Identification of three internalization sequences in the cytoplasmic tail of the 46 kDa mannose 6-phosphate receptor

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The cytoplasmic tail of the human 46 kDa mannose 6-phosphate receptor (MPR 46) is necessary for rapid internalization of the receptor and sufficient to mediate internalization of a resident plasma membrane protein. To localize the internalization sequences within the 67 amino acids of the cytoplasmic tail, the tail was progressively shortened from its C-terminus, internal deletions of between four and eight amino acids were introduced into the tail, and individual residues were substituted by alanine, glycine or serine. Three sequences were identified that contribute to the internalization of MPR 46. The first is located within the 23 juxtamembrane cytoplasmic residues of the tail. It contains four essential residues within a heptapeptide and does not resemble known internalization signals. The second sequence contains as a critical residue Tyr-45. The third region is located within the C-terminal seven residues and contains a di-leucine pair as essential residues. The first and third sequences were shown to function as autonomous internalization sequences. Substitution of critically important residues within a single internalization sequence was tolerated, with no or only a moderate decrease in the internalization rate. When essential residues from two or all three internalization sequences were substituted, however, the internalization rate was decreased by more than 60 % and 90 % respectively. This indicates that the autonomous internalization signals in the cytoplasmic tail of MPR 46 function in an additive manner, but are partly redundant.

INTRODUCTION

The 46 kDa mannose 6-phosphate receptor (MPR 46) is one of two receptors known to bind mannose-6-phosphate-containing lysosomal proteins [1]. The receptor binds newly synthesized lysosomal enzymes in the Golgi and transfers its ligands to the endosomal compartment, where ligands and receptors dissociate. From there the ligands are transferred to lysosomes or released into the medium [2,3]. The receptors either return to the Golgi to undergo a new round of transport or are transferred to the cell surface. The cell-surface receptors are internalized rapidly, but do not mediate endocytosis due to their poor affinity for ligands at the cell surface [4].

MPR 46 is a transmembrane protein with a single membrane-spanning domain. The N-terminal luminal domain is sufficient for ligand binding [5,6]. The C-terminal cytoplasmic tail of 67 amino acids is necessary for the trafficking of the receptor. Deletion of the cytoplasmic tail results in the accumulation of the truncated receptor at the cell surface [7]. The first trafficking signals to be identified in the cytoplasmic tail of MPR 46 were two separate signals for rapid internalization, one including Phe-13 and Phe-18 and the other including Tyr-45 [8]. A His-Leu-Leu sequence near the C-terminus was shown to be critical for the lysosomal enzyme sorting function of the receptor, presumably by determining trafficking in the Golgi [9]. Palmitoylation of Cys-34 is required to prevent the misrouting of MPR 46 to lysosomes, suggesting that signals exist that promote the recycling of the receptor from endosomes to the Golgi [10].

In the present study we have attempted to define the sequences that are critical for the rapid internalization of MPR 46 and to examine their function as autonomous endocytosis signals. We were able to identify three sequences that contribute to the rapid internalization of MPR 46 and which function in a co-operative manner. Two of these sequences, one containing a critical phenylalanine residue but otherwise not resembling aromatic acid residue-based endocytosis signals, and the other containing a di-leucine motif, were shown to function as autonomous internalization sequences.

MATERIALS AND METHODS

Recombinant DNA

Human wild-type MPR 46 cDNA was subcloned into the expression vector pBEH, resulting in the plasmid pBEH-29Δ5′ [5]. Individual substitutions of cytoplasmic tail residues 61–67 by serine, mutation of the codons of amino acids 6, 12, 20, 30, 48, 50, 55 and 61 to stop codons (to give mutants designated Stop6 etc.), and construction of the deletion mutants Δ13–19, Δ20–23, Δ24–29, Δ42–49, Δ50–54 and Δ55–60 (i.e. with sequences lacking the indicated residues) was carried out as reported previously [11,12]. It should be noted that the cDNAs for the Ser mutants and the deletion mutants (apart from Δ13–19 and Δ42–49) possess an *Xho*I site at position 745 that changes Gln-196 to Ser. This amino acid exchange has no effect on the distribution and internalization of the encoded protein (results not shown). For

Abbrevations used: MPR 46, 46 kDa mannose 6-phosphate receptor; mpr$^-$ cells, MPR-deficient mouse embryonic fibroblasts; LAP, lysosomal acid phosphatase; LAP-RP3, tail-less form of LAP; TGN, *trans*-Golgi network; Δ13–19 (etc.), mutant form of MPR 46 lacking residues 13–19 of the cytoplasmic tail (etc.); Stop6 (etc.), mutant form of MPR 46 in which the codon for amino acid 6 of the cytoplasmic tail has been mutated to give a stop codon (etc.).

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the construction of the chimaeras between lysosomal acid phosphatase (LAP) and MPR 46, *Xho*I sites were introduced into the cDNA of LAP [15] at position 1209 and into the cDNA of MPR 46 at nucleotide 745 or 906 [14]. The fragment encoding the luminal part and the transmembrane domain of LAP and the DNA fragments encoding residues 2–67 or 47–67 of the cytoplasmic tail of MPR 46 were fused at the introduced *Xho*I sites. This manipulation changed the final amino acid of the LAP transmembrane domain, Phe-404, to serine. The DNAs encoding the chimaeras were subcloned into the expression vector pBEH by using *Eco*RI and *Bam*HI sites.

