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Cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain

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ABSTRACT **The signals required for rapid internalization of the bovine cation-dependent mannose 6-phosphate receptor have been localized to two distinct regions of the 67-amino acid cytoplasmic domain. One signal includes phenylalanine 13 and phenylalanine 18, while the other involves tyrosine 45. The former signal is more potent than the latter, but both must be present for the maximal rate of receptor internalization. Each signal shares similarities with the known internalization signals of other recycling receptors.**

The 46-kDa cation-dependent mannose 6-phosphate receptor (CD-MPR) cycles between the Golgi complex, endosomes, and cell surface (1). In the Golgi complex the receptor binds newly synthesized lysosomal enzymes and transports them to acidified endosomal compartments, where the lysosomal enzymes are discharged for packaging into lysosomes (2). The receptor may return to the Golgi complex to mediate another round of lysosomal enzyme targeting, or it may move to the cell surface. While receptor molecules on the cell surface bind lysosomal enzymes poorly, the receptor is nevertheless rapidly internalized (3).

Previous studies of the cation-independent (CI-)MPR (4) and the receptors for low density lipoprotein (LDL) (5, 6), polymeric immunoglobulin (7), transferrin (8, 9), and IgG (10), as well as studies with a mutant influenza viral hemagglutinin (11), have shown that the determinants for rapid internalization of these receptors from the plasma membrane are contained within a short stretch of amino acids of the cytoplasmic domain which includes an essential tyrosine residue. On the basis of this information it seemed likely that the determinants for the rapid internalization of the CD-MPR would also be localized to its cytoplasmic domain.

The mature bovine CD-MPR is a type I membrane-spanning glycoprotein with a 165-amino acid residue extracytoplasmic region, a 25-residue transmembrane region, and a 67-residue carboxyl-terminal cytoplasmic region (12). In the present study, we have transfected mouse L cells with cDNAs that encode mutant bovine CD-MPRs with deletions or amino acid substitutions in the cytoplasmic domain. Stable clones of the transfected cell lines have been isolated and analyzed for the cellular distribution of the mutant CD-MPRs and the rate of internalization of the receptors from the cell surface. Our results indicate that the cytoplasmic domain of the CD-MPR contains two separate signals for rapid internalization. One signal contains two phenylalanine residues while the other contains a tyrosine residue.

MATERIALS AND METHODS

Reagents. Bovine milk galactosyltransferase and all chemicals were from Sigma. *Vibrio cholerae* neuraminidase was from Calbiochem. *Diplococcus pneumoniae* β-galactosidase was purified as described (13). UDP-[^{3}H]galactose (20–40 mCi/mmol; 1 Ci = 37 GBq) and Na^{125}I (100 mCi/ml) were from Amersham. Restriction endonucleases, T4 DNA polymerase, and ligase were from New England Biolabs. Phosphopentamannosyl-Sepharose and mannose 6-phosphate were provided by Walter Gregory of this laboratory. Oligonucleotides were synthesized by the protein chemistry facility at Washington University, St. Louis. Affinity-purified rabbit anti-bovine CD-MPR antibody was isolated as described (14).

Plasmid Constructs. The full-length CD-MPR cDNA clone (12) was inserted into the expression vector pSV2-neo to form pSV2-neo-cdmpr. pSV2-neo (15) was provided by Steven Fine (Washington University, St. Louis). Receptor deletion mutants were generated by using oligonucleotide-directed mutagenesis. A restriction fragment encoding the entire CD-MPR was subcloned in the vector pGBT518 (Gold Biotechnology, St. Louis, MO), and single-stranded template was generated by using helper phage M13K07. The truncated receptors were constructed by using oligonucleotide primers that introduced two sequential stop codons into the receptor cDNA. Following primer extension, the plasmids were used to transform *Escherichia coli* strain JM109. Colonies were selected by the AlterGene method (Gold Biotechnology). The region of the cDNA that had been through a single-stranded intermediate was sequenced. A restriction fragment containing the mutated cDNA was then subcloned in pSV2-neo.

