

A Determinant in the Cytoplasmic Tail of the Cation-dependent Mannose 6-Phosphate Receptor Prevents Trafficking to Lysosomes

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Abstract. The bovine cation-dependent mannose 6-phosphate receptor (CD-MPR) is a type I transmembrane protein that cycles between the *trans*-Golgi network, endosomes, and the plasma membrane. When the terminal 40 residues were deleted from the 67-amino acid cytoplasmic tail of the CD-MPR, the half-life of the receptor was drastically decreased and the mutant receptor was recovered in lysosomes. Analysis of additional cytoplasmic tail truncation mutants and alanine-scanning mutants implicated amino acids 34–39 as being critical for avoidance of lysosomal degradation. The

cytoplasmic tail of the CD-MPR was partially effective in preventing the lysosomal membrane protein Lamp1 from entering lysosomes. Complete exclusion required both the CD-MPR cytoplasmic tail and transmembrane domain. The transmembrane domain alone had just a minor effect on the distribution of Lamp1. These findings indicate that the cytoplasmic tail of the CD-MPR contains a signal that prevents the receptor from trafficking to lysosomes. The transmembrane domain of the CD-MPR also contributes to this function.

IN contrast to proteins that are stable constituents of subcellular organelles, the recycling receptors move constitutively between compartments. The trafficking of these receptors is regulated by sorting signals that are recognized at different stages of the pathway. Studies of the mannose 6-phosphate receptors (MPRs)¹ have proved useful in identifying some of these signals (Kornfeld, 1992). These receptors cycle between the Golgi complex, endosomes, and the plasma membrane (Duncan and Kornfeld, 1988). The MPRs initially bind newly synthesized lysosomal enzymes in the TGN. The receptor–ligand complexes then concentrate in clathrin-coated pits and exit the Golgi complex in clathrin-coated vesicles which fuse with acidified endosomal/prelysosomal compartments. The low pH of these compartments causes dissociation of the acid hydrolyses that are subsequently packaged into lysosomes. The MPRs either recycle back to the Golgi complex to mediate another round of sorting or move to the plasma membrane where they are rapidly internalized via plasma

membrane clathrin-coated pits. Importantly, the receptors avoid being transported from the prelysosomal endosomal compartments to lysosomes, whereas lysosomal membrane proteins are delivered efficiently from endosomes to lysosomes.

Two distinct MPRs have been characterized, the 275-kD mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGF-II receptor) and the 46-kD cation-dependent mannose 6-phosphate receptor (CD-MPR). Both are type I integral membrane glycoproteins (Kornfeld, 1992). The signals on the MPRs needed for endocytosis from the cell surface and efficient incorporation into clathrin-coated pits in the TGN reside in the cytoplasmic tails of these proteins. The signal for the rapid internalization of the Man-6-P/IGF-II receptor at the plasma membrane has been localized to the Y²⁶SKV²⁹ sequence in the 163-residue cytoplasmic tail (Lobel et al., 1989; Canfield et al., 1991; Jadot et al., 1992). The CD-MPR contains two distinct signals for rapid internalization in its 67-amino acid cytoplasmic domain (Johnson et al., 1990). One signal includes Phe13 and Phe18, while the second signal (which is less potent) involves Tyr45. Both receptors have dileucine-containing sequences near the carboxyl termini of their cytoplasmic tails that are required for efficient entry into Golgi clathrin-coated pits (Johnson and Kornfeld, 1992a,b; Chen et al., 1993).

In contrast to the sorting steps in the TGN and at the cell surface, it is presently unclear how the MPRs are sorted in endosomes and avoid degradation in lysosomes.

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1. *Abbreviations used in this paper:* CD-MPR, cation-dependent mannose 6-phosphate receptor; HB, homogenization buffer; Man-6-P/IGFII receptor, mannose 6-phosphate/insulin-like growth factor II receptor; MPR, mannose 6-phosphate receptor.