For construction of the other mutants, human MPR 46 wild-type cDNA was subcloned into the expression vector pMPSVHE [13] to yield pMPSVHE-29Δ5′. PCR mutagenesis on this vector was used to generate the Stop24, L64A/L65A and F13A mutants, and to construct the alanine or glycine substitution mutants of the MPR 46 Stop24 construct. The final PCR products were digested with *Bgl*II/*Eco*RI or with *Pfl*MI/*Eco*RI (F13A) and used to replace the corresponding fragments of the wild-type vector DNA pMPSVHE-29Δ5′. The PCR products carrying the Y45A and Y45A/L64A/L65A mutations were digested with *Pst*I/*Eco*RI and the purified fragments were assembled with the *Bgl*II/*Pst*I and *Bgl*II/*Eco*RI fragments of pMPSVHE-29Δ5′ in a three-part ligation. For generation of F13A/Y45A and F13A/Y45A/L64A/L65A, a similar three-part ligation was performed, replacing the *Bgl*II/*Pst*I fragment of pMPSVHE MPR 46 Y45A or of pMPSVHE MPR 46 Y45A/L64A/L65A respectively with a fragment including the mutation F13A. For construction of the deletion mutants Δ3–46 and Δ3–46/L64A/L65A, the final PCR products were digested with *Bgl*II/*Pvu*II and the purified fragments were assembled with the *Sph*I/*Pvu*II and *Sph*I/*Bgl*II fragments of pMPSVHE-29Δ5′ in a three-part ligation.

The sequences of primers used in this study are available upon request. All fragments synthesized by PCR were verified by dideoxy sequencing using the ABI 373A system.

Transfection

Transfection of the baby hamster kidney cell clone BHK-21 [5] and of mpr$^-$ cells (MPR-deficient mouse embryonic fibroblasts) was as reported previously [16].

Expression of MPR 46 and MPR 46 mutants

For determination of the relative amounts of human MPR 46, iodinated monoclonal antobody 21D3 was used [3]. Total MPR 46 was determined by binding of ^{125}I-labelled 21D3 in the presence of 0.1 % saponin, and surface-associated MPR 46 was determined by binding in the absence of saponin [11]. The expression levels of MPR 46 mutants in the cell lines used in this study was 0.3–3.1-fold that of wild-type MPR 46. To determine the expression of transfected wild-type MPR 46 relative to that of endogenous MPR 46 in BHK-21 cells, cells were metabolically labelled for 16 h with [^{35}S]methionine. The [^{35}S]MPR 46 was isolated either by immunoprecipitation with rabbit antiserum against the human receptor or by affinity chromatography on a (phosphomannan core)–Sepharose 4B column [11]. The [^{35}S]MPR 46 in the immunoprecipitates and eluates obtained with mannose 6-phosphate were quantified by densitometry after SDS/PAGE and fluorography.

Treatment with endoglucosaminidase H

Cell homogenate (25–50 μg of protein) in 10 mMTris/HCl, pH 7.5/0.9 % (w/v) NaCl/0.05 % Triton X-100, pH 7.4, was adjusted to pH 6.0 with 250 mM sodium phosphate buffer, pH 4.8, and incubated for 24 h at 37 °C in the absence or in the presence of 1 m-unit of endoglucosaminidase H (Boehringer, Mannheim).

Determination of internalization rate

Cells grown to confluency in 3 cm dishes were incubated for 1.5 h on ice in the presence of ^{125}I-21D3 (180000 c.p.m./0.6 ml of medium). Typically, between 1000 and 6000 c.p.m. was bound to the cells. Internalization of ^{125}I-21D3 was measured after incubation for up to 12 min at 37 °C [7]. Prior to harvesting, the surface-associated ^{125}I-labelled antibodies were removed by incubating the cells for 3×8 min on ice with 25 mM glycine/HCl, pH 2.5, and 0.15 M NaCl. The radioactivity that was resistant to the acid washing of cells warmed to 37 °C was corrected for the radioactivity that was resistant to the acid washing of cells that were not warmed to 37 °C (less than 5 % of the radioactivity bound during the 1.5 h incubation period).

Secretion of lysosomal enzymes

The secretion of β-hexosaminidase during a 24 h incubation period was measured using a fluorimetric assay [16]. For determination of secretion of newly synthesized β-glucuronidase, cells were metabolically labelled for 6 h and then chased for 16 h. β-Glucuronidase was immunoprecipitated from cells and medium, and quantified as described [16].

Other methods

Cross-linking with disuccinyl suberate [18], indirect immunofluorescence [11] and internalization of antibodies against LAP [17] were performed as described.

RESULTS

The cytoplasmic tail of MPR 46 is sufficient for rapid internalization of a reporter polypeptide

Human MPR 46 expressed in BHK cells is rapidly internalized from the cell surface. When cells that had been incubated for 1.5 h at 0 °C with monoclonal antibody 21D3, directed against the luminal domain of MPR 46, were warmed to 37 °C, a maximal internalization rate of up to 40 %/min was observed during the first 1 min, which levelled off within 6 min. The mean internalization rate calculated from a 3 min internalization period was 20 ± 2.8 %/min ($n = 42$) for wild-type MPR 46. As shown previously [8], internalization was dependent on the cytoplasmic tail of MPR 46 (Figure 1). Further experiments demonstrated that rapid internalization was not induced by the bivalent antibody and was not due to formation of hetero-oligomers with endogenous MPR 46 (results not shown). The cytoplasmic tail of MPR 46 is also sufficient to mediate rapid internalization when fused to a reporter polypeptide. As a reporter polypeptide the tail-less form of LAP (LAP-RP3) was used, which exhibits a basal rate of internalization of less than 3 %/min (Figure 1). Addition of the cytoplasmic tail of MPR 46 to LAP-RP3 [to give construct LAP–MPR 46 (2–67)] restored internalization to a value of $\geqslant 12$ %/min (Figure 1), supporting the notion that the cytoplasmic tail of MPR 46 is sufficient to induce rapid internalization of a reporter polypeptide.

