Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 14471–14476, December 1997
Cell Biology

Proper sorting of the cation-dependent mannose 6-phosphate receptor in endosomes depends on a pair of aromatic amino acids in its cytoplasmic tail

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Contributed by Stuart Kornfeld, October 28, 1997

ABSTRACT **The 67-amino acid cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor (CD-MPR) contains a signal(s) that prevents the receptor from entering lysosomes where it would be degraded. To identify the key residues required for proper endosomal sorting, we analyzed the intracellular distribution of mutant forms of the receptor by Percoll density gradients. A receptor with a $Trp^{19} \rightarrow Ala$ substitution in the cytoplasmic tail was highly missorted to lysosomes whereas receptors with either $Phe^{18} \rightarrow Ala$ or $Phe^{13} \rightarrow Ala$ mutations were partially defective in avoiding transport to lysosomes. Analysis of double and triple mutants confirmed the key role of Trp^{19} for sorting of the CD-MPR in endosomes, with Phe^{18}, Phe^{13}, and several neighboring residues contributing to this function. The addition of the Phe^{18}-Trp^{19} motif of the CD-MPR to the cytoplasmic tail of the lysosomal membrane protein Lamp1 was sufficient to partially impair its delivery to lysosomes. Replacing Phe^{18} and Trp^{19} with other aromatic amino acids did not impair endosomal sorting of the CD-MPR, indicating that two aromatic residues located at these positions are sufficient to prevent the receptor from trafficking to lysosomes. However, alterations in the spacing of the diaromatic amino acid sequence relative to the transmembrane domain resulted in receptor accumulation in lysosomes. These findings indicate that the endosomal sorting of the CD-MPR depends on the correct presentation of a diaromatic amino acid-containing motif in its cytoplasmic tail. Because a diaromatic amino acid sequence is also present in the cytoplasmic tail of other receptors known to be internalized from the plasma membrane, this feature may prove to be a general determinant for endosomal sorting.**

The biogenesis of lysosomes depends on sorting of newly synthesized acid hydrolases from the Golgi apparatus via endosomes to their final destination in lysosomes (1–3). The acid hydrolases are synthesized in the rough endoplasmic reticulum and transported to the Golgi apparatus where they specifically acquire mannose 6-phosphate residues, which act as recognition markers for the mannose 6-phosphate receptors (MPRs). The receptor–ligand complexes then exit the trans-Golgi network in clathrin-coated vesicles, which fuse with acidified endosomal compartments where the acid hydrolases are discharged for packaging into lysosomes. Subsequently, the MPRs either cycle back to the Golgi apparatus or move to the plasma membrane where they are rapidly internalized. The sorting of acid hydrolases is performed by two distinct MPRs, the 275-kDa mannose 6-phosphate/insulin-like growth factor-II receptor and the 46-kDa cation-dependent mannose 6-phosphate receptor (CD-MPR).

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The cycling of the CD-MPR between the trans-Golgi network, the plasma membrane, and endosomes is directed by at least four signals located in its 67-amino acid cytoplasmic tail (4–6). A dileucine-containing sequence (Leu^{64}-Leu^{65}) is required for sorting of the receptor in the Golgi apparatus, and this same motif also functions as one of the three independent internalization signals. Another internalization signal includes Phe^{13} and Phe^{18}, whereas the third involves Tyr^{45}. Recently, the cytoplasmic tail of the CD-MPR was shown to contain a fourth signal, which functions to prevent the receptor from trafficking from endosomal compartments to lysosomes where it would be degraded (7). A short stretch of 6 amino acids (Cys^{34}-Arg-Ser-Lys-Pro-Arg^{39}) within the cytoplasmic tail was identified as being necessary for avoidance of lysosomal degradation. The key residue in this sequence, Cys^{34}, was subsequently shown to be reversibly palmitoylated (8). Because the palmitoylated form of Cys^{34} is likely to be anchored to the lipid bilayer, this modification could have a dramatic effect on the conformation of the entire cytoplasmic tail. From these studies we could not distinguish whether Cys^{34} itself (or its palmitoylated form) was the crucial component of the endosomal sorting signal or whether this residue modulated an endosomal sorting signal(s) located elsewhere in the cytoplasmic tail.

The aim of the present study was to distinguish between these two possibilities. By analyzing the intracellular distribution of a variety of point mutants we demonstrate that proper endosomal sorting of the CD-MPR depends on a pair of aromatic amino acids (Phe-Trp) at positions 18 and 19 from the transmembrane domain. Within this sequence Trp^{19} is the key residue with Phe^{18} and possibly other neighboring residues contributing to this function. This finding is consistent with Cys^{34} modulating a signal that includes the Phe-Trp motif.