Based on kinetic studies in PC12 cells, Green and Kelly (1992) have postulated that receptor transport from endosomes to the Golgi complex is a constitutive process. According to this model, movement of membrane proteins from endosomes to lysosomes would require a positive signal. Some support for this notion has come from the study of Green et al. (1994) that demonstrated that *P*-selectin contains a signal in its cytoplasmic tail that is necessary for efficient delivery to lysosomes. In contrast, experiments with microinjected antibodies specific for the cytoplasmic tail of the CD-MPR have led to the suggestion that recycling from endosomes to the Golgi complex is signal mediated (Schulze-Garg et al., 1993). Consistent with this view, an immunoelectron microscopy study of HepG2 and BHK cells revealed that the CD-MPR and the asialoglycoprotein receptor are sequestered into separate tubular-vesicular structures associated with endosomes (Klumppermann et al., 1993). This provides direct evidence for sorting of these two receptor types.

The aim of the present study was to identify the determinants that mediate the sorting of the CD-MPR in endosomes. By analyzing a variety of truncation and alanine-scanning mutants, we demonstrate that the CD-MPR contains a signal that is required to avoid transport to dense lysosomes and subsequent proteolytic degradation.

Materials and Methods

Materials

Enzymes used in molecular cloning were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega Corp. (Madison, WI). α -MEM, Iscove's media, and FBS, as well as Lipofectin were from GIBCO BRL (Grand Island, NY); Percoll from Pharmacia Diagnostics AB (Uppsala, Sweden); nitrocellulose from Schleicher & Schuell, Inc. (Keene, NH); protease inhibitors from Sigma Chemical Corp. (St. Louis, MO); enhanced chemiluminescence Western blotting reagents from Amersham Corp. (Arlington Heights, IL); Expre^{35S} label from NEN (DuPont Co., Wilmington, DE); protein A-Sepharose beads from Repligen Corp. (Cambridge, MA); rabbit anti-mouse IgG from Zymed Laboratories, Inc. (San Francisco, CA); FITC goat anti-mouse IgG from Cappel (Organon Teknika Corp., Durham, NC); Permax multichamber slides from Nunc (Naperville, IL); cell culture dishes from Falcon Labware (Becton Dickinson and Co., Lincoln Park, NJ). Oligonucleotides were synthesized with a solid phase synthesizer (380A; Applied Biosystems, Inc., Foster City, CA) by the Protein Chemistry Facility of Washington University.

Recombinant DNA

All basic DNA procedures were as described (Sambrook et al., 1989). A cDNA encoding the cytoplasmic tail and transmembrane domain of the CD-MPR (Johnson et al., 1990) was subcloned into pBSK⁻ using EcoRI and HindIII restriction enzymes to give the plasmid pBSK-MPR^{TMD/tail}. The PCR procedure of Ho et al. (1989) was used to generate the alanine-scanning mutants and the MPR C30C34 construct with pBSK⁻ MPR^{TMD/tail} serving as template together with bp 170–193 and 1260–1241 of pBSK as the down- and upstream primers. Appropriate partial complementary pairs of oligonucleotides in which the desired alanine replacements had been incorporated were chosen as internal primers. The final PCR products were digested with BglII and MluI, and the purified fragments were assembled with the EcoRI-BglII fragment of pBSK(B⁻H⁻)-MPR(BglII⁻) (pBSK-MPR that has the BamHI and HindIII sites in its polylinker deleted and the BglII site at amino acid 112 of CD-MPR destroyed by a silent point mutation) and the EcoRI-MluI fragment of pSFFVneo in a three-part ligation.

Mutants His63Stop, Asp51Stop, and Asp28Stop have been described previously (Johnson et al., 1990; Johnson and Kornfeld, 1992b). The constructs Glu58Stop and Gly54Stop were created as described in Johnson et al. (1990).