To facilitate subsequent mutagenesis, a partial synthetic CD-MPR cDNA containing 15 restriction sites was assembled as follows: A series of synthetic oligonucleotides which encoded the entire transmembrane and cytoplasmic domains were annealed together in ligase buffer (26) by heating at 80°C for 10 min and then slowly cooling to 25°C. This double-stranded oligonucleotide was subcloned in the vector pGEM3ZF(+) (Promega) and sequenced. Substitutions of individual or groups of amino acids were made by cassette mutagenesis (16). Briefly, the parent plasmid was digested with appropriate restriction enzymes and the desired DNA fragments were purified from agarose by using Geneclean (BIO 101, La Jolla, CA) according to the manufacturer's specifications. Mutant oligonucleotides were annealed together as described above to generate a double-stranded cassette, which was then ligated to the desired DNA fragment from the parent plasmid and the resultant plasmid was used to transform *E. coli* strain JM109. Plasmids were isolated and screened by restriction enzyme mapping, and the identity of the constructs was confirmed by DNA sequencing of the mutated region. These mutant CD-MPR cDNAs were then subcloned in pSV2-neo to generate the various mutant receptors. Mutations are named by residue (one- or three-letter symbol) and position and the substitution (alanine or stop codon); Arom$^-$ indicates substitution of alanine for all aromatic residues.

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Abbreviations: MPR, mannose 6-phosphate receptor; CD-, cation-dependent; CI-, cation-independent; BSA, bovine serum albumin; LDL, low density lipoprotein.

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Transfection of Cell Lines. A CI-MPR-deficient mouse L cell line designated L(CI-MPR$^-$) was maintained as described (17). The L(CI-MPR$^-$) cells were transfected with 20 μg of *Xba* I-linearized pSV2-neo, pSV2-neo-cdmpr, or mutant pSV2-neo-cdmprs as described (4). For each construct, 20 neomycin sulfate (G418)-resistant colonies were removed 7 to 10 days after transfection and screened for receptor expression by their ability to bind ^{125}I-labeled anti-CD-MPR antibody (see below). Three to six colonies expressing the highest levels of receptor were selected and expanded. The subclones were maintained in α minimal essential medium (α-MEM) containing 10% heat-inactivated fetal bovine serum supplemented with G418 at 350 μg/ml.

Receptor Distribution. Confluent cells in six-well tissue culture dishes were rinsed with α-MEM and incubated with α-MEM containing 15 mM Hepes, pH 7.4, plus or minus 0.1% saponin for 1 hr on ice. Then 1×10^6 cpm of ^{125}I-labeled affinity-purified rabbit anti-CD-MPR antibody was added to the medium, and the incubation was continued for 2 more hr on ice. The antibody was iodinated as described (18). The cells were washed extensively with phosphate-buffered saline (26) containing bovine serum albumin at 10 mg/ml (PBS/BSA), and the cell-associated radioactivity was determined on a γ counter after solubilization of the cells with 0.1 M NaOH.