MATERIALS AND METHODS

Materials. Enzymes used in molecular cloning were obtained from Boehringer Mannheim, New England Biolabs, or Promega; α-minimal essential medium (α-MEM), fetal calf serum, and Lipofectin were from GIBCO/BRL; Percoll was from Pharmacia; nitrocellulose from Schleicher & Schuell; protease inhibitors from Sigma; ECL Western blotting reagents from Amersham; protein A-Sepharose beads from Repligen; and cell culture dishes from Falcon. Oligonucleotides were synthesized either by the Protein Chemistry Facility

Abbreviations: MPR, mannose 6-phosphate receptor; CD-MPR, cation-dependent mannose 6-phosphate receptor; MR, mannose receptor; PLA_2R, phospholipase A_2 receptor.

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of Washington University or Microsynth GmBH (Galbach, Switzerland). The mAb 22D4 specific for the CD-MPR and polyclonal antibodies specific for Lamp1 were generously provided by D. Messner (9) and M. Fukuda (Burnham Inst.) (10), respectively.

Recombinant DNA. All basic DNA procedures were as described (11). The PCR procedure of Ho *et al.* (12) was used to generate the W19A, MPR-FF, MPR-FY, MPR-YF, MPR-YW, MPR 10–12A, MPR 14–16A, MPR 20–21A, MPR 22–24A, MPR-S5, and MPR Δ6–9 constructs with pBSK-$MPR^{TMD/tail}$ (7) serving as a template together with bp 170–193 and 1260–1341 of $pBSK^-$ as the down- and upstream primers, respectively. Appropriate partial complementary pairs of oligonucleotides in which the desired amino acid replacement had been incorporated were chosen as internal primers. The final PCR products were subcloned into pSFFV-neo as described (7). The constructs LLL-M(FW) and LLL-M(AA) were created with PCR by using pBSK-Lamp1 (7) as a template. For this reaction bp 998-1018 of Lamp1 was used as a downstream primer together with an upstream primer containing either a wild-type (FW) or mutant (AA) sequence of the CD-MPR attached to the extreme C-terminal sequence of Lamp1. The PCR products were ligated into pSFFVneo. Mutants FFWY→A, FFW→A, FW→A, F13A, F18A, and F32A have been described previously (4). All coding sequences created by PCR were verified by sequencing with the Sanger dideoxy chain termination method (13).

Cell Culture and Transfection. A mannose-6-P/insulin-like growth factor-II receptor-deficient mouse L cell line designated D9 ($LRec^-$) was maintained in α-MEM containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% humidified CO_2 atmosphere (14). The cells were transfected with 20 μg of *Xba*I-linearized DNA with Lipofectin according to the manufacturer's directions. Selection for resistance to neomycin (G418) was as described previously (15) except that the final G418 concentration was 500 μg/ml. Resistant colonies were screened for either bovine CD-MPR or human Lamp1 expression by immunoblotting. Clones expressing similar amounts of receptor were expanded for further study and maintained in selective medium.

Percoll Gradient Fractionation. Confluent cells grown in a 100-mm Petri dish were incubated for 24 h in growth medium supplemented with 100 μM each of pepstatin A and leupeptin. The cells were then harvested, ruptured with a ball bearing homogenizer, and fractionated on 18% Percoll density gradients as described previously (7).

SDS/PAGE and Immunoblotting. Proteins were separated on 10% SDS/polyacrylamide minigels (Bio-Rad) by using the Laemmli system (16). After electrophoresis gels were transferred onto nitrocellulose membranes according to the method of Towbin *et al.* (17). The immunoblotting was performed as previously described (7). The autoradiographs were quantitated by using a personal densitometer (Molecular Dynamics).

Assays and Miscellaneous Methods. β-Hexosaminidase activity was determined as described (7). Protein concentration was determined with the Bio-Rad protein assay kit by using protein standard I. Affinity-purified anti-CD-MPR antibodies were iodinated by using Iodo-Gen (Pierce) according to the manufacturer's recommendation and separated from the free ^{125}I on NAP5 columns (Pharmacia).

Steady State Surface Distribution of CD-MPR. Confluent cells in 12-well plates were incubated for 24 h with 100 μM each of pepstatin A and leupeptin. The cells were then washed with PBS and incubated for 15 min on ice with either 10 mg/ml BSA in PBS (cell surface) or with PBS containing 10 mg/ml BSA and 0.1% saponin (total). The cells were next incubated with 1×10^6 cpm of ^{125}I-labeled antibodies to the CD-MPR in either 10 mg/ml BSA in PBS (cell surface) or PBS containing 10 mg/ml BSA and 0.1% saponin (total) on ice. After 2–4 h, the cells were washed 5 times with 10 mg/ml BSA in PBS or 10 mg/ml BSA/0.1% saponin in PBS and solubilized in 1 ml of 0.1 M NaOH. Cell-associated radioactivity was determined with a γ-counter.

The steady state surface distribution of Lamp1 and the mutants LLL-M(FW) and LLL-M(AA) was determined with sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate (Pierce) as described previously (18).