The plasmid-encoding human cDNA of Lamp1, pDSR α -hLamp1 (Williams and Fukuda, 1990) was cut with EcoRI, and the fragment was subcloned into the EcoRI site of the polylinker of pBSK⁻, resulting in the plasmid pBSK-Lamp1. Chimeric mutants between CD-MPR and Lamp1 were created using standard PCR protocols with the overlap extension technique (Ho et al., 1989). For the construction of LLM and LMM, which contain the luminal, transmembrane, and cytoplasmic domains from Lamp1 (L) or CD-MPR (M) in the order shown, plasmids pBSK-Lamp1 and pBSK-MPR^{TMD/tail} were used as templates with bp 1260–1241 of pBSK and bp 998–1018 of Lamp1 as flanking primers. An appropriate partial complementary pair of oligonucleotides in which the desired overlap between the Lamp1 and CD-MPR cDNAs had been incorporated was chosen as internal primers. The resulting PCR fragments were cut with Eco47III and XbaI and used to replace the Eco47III-XbaI fragment of pBSK-Lamp1. The resulting plasmids were designated pBSK-LMM and pBSK-LLM, respectively. The switch from the Lamp1 to the CD-MPR sequence was made at T354 (Lamp1) to L188 (CD-MPR), and G378 (Lamp1) to Q213 (CD-MPR) to create the chimeras LMM and LLM, respectively. To construct the chimera LML plasmids, pBSK-LMM and pBSK(B⁻H⁻)-MML were used as templates for the PCR, bp 998–1018 of Lamp1 and bp 1260–1241 of pBSK were used as flanking primers, and internal primers were chosen as described above. The PCR product was digested with Eco47III and XbaI to replace the corresponding fragment of pBSK-Lamp1 generating the plasmid pBSK-LML. The plasmids pBSK-LLM, pBSK-LMM, and pBSK-LML were cut with EcoRI and ligated into pSFFVneo (Fuhlbridge et al., 1988; Johnson et al., 1990) resulting in the final plasmids used for transfection.

The final plasmids were sequenced using the Sanger dideoxy chain termination method (Sanger et al., 1977) to verify that the mutant constructs were correct.

Cell Culture and Transfection

A Man-6-P/IGF-II receptor-deficient mouse L cell line designated D9 (LRec⁻) was maintained in (α -MEM) containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% humidified CO₂ atmosphere (Gabel et al., 1983). The cells were transfected with 20 μ g of XbaI-linearized DNA using Lipofectin (GIBCO BRL) according to the manufacturer's directions. Selection for resistance to neomycin (G418) was as described previously (Lobel et al., 1989) except that the final G418 concentration was 500 μ g/ml. Resistant colonies were screened for expression by immunoblotting. Selected clones were expanded for further study and maintained in selective medium.

Antibodies

mAb G1/139 against human Lamp1 (Schweizer et al., 1988) and rabbit polyclonal antisera 931-A against human Lamp1 (Carlsson et al., 1988) were kind gifts of Dr. H.-P. Hauri (Biozentrum, Basel, Switzerland) and Dr. M. Fukuda (Cancer Research Center, La Jolla, CA), respectively. mAb 22D4 specific for the bovine CD-MPR was generously provided by Dr. D. Messner (Messner, 1993).

Confocal Immunofluorescence Microscopy

Cells were grown in eight-well Permax multichamber slides. The immunofluorescence procedure for permeabilized cells was that of Schweizer et al. (1988). In brief, formaldehyde-fixed and saponin-permeabilized cells were incubated with mAb G1/139 against Lamp1 or mAb 22D4 against CD-MPR followed by goat anti-mouse FITC. The specimens were examined with a confocal laser scanning microscope system (MRC 1000; Bio-Rad Laboratories, Hercules, CA) attached to a microscope (Carl Zeiss, Inc., Thornwood, NY).