Internalization Assay. Confluent cells in 60-mm tissue culture dishes were rinsed with α-MEM containing BSA at 1 mg/ml and 50 mM Hepes, pH 7.4 (buffer A). The cells were then incubated with *V. cholerae* neuraminidase at 0.02 unit/ml and *D. pneumoniae* β-galactosidase at 0.01 unit/ml in 1.5 ml of buffer A for 1 hr at 37°C. The cells were washed four times with warm buffer A followed by two rinses with ice cold α-MEM lacking phosphate, bicarbonate, and glucose but containing BSA at 1 mg/ml, 50 mM Hepes at pH 7.4, and 2 mM $MnCl_2$ (buffer B). Surface glycoproteins were labeled by adding 1.0 ml of buffer B containing UDP-[^{3}H]galactose at 20 μCi/ml, 10 mM galactonolactone (Sigma), and galactosyltransferase at 0.2 unit/ml to the cells and incubating for 10 min on ice. The cells were rapidly washed four times with cold buffer A followed by the addition of α-MEM/10% fetal bovine serum and incubation at 37°C for 2–20 min ("chase") to allow internalization of surface CD-MPR. The cells were then chilled to 4°C, harvested with trypsin/EDTA, and incubated at 4°C for 12–16 hr with β-galactosidase at 0.03 unit/ml. The cells were washed with PBS and sonicated in lysis buffer (50 mM imidazole·HCl/150 mM NaCl/2% Triton X-100/0.5% deoxycholate/20 mM $MnCl_2$, pH 6.5, and 2 μg/ml each of pepstatin A, leupeptin, chymostatin, and antipain and 10 trypsin inhibitory units of aprotinin per ml), followed by centrifugation at 50,000 × *g* for 30 min at 4°C. The supernatant containing the solubilized cellular CD-MPR was applied to a phosphopentamannose-Sepharose column. The column was washed with buffer containing 50 mM imidazole·HCl, 150 mM NaCl, 0.05% Triton X-100, BSA at 0.1 mg/ml, and 20 mM $MnCl_2$, and the bound CD-MPR was eluted with 10 mM mannose 6-phosphate and its radioactivity was measured. The proportion of internalized CD-MPR was calculated by dividing the radioactivity eluted with mannose 6-phosphate at each time point (β-galactosidase-resistant CD-MPR) by the radioactivity eluted with mannose 6-phosphate from lysates of cells not stripped with β-galactosidase after labeling (total CD-MPR). The amount of radioactivity recovered in the latter fraction (total CD-MPR) was greater than 900 cpm in all instances except for the wild-type receptor (270, 370, and 1095 cpm recovered in three separate determinations) and the $F^{18}W^{19}$→A mutant receptor (349 cpm recovered in a single determination).

RESULTS

Effect of Cytoplasmic Domain Deletions on Receptor Distribution. We initially analyzed the effect of cytoplasmic domain deletions on the cell surface/cytoplasmic distribution of the receptor. This measurement serves as a useful screen for defects in receptor internalization, since receptors that fail to undergo rapid internalization accumulate on the cell surface. Stable clones of mouse L cells that had been transfected with cDNAs encoding wild-type or mutant bovine CD-MPRs containing 50 amino acids (Asp^{51} → Stop), 27 amino acids (Asp^{28} → Stop), or 7 amino acids (Lys^8 → Stop) of the 67-amino acid cytoplasmic tail were analyzed. (We have numbered the amino acid residues of the cytoplasmic domain 1–67, starting with the glutamine residue immediately following the transmembrane domain. See Table 2.) The level of receptor expression in the various clones was similar, and the concentration of mutant receptor was never greater than twice that obtained with transfection of the normal CD-MPR cDNA. This ensured that any observed differences in distribution (and internalization rates) were not due to nonspecific saturation of the internalization machinery (8).

The distribution of the receptor was determined by measuring the binding of ^{125}I-labeled anti-CD-MPR antibody to intact cells (surface receptor) versus binding to cells permeabilized with 0.1% saponin (total receptor) (19). As summarized in Table 1, about 10% of the normal bovine CD-MPR was judged to be on the cell surface when this technique was used. Deletion of 17 amino acids (Asp^{51} → Stop) and 40 amino acids (Asp^{28} → Stop) of the cytoplasmic domain resulted in modest increases in surface molecules (31% and 35%, respectively), whereas deletion of all but 7 amino acids of the cytoplasmic tail (Lys^8 → Stop) was associated with a striking accumulation of receptor on the surface (80%).

Direct Assay of Receptor Internalization. To measure the rate of receptor internalization directly, we took advantage of the fact that each receptor dimer contains six complex-type asparagine-linked oligosaccharides with sialic acid→galactose sequences at their nonreducing termini (20). This allowed the labeling of surface molecules in the following manner: Monolayers of cells were treated with neuraminidase and β-galactosidase to remove sialic acid and galactose residues and then incubated with galactosyltransferase and UDP-[^{3}H]galactose for 10 min on ice. Under these conditions