RESULTS

Trp^{19} of the CD-MPR Cytoplasmic Tail Is Essential for Avoiding Receptor Accumulation in Dense Lysosomes. In our studies to identify the amino acids in the cytoplasmic tail of the CD-MPR responsible for endosomal sorting, we initially focused on the aromatic residues. To this end, we determined the intracellular distribution of four mutant receptors that have Phe^{13}, Phe^{18}, Trp^{19}, and Phe^{32} individually changed to alanine (F13A, F18A, W19A, and F32A; Fig. 1*A*). Mouse L cells stably expressing these mutant receptors were preincubated for 24 h in the presence of pepstatin A and leupeptin to inhibit degradation of receptors that had entered lysosomes (7). The cells were then harvested and homogenized, and the lysosomes were separated from other organelles on 18% isoosmotic Percoll density gradients (18, 19). The gradient fractions were analyzed in three pools: pool I containing dense lysosomes, pool II containing intermediate density membranes, and pool III containing low density membranes including endosomes, Golgi apparatus, plasma membrane, and endoplasmic reticulum. The receptor distribution in the three pools was determined by SDS/PAGE followed by Western blotting (Fig. 1*B* and Table 1 for quantitation of multiple experiments). As reported previously, the wild-type CD-MPR was almost completely excluded from dense lysosomes (4 ± 1% in pool I). In contrast, 33 ± 10% of the mutant receptor with the W19A substitution accumulated in dense lysosomes during the time of the experiment. Replacement of either Phe^{18} or Phe^{13} with alanine resulted in intermediate accumulations of receptor in

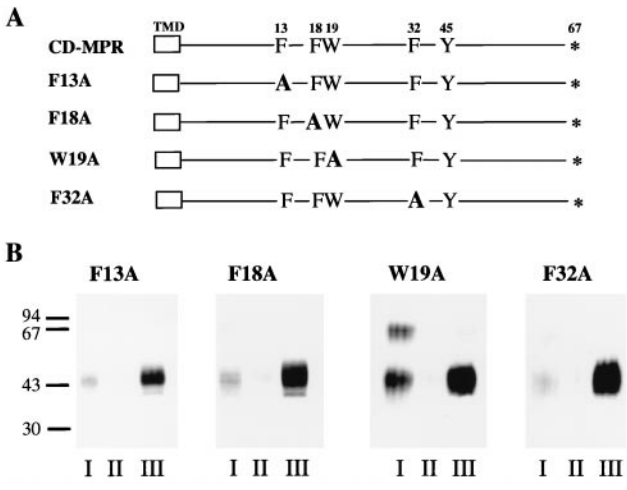

FIG. 1. Trp^{19} is essential for avoiding receptor trafficking to dense lysosomes. (*A*) Schematic illustration of the point mutants within the cytoplasmic tail of the CD-MPR. Amino acids replacing the wild-type sequence are shown in bold letters. (*B*) Mouse L cells stably expressing F13A, F18A, W19A, and F32A were preincubated with pepstatin A and leupeptin for 24 h. The cells were then homogenized with a ball bearing homogenizer, and postnuclear fractions were subjected to Percoll density gradient centrifugation (18% Percoll). The collected fractions were combined into pools I, II, and III (three each) and further analyzed by SDS/PAGE and immunoblotting with mAb 22D4. The upper band at ≈90 kDa is the dimeric form of the W19A receptor. The recovery of dimer varied with the different constructs. Both the monomeric and dimeric forms of the receptor were included in the quantitation summarized in Table 1.

Table 1. Summary of the cellular distribution of wild-type and mutant CD-MPR

Construct	% of surface*	Gradient, % in lysosomes†	% of internal pool in lysosomes‡
ML4 (wild-type) (CD-MPR)	17 ± 5 (n = 4)	4 ± 1	5
F13A	6 ± 2 (n = 3)	9 ± 4 (n = 5)	10
F18A	33 ± 8 (n = 3)	13 ± 2 (n = 5)	19
F32A	16 ± 2§	2 ± 1 (n = 3)	2
W19A	24 ± 6 (n = 7)	33 ± 10 (n = 7)	43
FW→A	48 ± 2 (n = 3)	29 ± 8 (n = 5)	56
FFW→A	56 ± 3 (n = 3)	29 ± 8 (n = 7)	66
FFWY→A	67 ± 5 (n = 5)	22 ± 4 (n = 5)	67
MPR-FF	29 ± 19 (n = 9)	5 ± 3 (n = 4)	7
MPR-FY	18 ± 7 (n = 9)	4 ± 2 (n = 3)	5
MPR-YF	27 ± 18 (n = 9)	8 ± 5 (n = 7)	11
MPR-YW	18 ± 8 (n = 9)	3 ± 2 (n = 5)	4

*Percentage of the various mutant receptors that were present at the cell surface at steady state. The values are expressed as mean ± SE; n is the number of determinations.
†Percentage of the various mutant receptors recovered in dense lysosomes on Percoll gradients as described in Fig. 1. The values are expressed as mean ± SE; n is the number of determinations.
‡The percentage of the internal pool of receptor that is in lysosomes was calculated using the numbers for the percentage on the surface and in lysosomes.
§Taken from ref. 4.

dense lysosomes (F18A, 13 ± 2% and F13A, 9 ± 4% in pool I) whereas the receptor with the F32A mutation behaved the same as the wild-type receptor (2 ± 1% in pool I).