Internalization of MPR 46 mutants with truncated forms of the cytoplasmic tail

To localize sequences determining the internalization rate, the cytoplasmic tail of MPR 46 was progressively shortened by

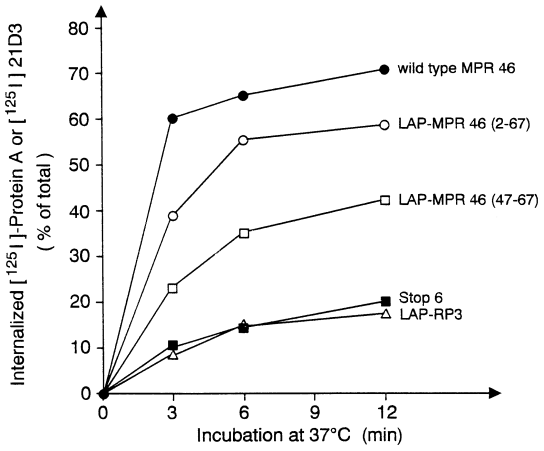

Figure 1 Internalization of LAP–MPR 46 chimaeras

BHK cells expressing LAP-RP3 or a chimaeric polypeptide in which residues 2–67 [LAP-MPR 46 (2–67)] or 47–67 [LAP-MPR 46 (47–67)] of the cytoplasmic tail of MPR 46 replaced the cytoplasmic tail of LAP were incubated with antiserum against LAP (open symbols). The internalization of 21D3 antibodies at 37 °C was followed after tagging with ^{125}I-labelled Protein A (see the Materials and methods section). The values represent the means of two or three independent experiments. For comparison, the internalization of ^{125}I-21D3 by BHK cells expressing MPR 46 or the MPR 46 Stop6 mutant (filled symbols) was determined as described in the Materials and methods section.

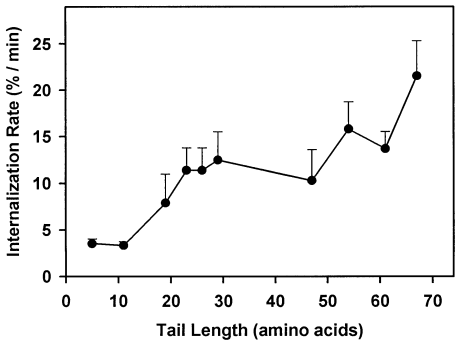

Figure 3 Internalization rate of wild-type MPR 46 and of receptor mutants with truncated tails as a function of tail length

The internalization of ^{125}I-21D3 was determined as described in the Materials and methods section. The internalization rate was calculated from the 3 min values. The means ± range of four to eight independent experiments are given.

introducing stop codons at positions 61, 55, 48, 30, 24, 20 and 12 of the tail (Figure 2B). When internalization was plotted against the length of the tail, it became apparent that shortening the tail from 67 to 60, from 54 to 47 or from 23 to 11 residues decreased the internalization rate, whereas shortening the tail from 60 to 54, from 47 to 23 or from 11 to five residues had no effect, or even increased internalization (Figure 3). We conclude from these results that multiple sequences between residues 12 and 23, 48 and 54, and 61 and 67 contribute to the rapid internalization of MPR 46. Other sequences, such as the N-terminal 11 residues of the tail and the 24 residues from Asn-24 to Gly-47, appear to lack information required for endocytosis, or can function only in combination with sequences distal to residues 11 and 47 respectively.

Internalization of receptor mutants with deletions in the cytoplasmic tail

The internalization kinetics of the truncated receptors had indicated that, in addition to the seven C-terminal residues, internal sequences between residues 12 and 23 and residues 47 and 54 may contain information required for rapid internalization. We introduced, therefore, a total of six deletions, each comprising four to eight residues, which covered the sequences from residues 13 to 29 and from residues 42 to 60 (see Figure 2C). Except for mutant Δ42–49, none of the deletions decreased the

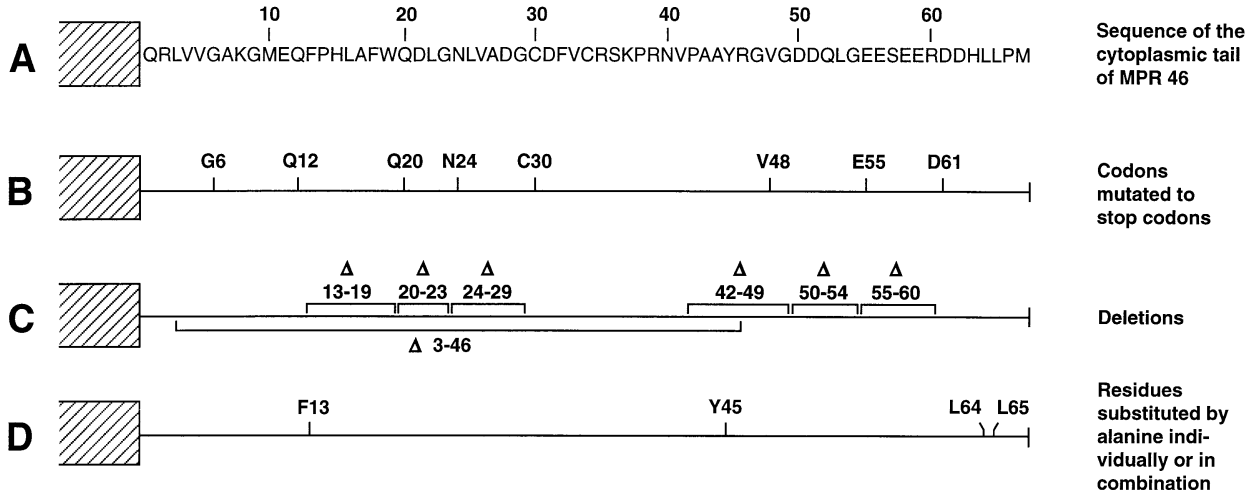

Figure 2 Amino acid sequences of the MPR 46 tail and of the tail mutants

The numbering follows that of Johnson et al. [8], and starts with the glutamine residue following the transmembrane domain. This corresponds to Gln-211 of the human MPR 46 full-length protein [14].

Table 1 Internalization of MPR 46 mutants with deletions in the cytoplasmic tail

Values are means ± S.D. The numbers in parentheses refer to the numbers of independent determinations. The value for wild-type MPR 46 was obtained in parallel with those for the deletion mutants.