Table 1. Distribution of mutant CD-MPR in transfected L cells

Mutations	No. of foci	No. of determinations	% CD-MPR on surface
Wild type	1	10	9.6 ± 0.6
Asp^{51} → Stop	1	4	30.6 ± 1.5
Asp^{28} → Stop	1	5	34.5 ± 2.0
Lys^8 → Stop	1	7	80.4 ± 5.3
$F^{13}F^{18}W^{19}$ → A: (Asp^{28} → Stop)	3	10	70.4 ± 2.1
F^{13} → A	3	8	6.3 ± 0.8
F^{18} → A	3	9	35.5 ± 2.5
$F^{18}W^{19}$ → A	2	6	31.7 ± 2.2
$F^{13}F^{18}W^{19}$ → A	6	19	55.2 ± 4.5
F^{32} → A	3	12	15.9 ± 2.2
Y^{45} → A	5	14	17.6 ± 2.6
$F^{32}Y^{45}$ → A	4	15	27.2 ± 6.0
$F^{13}F^{18}W^{19}Y^{45}$ → A	4	12	59.4 ± 3.6
$F^{13}F^{18}W^{19}F^{32}$ → A	5	15	41.4 ± 2.4
Arom$^-$	3	11	65.1 ± 5.0

The total binding of ^{125}I-labeled anti-bovine CD-MPR antibody (in the presence of saponin) was 45,000–200,000 cpm. The values for the percent CD-MPR on the surface represent the mean ± SEM for the number of individual foci and the number of determinations indicated.

the cell surface glycoproteins, including the CD-MPR, are labeled with [^{3}H]galactose. The labeled cells were warmed to 37°C for 2–20 min to allow receptor internalization to occur and then rapidly chilled to 4°C to stop receptor movement. The cells were next retreated with β-galactosidase to remove the [^{3}H]galactose from receptors that remained on the cell surface. Therefore only the receptor that was internalized would retain the [^{3}H]galactose label. Finally, the CD-MPR was isolated and the content of [^{3}H]galactose was determined and compared with the content of [^{3}H]galactose on receptor from control cells not reincubated with the β-galactosidase (i.e., total receptor). In this way it was possible to determine the percentage of surface receptors that were internalized at each time point.

The data from this analysis are shown in Fig. 1. It is apparent that the wild-type CD-MPR is internalized rapidly, with a $t_{1/2}$ of less than 2 min, whereas the tailless receptor ($Lys^8 \rightarrow$ Stop) is internalized extremely slowly. In fact, the rate of internalization of the tailless receptor is indistinguishable from the rate at which total cell surface glycoproteins are internalized and likely represents nonspecific pinocytosis. The truncated receptors with 50 ($Asp^{51} \rightarrow$ Stop) and 27 ($Asp^{28} \rightarrow$ Stop) amino acids of the cytoplasmic tail are internalized at intermediate rates.

Role of Phe^{13}, Phe^{18}, and Trp^{19} in CD-MPR Internalization. The above results indicated that a signal sufficient for rapid internalization of the CD-MPR was located between residues 8 and 27 of the cytoplasmic tail. This region of the cytoplasmic tail contains three aromatic residues, Phe^{13}, Phe^{18}, and Trp^{19} (see Table 2 for sequences). To determine if these residues are required for rapid internalization, the $Asp^{28} \rightarrow$ Stop construct was further mutated to convert the three aromatic residues to alanines [construct $F^{13}F^{18}W^{19} \rightarrow A$:($Asp^{28} \rightarrow$ Stop)]. When this construct was transfected into L cells and analyzed, 70% of the expressed receptor molecules were found to be present on the cell surface, consistent with the loss of the signal for rapid internalization (Table 1). This mutant receptor was internalized slightly faster than the tailless receptor ($Lys^8 \rightarrow$ Stop) but significantly slower than the $Asp^{28} \rightarrow$ Stop truncated receptor (Fig. 1).