We next examined the effect of mutating Trp^{19} along with one to three of the other aromatic residues. The constructs are shown in Fig. 2*A*, and the effect on receptor subcellular distribution is given in Fig. 2*B* and Table 1. All three mutant proteins accumulated in dense lysosomes to about the same extent as the W19A mutant (29 ± 8% of FFW→A and FW→A and 22 ± 4% of FFWY→A were recovered in pool 1). However, because the Phe and Tyr mutations impair the internalization signals of the CD-MPR (4, 6), a greater fraction of these mutant receptors is present on the cell surface at steady state and not available for mislocalization to lysosomes. To take this skewed distribution into account, we calculated the accumulation of the different mutant receptors in lysosomes as a percentage of the internal pool. First we determined what fraction of the mutant receptors is present at the cell surface at steady state by measuring the binding of ^{125}I-labeled anti-CD-MPR antibody to intact cells (surface receptor) vs. binding to cells permeabilized with 0.1% saponin (total receptor). Consistent with previous results (4), 67 ± 5% of FFWY→A, 56 ± 3% of FFW→A, and 48 ± 2% of FW→A were present on the plasma membrane compared with 17 ± 5% of the wild-type receptor (Table 1). The cells expressing the W19A and F18A mutant receptors exhibited a modest increase in surface molecules (24 ± 6 and 33 ± 8%, respectively), whereas the F13A mutant did not accumulate on the cell surface (6 ± 2%). These values were then used to calculate the percentage of the internal receptor pool that accumulated in lysosomes during the period of treatment with pepstatin A and leupeptin. As summarized in Table 1, 5% of the intracellular wild-type receptor was recovered in the lysosomal fraction. The F13A and F18A mutants showed modest increases in lysosomal accumulation (10 and 19%, respectively). A much greater fraction of the intracellular W19A mutant accumulated in lysosomes (43%), and this increased to 56% when Phe^{18} was also mutated (FW→A). A slightly larger accumulation of mutant receptor in lysosomes was found with the FFW→A and FFWY→A mutants (66 and 67%, respectively).

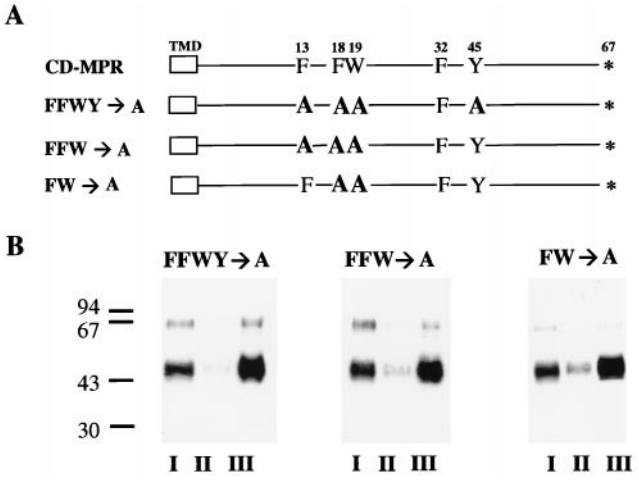

FIG. 2. FFWY→A, FFW→A, and FW→A accumulate in dense lysosomes. (*A*) Schematic illustration of the point mutants within the cytoplasmic tail of the CD-MPR. Amino acids replacing the wild-type sequence are shown in bold letters. (*B*) Mouse L cells stably expressing FFWY→A, FFW→A, and FW→A were preincubated with pepstatin A and leupeptin for 24 h and then fractionated as described in Fig. 1.

To exclude the possibility that these mutations inhibit the palmitoylation of Cys^{34} and thereby cause secondary effects on the conformation of the cytoplasmic tail, the cells with the FW→A mutant receptor were labeled with [^{3}H]palmitate, and the extent of palmitoylation of the CD-MPR was determined as described (8). This mutant receptor was labeled to the same extent as the wild-type receptor, excluding a secondary effect on palmitoylation (data not shown). Taken together, these results indicate that Trp^{19} is the key residue for endosomal sorting of the CD-MPR with Phe^{18} and possibly Phe^{13} serving as secondary components of the signal.