MPR 46	Internalization at 37 °C (%/min)
Δ13–19	22.8 ± 3.5 (3)
Δ20–23	23.6 ± 1.5 (2)
Δ24–29	24.0 ± 4.4 (2)
Δ42–49	19.6 ± 3.0 (5)
Δ50–54	23.2 ± 2.6 (2)
Δ55–60	22.8 ± 0.3 (2)
Wild type	22.1 ± 3.3 (5)

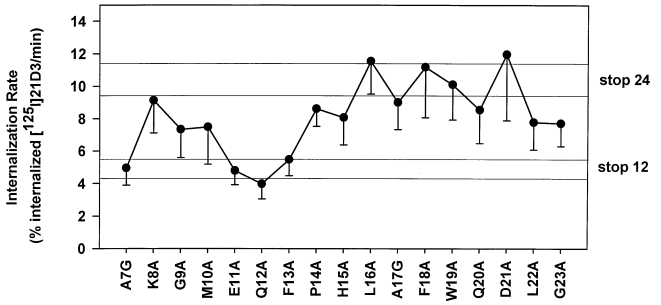

Figure 4 Internalization of MPR 46 Stop24 mutants with individual residues replaced by alanine

The internalization of ^{125}I-21D3 was determined as described in the Materials and methods section. The incubation at 37 °C was for 2, 4 and 6 min. The internalization rate was calculated from the 4 min values. The means ± S.D. of four to six independent experiments using two to four clones for each mutant are given.

internalization rate (Table 1). Internalization of the Δ42–49 mutant was slightly decreased, to 89 % of control. This deletion includes Tyr-45, the substitution of which by alanine was shown previously to decrease the internalization rate moderately [8]. When we tested a Y45A mutant, the internalization rate was slightly decreased to 87 ± 4 % (results not shown). We conclude from these results that, in MPR 46, the loss of a single sequence that can mediate rapid internalization is compensated for by the remaining internalization sequences. An exception appears to be the C-terminal sequence comprising residues 61–67, the deletion of which decreased the internalization rate by about one-third (Figure 3). We therefore restricted our search for residues that are critical for internalization to those mediating rapid internalization of the Stop24 mutant (see Figure 3) and those that are located between residues 61 and 67.

Amino acids critical for internalization in the 23 juxtamembrane cytoplasmic residues

A sequence corresponding to the N-terminal 23 residues of the cytoplasmic tail of MPR 46 mediated internalization at about one-third of the rate of wild-type MPR 46 (Figure 3). To identify the residues critical for internalization, residues 7–23 of the MPR 46 Stop24 mutant were individually substituted by alanine or glycine. The mutants were expressed in BHK cells and analysed for their internalization rate (Figure 4). Of the 17 residues tested, four turned out to be critical for internalization of the MPR 46 Stop24 mutant. Their individual substitution by alanine (Glu-11, Gln-12 and Phe-13) or glycine (Ala-7) reduced the internalization rate to a basal level. The majority of the substitution mutants showed a partial decrease of the internalization rate, and at four positions substitution by alanine did not affect internalization. It should be noted that the latter included the aromatic residues Phe-18 and Trp-19.

The C-terminal third of the cytoplasmic tail contains an autonomous internalization signal with a critical di-leucine motif

The truncation of the C-terminal seven residues resulted in a decrease in the internalization rate of about one-third (Figure 3). To identify the critical amino acids, residues 61–67 were individually substituted by serine. The substitution of Asp-61, Asp-62, His-63, Pro-66 or Met-67 affected the internalization rate by less than 10 %, whereas substitution of Leu-64 and Leu-65 decreased the internalization rate by 21 and 56 % respectively (Figure 5A). Substitution of Leu-64 and Leu-65 by alanine decreased internalization by 23 and 31 % respectively (results not shown).

The truncation of the seven C-terminal residues and their individual substitution had indicated that this sequence contributes to the rapid internalization of MPR 46. This could result either from the disruption of an autonomous internalization signal containing Leu-64 and Leu-65 as critical residues or from an indirect effect of residues 61–67 on internalization sequences located more proximally in the cytoplasmic tail. To differentiate between these two possibilities, residues 47–67 were fused to the reporter polypeptide LAP-RP3 (see Figure 1). Residues 47–60 were included to separate the putative internalization signal within residues 61–67 from the membrane, as several transport signals, including some for internalization, require a minimal distance from the membrane [19,20]. On the other hand, the transferred sequence excluded all aromatic residues previously identified to be critical for internalization of the MPR 46 tail [8]. When corrected for the basal internalization rate of LAP-RP3, the internalization rate of the LAP–MPR 46 chimaera containing the C-terminal one-third of the MPR 46 tail (residues 47–67) was 48 % of that of the LAP–MPR 46 chimaera containing the full-length tail of MPR 46 (Figure 1). We conclude from these results that residues 47–67 of MPR 46 contain a signal that is sufficient to mediate the rapid internalization of a reporter polypeptide.

An MPR 46 deletion mutant retaining residues 47–67 of the cytoplasmic tail (MPR 46 Δ3–46) was internalized with an intermediate internalization rate of 13.2 %/min. Substitution of Leu-64 and Leu-65 by alanine (MPR 46 Δ3–46/L64A/L65A) reduced the internalization rate to 2.6 %/min, which is comparable with the internalization rate of the Stop6 mutant (Figure 5B). This indicates that the di-leucine motif is critical for the internalization signal residing within the C-terminal one-third of the MPR 46 cytoplasmic tail.