The effect of mutating these three aromatic residues, either alone or in combination, in the full-length CD-MPR construct was also examined. Replacement of Phe^{18} with an alanine ($F^{18} \rightarrow A$) resulted in an increase in surface receptor (36%, Table 1) and an approximately 2-fold decrease in the rate of internalization (Fig. 2) as compared with the full-length wild-type CD-MPR. The double mutant ($F^{18}W^{19} \rightarrow A$) gave essentially the same results, indicating that the loss of the Trp^{19} residue does not result in further impairment of receptor internalization (Table 1; Fig. 2). Replacement of Phe^{13} with an alanine ($F^{13} \rightarrow A$) did not alter receptor distribution (Table 1), indicating that the loss of this residue, by itself, has little effect on the rate of receptor internalization. However, when Phe^{13} was removed from the full-length receptor that already had Phe^{18} and Trp^{19} replaced by alanine, the resultant receptor ($F^{13}F^{18}W^{19} \rightarrow A$) showed a greater accumulation on the cell surface (55%, Table 1) and a slower rate of internalization compared with the full-length double mutant ($F^{18}W^{19} \rightarrow A$, Fig. 2). However, this full-length $F^{13}F^{18}W^{19} \rightarrow A$ mutant receptor was not as impaired as the truncated $F^{13}F^{18}W^{19} \rightarrow A$:($Asp^{28} \rightarrow$ Stop) mutant.

Role of Phe^{32} and Tyr^{45} in CD-MPR Internalization. The finding that the full-length $F^{13}F^{18}W^{19} \rightarrow A$ mutant receptor was internalized more rapidly than the truncated $F^{13}F^{18}W^{19} \rightarrow A$:($Asp^{28} \rightarrow$ Stop) mutant receptor indicated that there might be a second internalization signal located distal to residue 27 of the cytoplasmic tail. Examination of the amino acid sequence in this region revealed the presence of two additional aromatic residues, Phe^{32} and Tyr^{45} (Table 2). To evaluate the role of these residues, a full-length construct ($Arom^-$) was made in which all five aromatic residues were replaced by alanines. When cells were transfected with this construct, the resultant receptor accumulated on the cell surface to a large extent (65%, Table 1) and was internalized extremely slowly, similar to the rate observed with the tailless receptor (Fig. 2). This indicated that either Phe^{32} or Tyr^{45} or both could function as components of an internalization signal.

Constructs replacing either amino acid alone or both together were generated and transfected into the L cells. Replacement of either residue alone ($F^{32} \rightarrow A$ and $Y^{45} \rightarrow A$) resulted in full-length receptors that showed slight accumulations on the cell surface (16% and 18%, respectively, Table 1). The full-length double mutant ($F^{32}Y^{45} \rightarrow A$) showed a somewhat greater surface accumulation (27%, Table 1). The $Y^{45} \rightarrow A$ mutant receptor exhibited a moderate impairment in

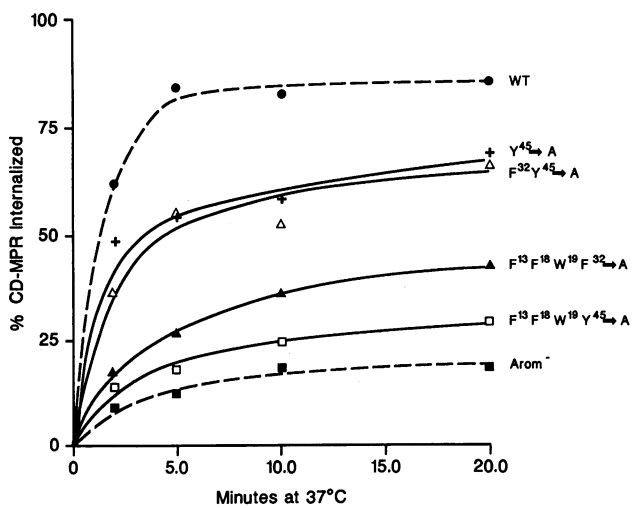

FIG. 1. Effect of cytoplasmic domain deletions on CD-MPR internalization. The values shown are the average of two or three independent determinations, except for the $Asp^{51} \rightarrow$ Stop mutant, which was measured only once. CD-MPRs: ●, wild-type (WT); +, $Asp^{51} \rightarrow$ Stop; △, $Asp^{28} \rightarrow$ Stop; ■, $F^{13}F^{18}W^{19} \rightarrow A$:($Asp^{28} \rightarrow$ Stop); ○, $Lys^8 \rightarrow$ Stop. ▲, Total cell surface glycoproteins.