The Phe^{18}-Trp^{19} Sequence of the CD-MPR Partially Impairs Delivery of Lamp1 to Lysosomes. Having established that the Phe^{18}-Trp^{19} sequence is necessary to prevent the CD-MPR from entering lysosomes, we asked whether this sequence is sufficient to impair sorting of a lysosomal membrane protein to lysosomes. A chimeric construct was created, which has amino acids 10–21 of the CD-MPR cytoplasmic tail (MEQFPHLAFWQD) attached to the C terminus of wild-type Lamp1 (Fig. 3*A*, LLL-M(FW)). The cytoplasmic tail of Lamp1 was left intact to ensure delivery of the chimeric molecule to endosomes. As a control, a construct was generated with residues 10–21 of the FW→A mutant (Fig. 3*A*, LLL-M(AA)). Pulse–chase experiments with cells expressing these constructs established that each of the chimeric proteins moved from the endoplasmic reticulum to the Golgi at the same rate as Lamp1, as judged by Asn-linked oligosaccharide processing. When postnuclear supernatants of these cells were analyzed on Percoll density gradients following preincubation with pepstatin A and leupeptin, the recovery of LLL-M(FW) protein in the dense lysosomal fraction was significantly decreased compared with wild-type Lamp1 (44 vs. 62%, $P < 0.001$; Fig. 3*B*). In contrast, LLL-M(AA) protein accumulated in dense lysosomes to the same extent as the wild-type Lamp1 (58 and 62%, respectively). The accumulation of LLL-M(FW) protein in dense lysosomes was only moderately greater than the 33% accumulation of the LLM chimera (Fig. 3 *A* and *B*), which has the cytoplasmic tail of Lamp1 replaced by the complete wild-type tail of the CD-MPR (7).

We next determined the fraction of the chimeric molecules present at the cell surface by using a surface biotinylation technique (18). As shown in Fig. 3*C*, very low amounts of Lamp1, LLL-M(FW), and LLL-M(AA) (1, 7, and 4%, respectively) were present at the cell surface at steady state. These results demonstrate that neither LLL-M(FW) nor LLL-M(AA) is mislocalized to the plasma membrane and therefore

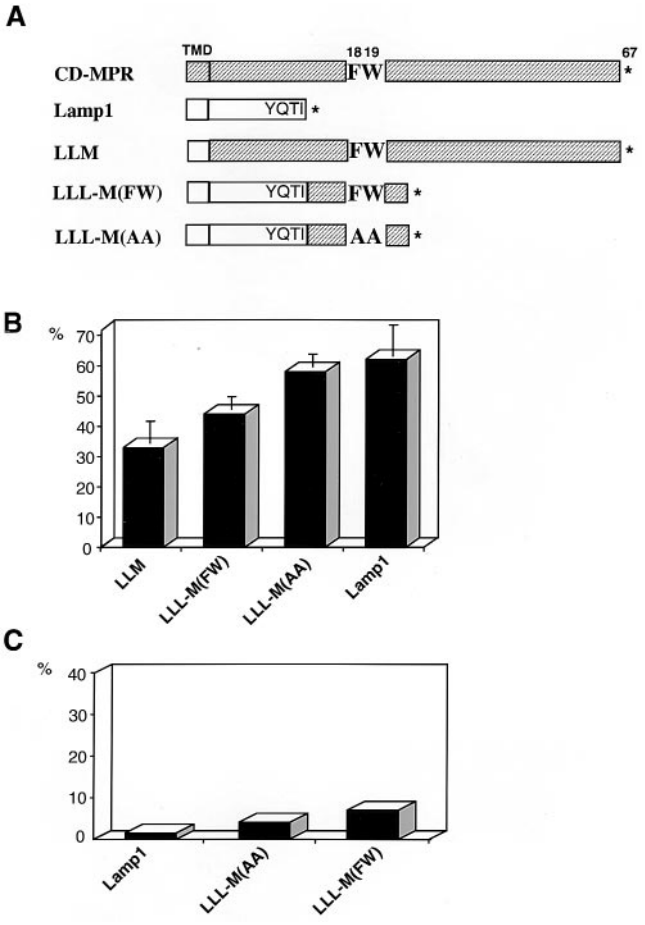

FIG. 3. The Phe[18]-Trp[19] sequence of the CD-MPR is sufficient to impair delivery of Lamp1 to lysosomes. (*A*) Schematic illustration of wild-type CD-MPR, wild-type Lamp1, and the chimeras LLM, LLL-M(FW), and LLL-M(AA). (*B*) Subcellular distribution of wild-type Lamp1, LLM, LLL-M(FW), and LLL-M(AA) on Percoll density gradients. Mouse L cells stably expressing wild-type Lamp1, LLM, LLL-M(FW), and LLL-M(AA) were preincubated with pepstatin A and leupeptin for 24 h and then fractionated as described in Fig. 1. Immunoblots of multiple gradients were quantitated, and the value of pool I (dense lysosomes) is expressed as a percentage of the sum of all three pools. The value for wild-type Lamp1 is from Reference 7. (*C*) Steady state cell surface distribution of wild-type Lamp1, LLL-M(FW), and LLL-M(AA). Cell surface proteins of mouse L cells stably expressing wild-type Lamp1, LLL-M(FW), and LLL-M(AA) were derivatized by using sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate. The cells were lysed, and the surface-biotinylated and internal Lamp1 molecules were immunoprecipitated by using a polyclonal anti-Lamp1 antibody (10). After solubilization of the first immunoprecipitate the samples were incubated with streptavidin–agarose beads to precipitate surface-biotinylated molecules. The proteins in the supernatant that did not bind to streptavidin (unbound, internal) and the proteins in the precipitate that did bind to streptavidin (bound, surface) were analyzed by SDS/PAGE and immunoblotting. Autoradiographs from multiple experiments were quantitated by scanning densitometry. The values presented in the graph represent the percentage of the different constructs detected at the cell surface (bound) relative to the total amount detected (bound and unbound combined).

support the conclusion that the diaromatic motif of the CD-MPR impairs the entry of Lamp1 into lysosomes.