Role of Phe-13, Tyr-45, Leu-64 and Leu-65 in internalization of MPR 46

The previous experiments had identified Phe-13, Leu-64 and Leu-65 as critical elements of two autonomous internalization signals in the MPR 46 tail. In addition, substitution of Tyr-45 by alanine had identified this residue as contributing weakly to the internalization efficiency of the MPR 46 tail ([8]; the present study). To assess the roles of Phe-13, Tyr-45, Leu-64 and Leu-65 in the internalization of MPR 46, these residues were substituted either individually or in different combinations in the context of the full-length tail of MPR 46. The mutants were expressed in mpr$^-$ cells. The use of these cells, which had become available

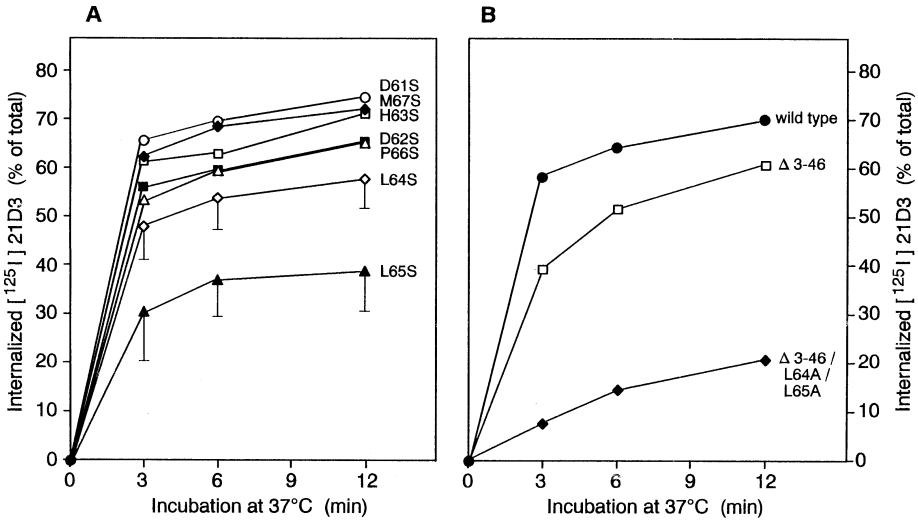

Figure 5 Internalization of MPR 46 mutants having substitutions within the C-terminal heptapeptide

(**A**) Residues 61–67 were individually substituted by serine. The internalization rate was determined as described in the Materials and methods section. The values represent the means of three to ten independent experiments. For the L64S and L65S mutants, the range is indicated. (**B**) Internalization of a mutant carrying deletion of residues 3–46 (Δ3–46) and the effect of substitution of leucine residues 64 and 65 by alanine on the internalization of this deletion mutant (Δ3–46/L64A/L65A). The internalization of wild-type MPR 46 was determined in parallel with the experiments shown in (**A**) and (**B**).

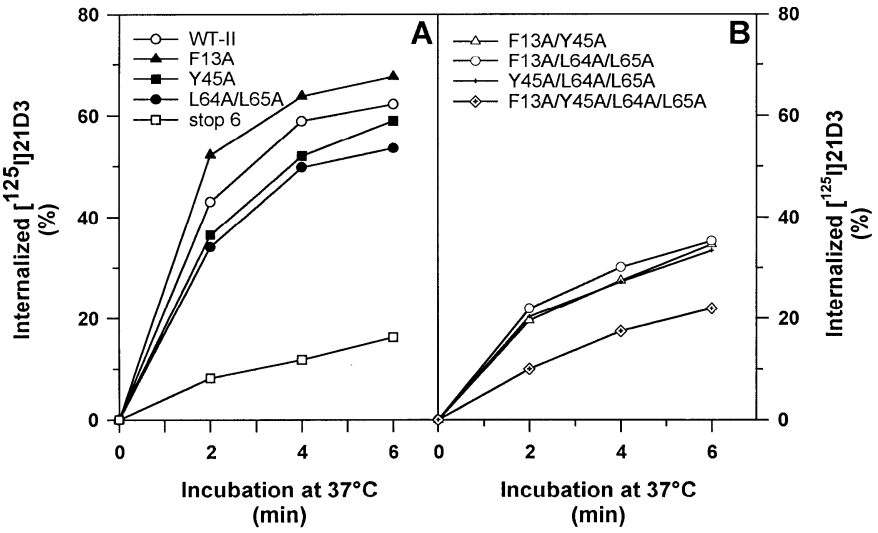

Figure 6 Internalization of MPR 46 mutants with substitutions of Phe-13, Tyr-45 and leucine residues 64 and 65, alone or in combination

mpr$^-$ cells were used for the expression of wild-type MPR 46, the Stop6 mutant and mutants carrying substitutions of Phe-13, Tyr-45 and the di-leucine motif, alone or in combination. Internalization of ^{125}I-21D3 was determined as described in the Materials and methods section. The values represent the means of 5–10 independent experiments. The S.D.s were within $\pm$15% of the individual values in all cases.

only in the later stages of the present study [21] and which internalize wild-type MPR 46 and the Stop6 mutant with similar kinetics as in BHK cells (17%/min and 3%/min respectively; Figure 6A), excluded any interference of the internalization kinetics of mutant MPR 46 by endogenous MPR and facilitated the analysis of the sorting function of the mutant receptors (see below).

Substitution of Phe-13 by alanine led to a slight increase in the internalization rate (19%/min; Figure 6A), indicating that loss of this residue can be compensated fully in the context of the entire MPR 46 tail. Substitution of Tyr-45 or of Leu-64 and Leu-65 led to moderate decreases in the internalization rate (15 and 14%/min respectively), indicating that their loss is less well tolerated than that of Phe-13, but still does not critically affect the internalization rate (Figure 6A).