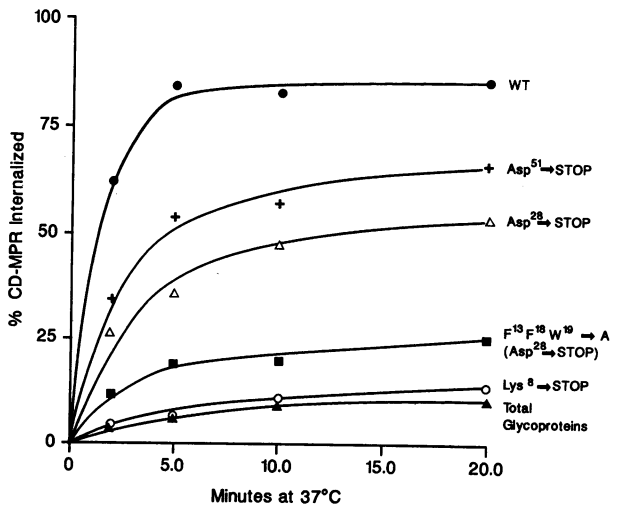

FIG. 2. Effect of mutating Phe^{13}, Phe^{18}, and Trp^{19} on CD-MPR internalization. The values plotted are the average of two independent determinations, except for $F^{18}W^{19} \rightarrow A$, which was measured only once. Mutant CD-MPRs: +, $F^{18} \rightarrow A$; □, $F^{18}W^{19} \rightarrow A$; ▲, $F^{13}F^{18}W^{19} \rightarrow A$; ■, $Arom^-$. The broken lines represent the data shown in Fig. 1 for the wild-type (●) and the $Lys^8 \rightarrow$ Stop (○) CD-MPRs.

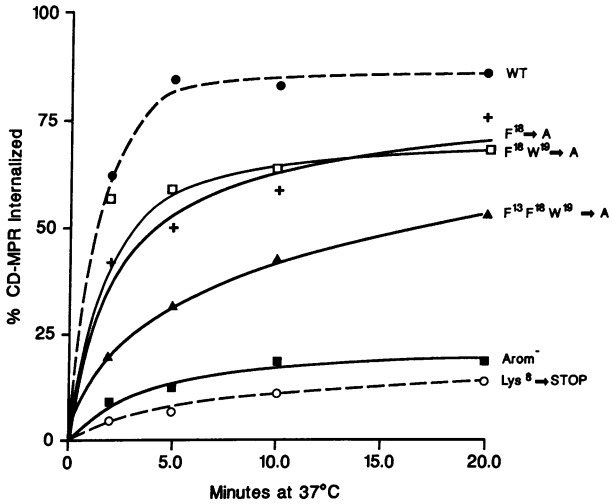

FIG. 3. Effect of mutating Phe[32] and Tyr[45] on CD-MPR internalization. The values plotted are the average of two independent determinations. Mutant CD-MPRs: △, $F^{32}Y^{45} \rightarrow A$; +, $Y^{45} \rightarrow A$; ▲, $F^{13}F^{18}W^{19}F^{32} \rightarrow A$; □, $F^{13}F^{18}W^{19}Y^{45} \rightarrow A$. The broken lines represent the data shown in Fig. 1 (●, wild-type CD-MPR) and Fig. 2 (■, Arom⁻ CD-MPR).

internalization, and the double mutant ($F^{32}Y^{45} \rightarrow A$) behaved almost identically in the direct internalization assay (Fig. 3).

The relative contribution of Phe[32] and Tyr[45] to the rate of receptor internalization was further evaluated by adding these residues back to the Arom⁻ construct. The addition of Phe[32] to the Arom⁻ construct ($F^{13}F^{18}W^{19}Y^{45} \rightarrow A$) resulted in only a slight decrease in the accumulation of the receptor on the cell surface (from 65% for Arom⁻ to 59%; Table 1) and had a very small effect on the rate of receptor internalization (Fig. 3). In contrast, the addition of Tyr[45] (construct $F^{13}F^{18}W^{19}F^{32} \rightarrow A$) decreased the amount of receptor on the cell surface to 41% (Table 1) and significantly enhanced the rate of internalization (Fig. 3). However, this mutant receptor ($F^{13}F^{18}W^{19}F^{32} \rightarrow A$) was not internalized as rapidly as the receptor containing Phe[13], Phe[18], and Trp[19] but lacking Phe[32] and Tyr[45] ($F^{32}Y^{45} \rightarrow A$) (Fig. 3). Taken together, these findings indicate that Tyr[45] functions as a component of an internalization signal whereas Phe[32] probably does not.