Two Aromatic Amino Acid Residues at Positions 18 and 19 of the Cytoplasmic Tail Are Sufficient for Correct Endosomal Sorting of the CD-MPR. To determine whether the signal was specifically Phe-Trp or whether the aromatic nature of these residues was sufficient for sorting, we tested whether replacement of Phe[18] and/or Trp[19] with other aromatic amino acid residues affected endosomal sorting of the receptor. Two constructs were prepared in which Trp[19] was replaced by either a phenylalanine or a tyrosine (MPR-FF and MPR-FY, respectively). A third construct had Phe[18] and Trp[19] changed to tyrosine and phenylalanine, respectively (MPR-YF), whereas a fourth construct had only Phe[18] substituted by a tyrosine (MPR-YW). As summarized in Table 1, the three mutant receptors with a single aromatic residue changed were excluded from lysosomes to the same extent as the wild-type CD-MPR, whereas the construct with both residues changed (MPR-YF) showed a slight increase (8 ± 5%) in lysosomal accumulation. And even though the MPR-YF and MPR-FF mutants accumulated to a small extent at the plasma membrane (29 and 27%, respectively), the percentage of the internal pool that was present in lysosomes remained low (11% for MPR-YF and 7% for MPR-FF).

These data indicate that two aromatic amino acid residues at positions 18 and 19 of the cytoplasmic tail of the CD-MPR are sufficient for correct endosomal sorting.

Effect of Amino Acids Surrounding Phe[18] and Trp[19] on Receptor Targeting. To analyze whether the amino acids near the diaromatic sequence (Phe[18]-Trp[19]) influence the accumulation of the receptor in dense lysosomes, constructs were created in which amino acids 10–12 (MPR10–12A), 14–16 (MPR 14–16A), 20–21 (MPR 20–21A), and 22–24 (MPR 22–24A) were changed to alanine (Table 2). When the receptor distribution was determined in cells expressing these various constructs, MPR 10–12A and MPR 20–21A were found to be excluded from dense lysosomes (2 ± 1 and 2 ± 1%), whereas 19 ± 6% of MPR 14–16A and 17 ± 10% of MPR 22–24A were recovered in this organelle (Table 2). Because none of these mutant receptors accumulated at the cell surface (Table 2), the percentage of the internal pool present in lysosomes was equal to the percentage of the mutant receptors found in lysosomes on the Percoll gradients. These results indicate that amino acids 10–12 and 20–21 are not involved in preventing the receptor from accumulating in lysosomes, whereas residues 14–16 and 22–24 have a modest effect on endosomal sorting.

Addition or Removal of Amino Acids Within the Cytoplasmic Tail of the CD-MPR Impairs Endosomal Sorting. We have demonstrated that the spacing of the sorting signal in the cytoplasmic tail of Lamp1 relative to its transmembrane domain is critical for efficient endosomal sorting (18). To determine whether addition or removal of amino acids upstream of the Phe[18]-Trp[19] sequence affects the endosomal sorting of the CD-MPR, two constructs were prepared in which either five alanine residues were introduced between amino acids Ala[7] and Lys[8] (MPR-S5) or four residues (Gly[6] to Gly[9]) were deleted (MPR Δ6–9) (Table 2). As summarized in the table, both MPR-S5 and MPR Δ6–9 accumulated to a significant extent in dense lysosomes (33 ± 11 and 24 ± 8%, respectively). Because neither of these mutant receptors was mislocalized to the cell surface, the percentage of the internal pool of MPR-S5 and MPR Δ6–9 in lysosomes was 38 and 28%, respectively.

These results show that addition or removal of amino acids upstream of the Phe[18]-Trp[19] sequence leads to a mislocalization of the CD-MPR to lysosomes.