The simultaneous substitution of the critical residues present in two of the three internalization sequences decreased the internalization rate to about 8%/min, irrespective of whether Phe-13 was substituted together with Tyr-45 or with Leu-64 and Leu-65, or whether Tyr-45 was substituted together with Leu-64 and Leu-65 (Figure 6B). When corrected for the basal internalization rate of the Stop6 mutant, the internalization rate mediated by

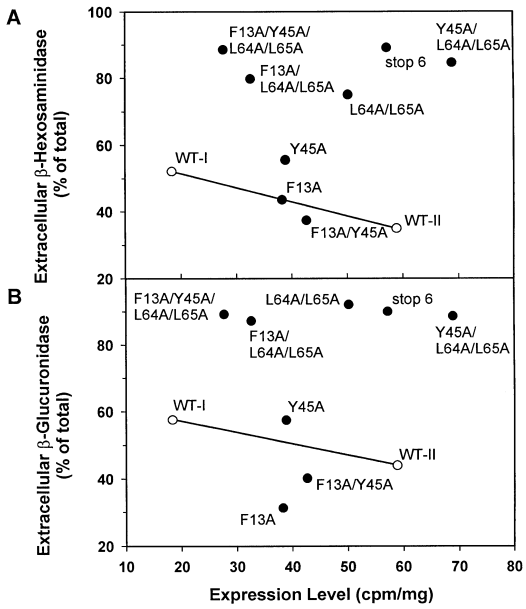

Figure 7 Sorting function of MPR 46 mutants lacking internalization sequences

The extracellular activity of β-hexosaminidase (**A**) and of β-glucuronidase (**B**) is shown as a function of the expression level for the various mutant forms of MPR 46. The values for β-hexosaminidase represent the means of six determinations in two independent experiments, and were measured using a fluorimetric assay. For β-glucuronidase the ratio of extracellular precursor (72 kDa) to the intracellular mature form (69 kDa) was detected by metabolic labelling and quantification of radioactivity incorporated into the immunoprecipitated polypeptides. WT-I and WT-II, wild-type MPR 46.

the mutant tails retaining only Phe-13 or Tyr-45 or the di-leucine motif was about one-third of that mediated by the wild-type tail.

The simultaneous substitution of Phe-13, Tyr-45, Leu-64 and Leu-65 decreased the internalization rate (4 %/min) to close to that of the Stop6 mutant (Figure 6A), indicating that a mutant tail lacking Phe-13, Tyr-45 and the di-leucine motif has lost more than 90 % of its internalization efficiency.

Sorting function and surface expression of MPR 46 mutants lacking internalization sequences

The loss of sequences critical for internalization may impair the sorting function of MPR 46 and alter its steady-state distribution. The sorting function was analysed in mpr$^-$ cells. These cells secrete more than 90 % of newly synthesized lysosomal enzymes. Re-expression of MPR 46 reduces this level of secretion to $\sim$ 40 %, depending on the level of receptor expression [16]. Sorting of lysosomal enzymes was monitored by determining the β-hexosaminidase activity that had accumulated in the culture medium during a 24 h incubation period, and by determining the fraction of β-[^{35}S]glucuronidase that had been secreted during metabolic labelling of cells with [^{35}S]methionine for 6 h followed by a chase of 16 h. The fraction of secreted β-hexosaminidase and β-[^{35}S]glucuronidase was referred to the level of MPR 46 expression. The latter was determined by measuring binding of ^{125}I-21D3 to cells after permeabilization with 0.5 % saponin. The secretion of β-hexosaminidase (Figure 7A) and of β-glucuronidase (Figure 7B) by cells expressing MPR 46 mutants in which Phe-13 and/or Tyr-45 was substituted by alanine was as low as in cells expressing wild-type MPR 46. Substitution of the di-leucine motif alone or in combination with Phe-13 and/or Tyr-45 severely impaired sorting: the fraction of β-hexosaminidase and β-glucuronidase secreted was almost as high as in cells lacking any MPR or expressing the Stop6 mutant.

Table 2 Expression levels and surface expression of MPR 46 constructs in mpr$^-$ cells

The level of endogenous MPR 46 (1.83 μg/mg of cell protein) in mouse embryonic fibroblasts corresponds to a binding of 18500 c.p.m. of ^{125}I-21D3/mg to the re-expressed human MPR 46 protein (see [16]).

MPR 46 construct	Expression level (c.p.m. of ^{125}I-21D3/mg)	Surface-localized MPR 46 (% of total)
Wild type clone I	18500	11
Wild type clone II	59000	8
Stop6	57000	21
F13A	38500	10
Y45A	39000	11
L64A/L65A	50000	14
F13A/Y45A	42500	19
F13A/L64A/L65A	32500	13
Y45A/L64A/L65A	69000	14
F13A/Y45A/L64A/L65A	27500	15

These results clearly demonstrate that a decrease in the internalization rate is not associated with a decrease in sorting efficiency. An example of the dissociation of the two parameters is the F13A/Y45A mutant, which has a normal sorting efficiency, whereas the internalization rate is reduced to one-third.

A decrease in the internalization rate would be expected to result in an increase in the fraction of the receptor that is present at the cell surface. The fraction of MPR 46 expressed at the cell surface was monitored by quantifying the amounts of ^{125}I-21D3 bound to non-permeabilized and saponin-permeabilized cells. Between 8 and 11 % of wild-type MPR 46 was expressed at the cell surface. This fraction was increased up to 19 % for the various alanine substitution mutants and to 21 % for the Stop6 mutant (Table 2). The rather small fraction of MPR 46 mutants present at the cell surface could reflect impaired access to the cell surface, e.g. by preventing their exit from the endoplasmic reticulum. Retention in the endoplasmic reticulum would result in accumulation of immature MPR 46 forms, which contain high-mannose-type oligosaccharides, whereas in mature MPR 46 forms three of the four N-linked oligosaccharides are processed to complex-type structures. Immature and mature forms can easily be distinguished by their apparent sizes on SDS/PAGE and sensitivity to endoglucosaminidase H [22]. When receptor polypeptides synthesized during a 6 h labelling period were analysed, immature MPR 46 polypeptides (10–17 % of the total) were observed for mutants in which Phe-13 and/or the di-leucine motif were substituted (Figure 8). The proportion of immature forms increased to 40 % if all three internalization sequences were substituted (F13A/Y45A/L64A/L65A), and to 80 % if the cytoplasmic tail was deleted (Stop6 mutant). However, at steady state the proportion of immature forms was much lower. In Western blots of cell extracts, immature forms accounted for less than 10 % of the F13A/Y45A/L64A/L65A MPR 46 mutant (results not shown). Thus retention in the endoplasmic reticulum cannot fully explain the low level of surface expression of MPR 46 mutants with decreased internalization efficiency. Whether retention in other organelles, such as lysosomes, accounts for the low surface expression remains to be determined. It should be noted that MPR 46 may accumulate in lysosomes of MPR-deficient cells due to their low levels of lysosomal proteinases.