DISCUSSION

These data demonstrate that the rapid internalization of the CD-MPR is mediated by two distinct and functionally independent signals in its cytoplasmic domain. One signal includes Phe[18] as the key residue, with Phe[13] serving as a secondary component, while the other signal involves Tyr[45]. The former signal is more potent than the latter, but both must be present to allow the maximal rate of receptor internalization. Preliminary evidence indicates that the polymeric immunoglobulin receptor may also contain two internalization signals (7), suggesting that the presence of multiple signals will not be limited to the CD-MPR.

The evidence for the role of Phe[18] in receptor internalization is straightforward. Replacement of this residue with an alanine results in a receptor molecule that is impaired in the rate of internalization and consequently accumulates on the cell surface. However, the situation with Phe[13] is more complex. When alanine was substituted for Phe[13] in an otherwise normal receptor, there was no effect on receptor distribution (the internalization rate could not be determined due to the low surface expression). Yet when Phe[13] was removed from a receptor that already had Phe[18] and Trp[19] replaced with alanine residues, the resultant receptor exhibited a greater accumulation on the cell surface and was internalized more slowly than the receptor with the Phe[18] and Trp[19] double substitution. This latter result supports the notion that Phe[13] is a component of an internalization signal that includes Phe[18]. The reason why Phe[13] substitution by itself has no apparent effect on receptor internalization is not clear. Since alanine substitution for Trp[19] did not result in further impairment of a receptor with a Phe[18] → A substitution, it seems unlikely that this residue is a significant part of the internalization signal. The Phe[13]Phe[18] signal is similar to internalization signals on other receptors in terms of containing critical aromatic residues. However, it differs in that it does not contain a tyrosine. This finding, however, is not totally unexpected because Davis and co-workers (5) have reported that phenylalanine can fully substitute for tyrosine in mediating rapid internalization of the LDL receptor.

A comparison of the amino acid sequence of the Phe[13]–Phe[18] region in the CD-MPR with the sequence in the cytoplasmic domain of the LDL receptor known to be involved in rapid internalization of that receptor is shown in Table 2. It is striking that the LDL receptor internalization signal includes a phenylalanine at position 13 and a tyrosine at position 18 of the cytoplasmic tail. Chen and co-workers (6) have demonstrated that both of these residues are required for rapid internalization of the LDL receptor. These investigators have also shown that Asn[15] and Pro[16] are components of the internalization signal, and they have pointed out that the sequence Asn-Pro-Xaa-Tyr is commonly found in receptors that undergo rapid internalization. Interestingly, the CD-MPR lacks this sequence. Our data, as well as the data of Chen and co-workers (6), are consistent with the complete internalization signal spanning a six-amino acid region with critical aromatic residues at the first and sixth positions. In this regard the recent cloning of the mannose receptor has

Table 2. Cytoplasmic domains of receptors endocytosed via coated pits share at least one consensus internalization motif

Receptor	Sequence
	10 A 20 30 40 B 50 60
CD-MPR (b)	QRLVV GAKGM EQFPH LAFWQ DLGNL VADGC DFVCR SKPRN VPAAY RGVGD DQLGE ESEER DDHLL PM
LDL-R (h)	KNWRL KNINS INFDN PVYQT (residues +1 to 20)
Man-R (h)	KKRRV HLPQE GAFEN TLYFN (residues +1 to 20)
CI-MPR (b)	VSYKY SKVNK (residues +22 to 31)
T-R (h)	EPLSY TRFSL (residues +16 to 25)*
pIg-R (rb)	ADLAY SAFLL (residues +77 to 86)

The entire amino acid sequence of the cytoplasmic domain of the bovine CD-MPR is shown (single-letter code). The aromatic residues are highlighted in boldface type. Partial amino acid sequences of the cytoplasmic domains of the other receptors are given along with their position relative to the transmembrane region. CD-MPR (12); CI-MPR (21); Man-R, mannose receptor (22); LDL-R, LDL receptor (6); T-R, transferrin receptor (9); pIg-R, polymeric immunoglobulin receptor (7). Species from which the receptors were obtained are as follows: b, bovine; h, human; rb, rabbit. A corresponds to the region of the six-amino acid internalization motif; B corresponds to the region of the four-amino acid internalization motif.