DISCUSSION

Previously we reported that truncation of the cytoplasmic tail of the CD-MPR or mutation of Cys[34] of the tail to an Ala resulted in the gradual accumulation of the receptor in dense lysosomes whereas normally it is excluded from this organelle (7, 8). We postulated that these alterations inactivated a signal(s) that is essential for the receptor to avoid trafficking from endosomes to lysosomes where it would be degraded. Because Cys[34] is palmitoylated and likely to be anchored to the

Table 2. Summary of the cellular distribution of mutant CD-MPRs

Construct	Sequence	% on surface*	Gradient, % in lysosomes†	% of internal pool in lysosomes‡
	10 24			
CD-MPR	-MEQFPHLAFWQDLGNL-	17 ± 5 (n = 4)	4 ± 1	5
MPR 10–12A	-AAAFPHLAFWQDLGNL-	23 ± 9 (n = 6)	2 ± 1 (n = 3)	3
MPR 14–16A	-MEQFAAAAFWQDLGNL-	10 ± 6 (n = 9)	19 ± 6 (n = 3)	21
MPR 20–21A	-MEQFPHLAFWAALGNL-	2 ± 1 (n = 6)	2 ± 1 (n = 3)	2
MPR 22–24A	-MEQFPHLAFWQDLAAA-	13 ± 6 (n = 9)	17 ± 10 (n = 3)	19
	5 19			
CD-MPR	-VGAKGMEQFPHLAFW-			
MPR-S5	-VGAAAAAAKGMEQF-	13 ± 4 (n = 6)	33 ± 11 (n = 9)	38
MPRΔ6–9	-VMEQFPHLAFW-	14 ± 7 (n = 6)	24 ± 8 (n = 9)	28

*Percentage of the various mutant receptors that were present at the cell surface at steady state. The values are expressed as mean ± SE; n is the number of determinations.

†Percentage of the various mutant receptors recovered in dense lysosomes on Percoll gradients as described in Fig. 1. The values are expressed as mean ± SE; n is the number of determinations.

‡The percentage of the internal pool of receptor that is in lysosomes was calculated using the numbers of the percentage on the surface and in lysosomes.

lipid bilayer (20–24), we reasoned that Cys^{34} may influence the conformation of the entire cytoplasmic tail and thereby modulate sorting signals located elsewhere in the tail rather than being an actual component of a sorting signal. Therefore we searched for other amino acids that may be components of an endosomal sorting signal(s).

The results presented in the present study indicate that the diaromatic sequence Phe^{18}-Trp^{19} is the key component of a motif that functions as a signal for sorting the CD-MPR in endosomes. Because replacement of the Phe^{18}-Trp^{19} sequence with other aromatic residues did not alter the intracellular distribution of the receptor, it appears that the important feature required for endosomal sorting is the presence of two aromatic residues in the correct context. The role of Phe^{13} and residues 14–16 and 22–24 in the vicinity of Phe^{18}-Trp^{19} is less clear. Replacement of these residues with alanines resulted in a 2- to 4-fold increased accumulation of receptor in lysosomes vs. the 11-fold increase that occurred with the FW→A mutation. This is consistent with these changes altering the conformation of the cytoplasmic tail and secondarily influencing the presentation of the Phe^{18}-Trp^{19} sequence. Alternatively, some of these residues could be components of a more extensive signal motif. Because mutation of the Phe^{18}-Trp^{19} sequence does not impair palmitoylation of Cys^{34}, the altered trafficking cannot be because of a secondary effect on palmitoylation of this residue.

In addition to being necessary for the proper sorting of the CD-MPR in endosomes, the Phe-Trp diaromatic motif is also sufficient to partially impair the entry of Lamp1 into dense lysosomes. Thus, attachment of a 12-amino acid stretch of the CD-MPR cytoplasmic tail, including Phe^{18}-Trp^{19}, to the 11-amino acid cytoplasmic tail of Lamp1 (the LLL-M(FW) construct) reduced the accumulation of the molecule in lysosomes from 62 to 44%. The control construct, LLL-M(AA), behaved the same as Lamp1. By way of comparison, when the Lamp1 cytoplasmic tail is replaced with the full-length CD-MPR cytoplasmic tail (the LLM construct), 33% of the chimeric molecules still localize in dense lysosomes at steady state. Complete exclusion of Lamp1 from lysosomes is only achieved when both the transmembrane and cytoplasmic domains of Lamp1 are replaced with those of CD-MPR (7). The difference in the efficiency in endosomal sorting of LLL-M(FW) compared with LLM could reflect the fact that LLL-M(FW) lacks Cys^{34} and possibly other elements of the CD-MPR cytoplasmic tail that might facilitate the proper presentation of the diaromatic motif, as discussed above. It is also possible that the CD-MPR cytoplasmic tail contains additional sorting motifs that function at the level of the endosome. Furthermore, LLL-M(FW), in contrast to LLM, retains the Lamp1 cytoplasmic tail, which might contain a positive sorting signal for delivery from late endosomes to lysosomes. If this is the case, the ultimate distribution of the construct would represent a balance between the two signals.

We have suggested that the CD-MPR endosomal sorting signal may function in the recruitment of the receptor into endosomal-derived vesicles destined for the trans-Golgi network, early endosomes, or the plasma membrane (7). The simplest mechanism would be that the diaromatic amino acid sorting motif interacts with coat proteins that are involved in vesicle formation on endosomes. When the sorting motif is mutated, the receptor has a longer residence time in endosomes, increasing the likelihood that it will be delivered to lysosomes. An alternative possibility is that receptor molecules are continually moving from endosomes to lysosomes and the diaromatic motif functions in the retrieval of these receptors from lysosomes. This would be consistent with the recent finding that clathrin-coated vesicles can assemble on lysosomes (25). In this case, mutation of the diaromatic motif would block transport of the CD-MPR out of lysosomes resulting in the observed accumulation in that organelle. We consider this the least likely possibility because the CD-MPR is rapidly degraded in lysosomes in the absence of protease inhibitors.