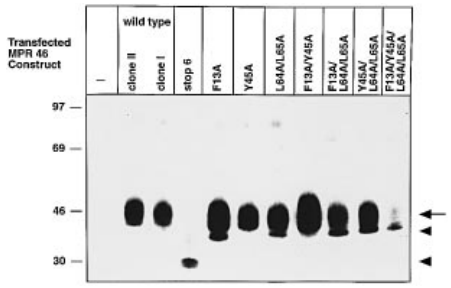

Figure 8 Expression of wild-type MPR 46 and internalization-defective mutants in mouse fibroblasts

mpr$^-$ cells stably transfected with the MPR 46 constructs indicated were metabolically labelled for 6 h. MPR 46 was immunoprecipitated from the cell extracts and separated by SDS/PAGE. The migration position of normally glycosylated MPR 46 polypeptides is indicated by the arrow, and those of MPR 46 polypeptides fully sensitive to endoglucosaminidase H are indicated by arrowheads. The positions of molecular mass markers (kDa) are shown on the left.

The low surface expression of internalization-deficient MPR 46 mutants appears to be a peculiarity of mpr$^-$ cells. In BHK cells, 37% of the MPR 46 Stop6 mutant was surface associated (results not shown), compared with 21% in mpr$^-$ cells. In mouse L-cells, 80% of an MPR 46 Stop8 mutant was found at the cell surface [8].

DISCUSSION

The cytoplasmic tail of MPR 46 contains three internalization sequences

The results of the present study led us to the conclusion that the cytoplasmic tail of MPR 46 contains three separate sequences which together mediate the rapid internalization of the receptor at a rate of up to 40%/min. Since loss of one of these internalization sequences was tolerated without an appreciable decrease in the internalization rate, identification of the sequences depended on constructs that encoded fragments of the cytoplasmic tail harbouring only one of the internalization sequences.

The 23 juxtamembrane cytoplasmic residues stimulate internalization 2–3-fold above the basal level. Individual substitution of residues 7–23 identified Ala-7, Glu-11, Glu-12 and Phe-13 as critical residues in the truncated tail. The heptapeptide A^{7}KGMEQF, which comprises the critical residues, has no obvious similarity to any of the known tyrosine-based internalization motifs. It may be related to the type II internalization signals defined by Naim and Roth [23], which contain a critically important aromatic residue at position 6.

A second internalization sequence contains as a critically important residue a tyrosine, which is part of the tetrapeptide Y^{45}RGV. This peptide resembles tyrosine-based internalization signals of the sequence YXXZ, where Z indicates a hydrophobic amino acid [24,25]. Tyrosine-based internalization signals function in a variety of membrane proteins as autonomous internalization sequences. It is therefore likely that the Y^{45}RGV tetrapeptide serves in MPR 46 as an autonomous internalization sequence, although this remains to be determined.

The third internalization sequence is represented by the C-terminal one-third (residues 47–67) of the MPR 46 cytoplasmic tail. This signal is autonomous and contains a di-leucine pair at positions 64 and 65 as critical residues. Di-leucine-based internalization motifs mediate the rapid internalization of several membrane proteins, including the γ-and δ-chains of the T-cell receptor [26], CD4 [27], the GLUT4 glucose transporter [28], the insulin receptor [29], the IgG F_c receptor [30], the MHC-associated invariant chain [31], the lysosomal membrane glycoprotein LIMP II [32], and the interleukin-6 signal transducer gp130 [33]. Di-leucine-based trafficking signals, however, are also implicated in the sorting of membrane proteins at the *trans*-Golgi network (TGN) into vesicles destined for the basolateral membrane of polarized cells [30] and for endosomes [34–36]. In fact, the di-leucine motif of the MPR 46 tail and a similarly positioned di-leucine motif in the cytoplasmic tail of the 300 kDa mannose 6-phosphate/insulin-like growth factor II receptor have been shown to be critical for the lysosomal enzyme-sorting function of both MPRs, presumably due their function in trafficking MPRs from the TGN to endosomes [9,37].

The critical residues of the three internalization sequences are separated from each other by more than 15 residues, suggesting that they are part of three distinct recognition motifs. In fact, for two of them it was demonstrated that they function independently of the other tail sequences.

The observation that the combined mutation of two or three critical residues in the full-length MPR 46 tail led to a decrease of internalization, whereas the single mutation of the same residues was without effect, does not necessarily indicate that these residues form part of the internalization sequences. The observed effect could also result from a conformational alteration of the cytoplasmic tail that inactivates its internalization sequence(s). However, three lines of argument can be put forward in favour of the assumption that the mutated residues indeed form part of internalization sequences. First, mutation of the same residues in tail fragments led to a loss of internalization activity. Secondly, for two of the critical residues (Phe-13 and Tyr-45) it was shown that their deletion together with that of six or seven adjacent residues did not alter or only minimally affected internalization (see Table 1). This indicates that which residue is substituted in combination with Phe-13 or Tyr-45 is important. Thirdly, deletions individually spanning four to eight residues and together covering more than 50% of the entire length of the tail did not affect internalization. This lessens the likelihood that the combined mutation of two single residues exerted its effect indirectly through a conformational change of the cytoplasmic tail. The observation that those residues that were identified as being critical for internalization mediated by tail fragments turned out to be also critical for internalization mediated by the full-length tail strengthens the view that these residues form part of the internalization sequences of the MPR 46 tail.