*The transferrin receptor is a type II membrane protein. The numbering is from the amino terminus rather than from the cytoplasmic tail–transmembrane junction. The Tyr (Y) is residue 41 from this junction.

revealed that its 45-amino acid cytoplasmic domain contains a phenylalanine at position 13 and a tyrosine at position 18, as well as a phenylalanine at position 19 (Table 2) (22). Since these are the only aromatic residues in the mannose receptor cytoplasmic tail, it is likely that they will be found to mediate the rapid internalization that this receptor undergoes. The mannose receptor does have an asparagine at position 15, but it lacks the Pro residue of the Asn-Pro-Xaa-Tyr sequence.

The second internalization signal in the CD-MPR involves the tyrosine residue at position 45. A comparison of the neighboring amino acid sequence with the internalization sequences of the CI-MPR (4), the transferrin receptor (9), and the polymeric immunoglobulin receptor (7) reveals a striking similarity (Table 2). All four receptors have an aromatic residue in the first position and a large hydrophobic residue in the fourth position, and three of the receptors have a basic amino acid in either the second or third position. The aromatic residue at the first position is an essential part of the internalization signal in all four receptors. Replacement of the valine with alanine in the fourth position of the CI-MPR causes a 75% decrease in the rate of receptor internalization, while replacement of the lysine at the third position with alanine results in a modest (35%) decrease in the internalization rate (W. Canfield, K.F.J., and S.K., unpublished results). Similarly the aromatic residue at the fourth position of the transferrin receptor is essential for rapid internalization, whereas replacement of the basic residue arginine at the second position with alanine has a lesser, but significant, effect on the rate of internalization of this receptor (9). The contribution of the second, third, and fourth residues in the CD-MPR and the polymeric immunoglobulin receptor have not been analyzed as yet. Taken together, these data indicate that a tetrapeptide sequence with an aromatic residue at the first position, a large hydrophobic residue at the fourth position, and a basic residue at either the second or third position is another motif that mediates internalization of recycling receptors.

One puzzling finding was that the $Asp^{51} \rightarrow$ Stop deletion mutant exhibited a modest decrease in the rate of internalization, almost as great as that observed with the $Asp^{28} \rightarrow$ Stop mutant. This was unexpected, since the 17 amino acids that are deleted in the $Asp^{51} \rightarrow$ Stop mutant do not involve either of the two identified internalization signals. One explanation for this finding is that the CD-MPR contains a third internalization signal somewhere within the last 17 amino acids of its cytoplasmic tail. However, this seems quite unlikely since the $Arom^-$ receptor, which contains these 17 amino acids, is almost as impaired in internalization as the tailless receptor. Another possible explanation for the finding is that the deletion of the terminal 17 amino acids somehow disrupts the function of the internalization signal that includes Tyr^{45} and presumably Arg^{46}, Gly^{47}, and Val^{48}. The $Asp^{51} \rightarrow$ Stop mutant contains just two amino acids beyond this signal, and it may be that more amino acids must be present for the signal to be fully functional.

It is of note that the Tyr^{45}-containing signal is less potent than the $Phe^{13}Phe^{18}$ signal. The reason for this is unknown, but one possibility is that the sequence is positioned in a less favorable context relative to the plasma membrane for optimal interactions with the "adaptor" proteins of the clathrin-coated pits (23). These proteins have been shown to interact with the cytoplasmic tails of the CI-MPR and LDL receptors and are thought to function in the concentration of receptors in the clathrin-coated pits (24, 25). Presumably the adaptor proteins can interact with both internalization sequences.

In summary, the cytoplasmic tail of the CD-MPR has two distinct internalization signals. One signal consists of six amino acids, while the other signal appears to consist of four amino acids. The two signals are similar, having aromatic and/or large hydrophobic residues in the first and last positions.

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