A question that arises is whether the CD-MPR cytoplasmic tail contains signals in addition to the diaromatic motif that serve to prevent receptor accumulation in lysosomes. We cannot exclude this possibility because of several findings. Deletion of the C-terminal 17 amino acids of the cytoplasmic tail (Asp^{51} stop) resulted in 26% receptor accumulation in dense lysosomes in the standard assay (7). Because this deletion is quite distant from both Cys^{34} and Phe^{18}-Trp^{19}, it is possible that another sorting signal may have been disrupted. Further, the rate of accumulation of the various mutants in dense lysosomes is considerably slower than that observed with a construct (MML) where the CD-MPR cytoplasmic tail was replaced with the Lamp1 cytoplasmic tail (unpublished data). One explanation for this difference is that the Lamp1 tail contains a positive signal for delivery from late endosomes to lysosomes. However, these findings can also be explained by postulating that the cytoplasmic tail of the CD-MPR contains two or more signals that participate in the trafficking of the receptor out of endosomes. This would not be surprising considering the complexity of the trafficking in the endosomal system.

Although the mannose-6-P/insulin-like growth factor-II receptor must also have a signal(s) that prevents trafficking from endosomes to lysosomes, it lacks a diaromatic amino acid motif in its cytoplasmic tail. This indicates that there must be additional mechanisms for avoiding transport to lysosomes. Interestingly, a comparison of the amino acid sequence of the Phe^{18}-Trp^{19} region in the CD-MPR with the sequences of the

Table 3. Cytoplasmic domains of receptors that contain a diaromatic amino acid sequence

Domain	Sequence
	10 20 30
CD-MPR tail	QRLVVGAKGMEQ**F**PHLA**FW**QDLGNLVADGC...
H-MR tail	KKRRVHL.PQEGA**F**ENTL**YF**NSQSSPGTSD...
M-MR tail	KKRHALHIPQEAT**F**ENTL**YF**NSNLSPGTSD...
H-PLA2-R tail	KHNGGFFRRLAG**F**RNP**YY**PATNFSTVYLEE...
B-PLA2-R tail	KHSHIIFGRLAQ**F**RNP**YY**PSANFSTVHLEE...
R-PLA2-R tail	KQNKGFFRRLAGVGNS**YY**PTTNFSTIHLEE...
ML-PLA2-R tail	KQKSDIFQRLTGSRGS**YY**PTLNFSTAHLEE...

The amino acid sequence of the cytoplasmic tails is shown in single-letter code. The diaromatic residues and the preceding phenylalanines are highlighted in boldface type. One sequence is shown for the CD-MPR since it is identical in all known species, two sequences for the mannose receptor (human and mouse) (26), and four sequences for the phospholipase A_2 receptor (human, bovine, rabbit, and mouse) (27–30).

cytoplasmic tails of the human and mouse mannose receptor (MR) and human, bovine, rabbit, and mouse phospholipase A_2 receptor (PLA_2R) reveals that all three receptors contain a diaromatic amino acid sequence at approximately the same distance from the transmembrane domain (Table 3). In addition, the CD-MPR and the MR have a phenylalanine five residues upstream of this sequence, whereas this distance is four residues for the human and bovine PLA_2R. Both the MR and the PLA_2R undergo rapid internalization, and the MR has been shown to recycle from the endosomal system back to the cell surface (31–34). It will be of interest to determine whether the diaromatic amino acid sequence in the cytoplasmic tails of these receptors also mediates sorting in endosomes.

Recently two groups reported that a subset of coatomer subunits binds a diphenylalanine-containing motif present in the cytoplasmic tails of members of the p24 family of putative cargo receptors (35, 36). These receptors have been implicated in protein trafficking between the endoplasmic reticulum and the Golgi complex. This raises the possibility that diaromatic amino acid motifs may be involved in trafficking between multiple organelles, similar to what has been observed with tyrosine-containing motifs.

We thank Dr. M. Fukuda and Dr. D. Messner for kindly providing antibodies against Lamp1 and CD-MPR, respectively; Dr. H. Riezman for allowing J. Rohrer to work in his laboratory during part of this study; and Dr. Rosalind Kornfeld and members of the Kornfeld laboratory for critically reading this manuscript. This research was supported by a Monsanto–Searle–Washington University School of Medicine biomedical research grant and National Institutes of Health Grant R01CA08759. J.R. was supported by Damon Runyon–Walter Winchell Cancer Postdoctoral Fellowship DRG 1216, by the Kanton Basel, and by a Prof. Max Cloetta Fellowship.

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