Johnson et al. [8], in an earlier study, identified in the cytoplasmic tail of MPR 46 two independent sequences mediating rapid internalization. One sequence included Phe-18 as the key residue and Phe-13 as a secondary component; the other, less potent, internalization sequence contained Tyr-45 as a critical residue. Deletion constructs suggested a third internalization sequence within the C-terminal 17 residues. The significance of this C-terminal signal, however, was questioned, as substitution of the aromatic residues, all of which are located within the N-terminal 45 residues of the tail, reduced the internalization rate to close to that of the tail-less receptor. The data presented here show that Phe-13 rather than Phe-18 is of critical importance for rapid internalization, and that the di-leucine pair at positions 64 and 65 functions as a third independent internalization sequence. The conclusion that Phe-18 is not part of an internalization sequence is supported by the normal internalization rate of three MPR 46 mutants in which Phe-18 was substituted by alanine in a truncated tail (Figure 4) or by serine in a full-length tail (results not shown), or in which residues Phe-13 to Tyr-19 were deleted (Table 1). This discrepancy suggests that the 2-fold decrease in

the rate of internalization observed for the F18A substitution [8] resulted from an indirect effect on an internalization signal that does not include Phe-18.

Partial redundancy of the three internalization sequences

The relative potencies of the three signals appear to be similar, if judged from the internalization rates of constructs in which the critical residues of two of the three signals had been substituted by alanine and only one internalization signal was left unchanged. When the relative potency was estimated from the decrease in the internalization rate caused by substitution of the critical residue(s) of a single internalization signal, the di-leucine-based signal appeared to be more potent than the Tyr-45-based signal, with the potency of the Phe-13-based signal being negligible. It should be noted that any estimate of the relative potency of individual internalization signals has to be taken with caution, since substitution of the critically important residue of one signal may not only abolish the activity of that signal, but could also indirectly affect the efficiency of the remaining signals.

Irrespective of the relative potencies of the individual internalization signals, they appear to function in an additive manner. Since the inactivation of two internalization signals leads to a much more pronounced decrease in the internalization rate than expected from the effects of their individual inactivation, it is concluded that the internalization signals in the MPR 46 cytoplasmic tail are partly redundant.

Multiple internalization signals have been identified in several membrane proteins, including the polymeric Ig receptor [38], the insulin receptor [29,39,40], the epidermal growth factor receptor [41], the γ-chain of the T-cell receptor [26] and the invariant chain of the MHC class II [31,34]. Where investigated, a partial redundancy of the multiple internalization motifs has been observed. In P-selectin, multiple residues distributed throughout the cytoplasmic tail of 34 residues contribute to rapid internalization. In spite of an extended site-directed mutagenesis study of the cytoplasmic tail, no short internalization signal could be identified in P-selectin [42].

The plasma membrane adaptor AP-2 can serve as a receptor for tyrosine- and di-leucine-based internalization signals, as well as for the clathrin lattice [43,44]. AP-2 is a heterotetramer containing two $\sim$ 100 kDa subunits (α- and β_2-adaptin) and two smaller subunits of 50 kDa (μ_2-chain) and 17 kDa (σ_2-chain). The β_2-adaptin and the μ_2-chain have been implicated in the binding of tyrosine-based internalization signals [45,46].

The presence of multiple internalization signals within a cytoplasmic tail raises several questions. Does binding involve multiple interactions between a cytoplasmic tail and a single AP-2 complex? Is a cytoplasmic tail in contact with several AP-2 complexes? Are only part of the internalization signals involved in establishing binding with AP-2, and are some of the internalization signals recognized by cytoplasmic receptors distinct from AP-2?

Epidermal growth factor receptors, which contain several internalization signals, form complexes with AP-2 in a stoichiometric 1:1 molar ratio [47]. Since receptors such as MPR 46 are present in membranes as homo-oligomers [18], a multimetric MPR-46–AP-2 complex may form, facilitating the interaction of a single cytoplasmic tail with several AP-2 complexes. Recent experiments using surface plasmon resonance analysis indicated that the interaction between the MPR 46 cytoplasmic tail and AP-2 indeed involves several separate sequences of the receptor tail. Short peptides representing distinct sequences of the MPR 46 cytoplasmic tail are capable of inhibiting the binding of AP-2 to the MPR 46 cytoplasmic tail immobilized to a sensor surface (S. Höning, unpublished work).

Sorting of lysosomal enzymes by internalization-defective MPR 46 mutants

The sorting of soluble lysosomal enzymes is thought to depend on the binding of newly synthesized lysosomal enzymes within the Golgi to MPR, the sorting of the complexes into AP-1- and clathrin-coated vesicles at the TGN, the fusion of these vesicles with endosomes, dissociation of receptors and lysosomal enzymes, and sorting of the receptors into vesicles trafficking back to the Golgi. The receptors recycling between the cell surface and endosomes are in equilibrium with those recycling between the TGN and endosomes [1]. Inactivation of an internalization signal could impair sorting by decreasing the steady-state concentration of receptors in the TGN compared with that at the plasma membrane. Sorting would also be impaired if the critically important residues of an internalization signal are also part of a signal involved in sorting at the TGN or endosomes. The normal sorting efficiency of the F13A/Y45A mutant, which is internalized only at 40 % of the rate of wild-type MPR 46, indicates that a decrease in internalization efficiency is not necessarily coupled with a decrease in sorting efficiency. The loss of the sorting function of mutants that lack the di-leucine motif suggests that these residues are also critically important for sorting at the TGN or endosomes. Johnson and Kornfeld [9] have reported that deletion of the C-terminal pentapeptide H^{63}LLPM or its substitution by alanine abolished the sorting function of MPR 46, whereas the return of the receptor from the cell surface to the Golgi was only moderately reduced. This was taken as evidence that the loss of the di-leucine pair impairs the sorting function of the receptor by impairing its trafficking at the Golgi. The present results are in agreement with this assumption, and indicate that the di-leucine motif functions both at the TGN and at the cell surface in the sorting of MPR 46 into clathrin-coated vesicles.

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