determined. Internalization rates per min were calculated, after subtraction of background values from non-expressing BHK-21 cells, as the difference of the ratio of intracellular and total cell associated radioactivity after 3 and 0 min of internalization divided by three.

Immunofluorescence based internalization assay

BHK-cells on coverslips were washed twice with ice cold PBS and once with PBS containing 0.2% gelatin (PBS-gelatin) for 5 min. Cells were incubated with LAP-specific antiserum, diluted 1:100 in PBS-gelatin, for 1 h at 4°C. After washing twice with PBS and once with MEM, 10 mM HEPES, pH 7.4 cells were incubated at 37°C for 3 min. Controls were kept at 4°C. Subsequently cells were washed twice with ice cold PBS and fixed in 3% paraformaldehyde in PBS (ice cold paraformaldehyde solution was added to the cells and allowed to warm up to room temperature during

the incubation period of 40 min). All subsequent steps were carried out at room temperature. Cells were washed twice with PBS, incubated in 50 mM NH_4Cl in PBS for 10 min and again washed twice with PBS. Cells were permeabilized by incubating twice with 0.3% Triton X-100 in PBS for 5 min. This step was omitted in the case of cell surface immunofluorescence. After washing twice with PBS and three times with PBS-gelatin, cells were incubated with a second antibody (goat anti-rabbit immunoglobulin, Cappel; diluted 1:200 in PBS-gelatin) conjugated to rhodamine. After washing three times with PBS-gelatin and once with distilled water cells were mounted in Mowiol and analyzed on a Zeiss confocal laser scaning microscope.

Internalization assay based on surface biotinylation

Expressing BHK cells on confluent 35 mm dishes were washed three times with ice cold PBS and incubated with 1.5 mg/ml sulfo-NHS-biotin (Pierce; Lisanti *et al.*, 1988) for 30 min at 4°C. After washing four times with ice cold PBS, cells were incubated in MEM at 37°C for 5 min. Controls were kept at 4°C. Subsequently cells were washed twice with ice cold PBS and incubated with an LAP-specific antiserum diluted 1:100 in PBS for 2 h at 4°C. Cells were washed twice with ice cold PBS, harvested in PBS and immunoprecipitation of cell surface located LAP was performed as described (Braun *et al.*, 1989). Immune complexes were separated by SDS−PAGE and proteins were transferred to nitrocellulose filters (Causin *et al.*, 1988; Braun *et al.*, 1989). Filters were blocked in 5% milkpowder in PBS containing 0.05% Triton X-100 at 4°C overnight and subsequently incubated with streptavidin−peroxidase (diluted 1:5000 in PBS containing 0.05% Tween 20) for 1 h at room temperature. Subsequently filters were washed once with 10 mM Tris−HCl, pH 7.4, 0.9% NaCl, 0.05% Tween 20, six times with 0.9% NaCl, 0.5% Triton X-100, 0.1% SDS and once with 10 mM Tris−HCl, pH 7.4, 0.9% NaCl, 0.05% Tween 20. LAP was detected with an enhanced chemiluminescence Western blotting system (ECL, Amersham). Filters emitting light were exposed to Kodak XAR film and signals were quantified by densitometry.

Peptide preparation

Peptides representing the cytoplasmic tail of LAP (residues 406−423 in Pohlmann *et al.*, 1988) and mutants, in which the tyrosine residue 413 is replaced by either phenylalanine or alanine, were synthesized and purified as described (Eberle *et al.*, 1991).

NMR data collection

Peptides were dissolved in 90% H_2O/10% D_2O. pH 2.5 was adjusted by addition of HCl. Peptide concentrations were 18, 15 and 6 mM for LAP-WT, LAP-F8 and LAP-A8, respectively. The temperature was 277 K. The chemical shifts refer to 2,2-dimethyl-2-silapentanesulfonate.

The one- and two-dimensional experiments were performed on a Bruker AMX 600 spectrometer at a proton resonance frequency of 600 MHz. For spin system identification, double quantum filtered correlated spectroscopy (DQF-COSY; Rance *et al.*, 1983; Bodenhausen *et al.*, 1984) and total correlated spectroscopy (TOCSY; Braunschweiler and Ernst, 1983) spectra with mixing times of 45 ms were recorded. Nuclear Overhauser enhancement spectroscopy (NOESY; Rance *et al.*, 1983; Bodenhausen *et al.*, 1984) experiments with mixing times of 200 ms were recorded for all peptides.

The FFT was performed on a Bruker X32 with the program UXNMR. The transformation sizes were 2 kb* 1 kb in w2 and w1, single-fold zero-filling in each dimension was applied. In the case of the NOESY spectra a cosine square filter function was applied in both dimensions. For volume integration of crosspeaks the program AURELIA was used.

$^3J_{NHH\alpha}$ coupling constants were extracted from 1D NMR spectra with 8 kb real datapoints zero-filled to yield 16 kb after Fourier transformation. A resolution enhancing Gaussian filter function was applied.

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