Percoll Gradient Fractionation

Confluent cells grown in a 100-mm petri dish were incubated for either 12 or 24 h in growth medium supplemented with 100 μ M each of pepstatin A and leupeptin. After two washes with PBS, the cells were scraped into 2 ml of homogenization buffer (HB) (0.25 M sucrose, 1 mM EDTA, pH 7.5) and centrifuged for 10 min at 140 g. The cells were resuspended in 850 μ l of HB, and passed 12 times through a ball bearing homogenizer (Balch and Rothman, 1985) with a clearance of 51.2 μ m. The homogenate was diluted with an additional 850 μ l HB, and centrifuged for 10 min at 400 g. The resulting postnuclear supernatant was layered over a discontinuous gradient consisting of a 1.2-ml cushion of 10 \times HB and 8.5 ml of an 18%

Percoll solution in $1 \times$ HB. The gradient was centrifuged for 30 min at 20,000 rpm in a Ti 50 rotor (Beckman Instruments Inc., Palo Alto, CA). The cushion followed by nine 1.2-ml fractions were collected from the bottom of the tube. For initial determinations of the β -hexosaminidase distribution, fractions 1–9 were processed individually. In subsequent experiments, the gradient fractions were combined as follows: fractions 1–3 (pool I), fractions 4–6 (pool II), and fractions 7–9 (pool III). The Percoll was removed by centrifugation twice for 30 min at 85,000 rpm in a TL 100.3 rotor (Beckman Instruments Inc.). The pelleted membranes were transferred into 1.5-ml ultracentrifugation tubes, diluted with HB to a final vol of 1 ml, and centrifuged for an additional 50 min at 70,000 rpm in the TL 100.3 rotor to remove the remaining Percoll. The sedimented membranes were transferred into 1.5-ml tubes and mixed with HB to a final vol of 300 μ l. The samples were adjusted to 0.5% Triton X-100, passed five times through a 25-gauge needle connected to a 1-ml syringe, and solubilized on ice for 30 min. An aliquot corresponding to 1/10 of the total volume was removed for the β -hexosaminidase assay. 300 μ l of $3 \times$ electrophoresis sample buffer (1.875 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.003% bromophenol blue) with (for detection with anti-Lamp1 mAb) or without (for detection with anti-CD-MPR mAb) 0.3 M DTT was added to the remaining sample and the mixture was boiled for 3 min. Aliquots (1/30 for analysis with anti-Lamp1 mAb, and 1/15 for analysis with anti-CD-MPR mAb) of the final sample volume were analyzed by SDS-PAGE and immunoblotting.

SDS-PAGE, Fluorography, and Immunoblotting

Proteins were separated on 6 and 10% SDS-polyacrylamide minigels (Bio-Rad Laboratories) using the Laemmli (1970) system. After electrophoresis, gels were either treated with Amplify, dried, and exposed to film XOMat AR; Eastman Kodak Co., Rochester, NY) (time course), or transferred onto nitrocellulose membranes according to the method of Towbin et al. (1979) (Percoll density fractionation). The nitrocellulose sheet was blocked with 3% nonfat dry milk powder (Schnucks, St. Louis, MO) in PBS. The blot was subsequently incubated with mAb G1/139 against Lamp1 (diluted 1:1,000 in PBS-3% powdered milk) or mAb 22D4 (diluted 1:500 in PBS-3% powdered milk) followed by an HRP-conjugated anti-mouse secondary antibody (Amersham Corp.). Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system according to the manufacturer's directions. The autoradiograph was quantitated using a personal densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Enzyme Assays

β -hexosaminidase activity was determined by dilution of the samples in 1.67 mM *p*-nitrophenyl *N*-acetyl- β -glucosaminide, 50 mM citrate, pH 4.5, 0.1% Triton X-100. The samples were incubated for 30–60 min at 37°C; the reaction was stopped with 0.2 M sodium carbonate, and the absorbance read at 400 nm.

Determination of Receptor Half-Lives

Confluent cells grown in six-well plates were rinsed twice with PBS and preincubated in methionine- and cysteine-free growth medium containing 10% dialyzed FCS and 20 mM Hepes, pH 7.4, for 30 min. The cells were then incubated for 60 min with 100 μ Ci of EXPRE^{35S} protein-labeling mixture in preincubation medium, and chased in normal culture medium in the presence of 10 mM unlabeled methionine for 0–24 h. For the 24-h time point, duplicate wells were incubated in the absence or presence of 100 μ M of pepstatin A and leupeptin. At the end of the chase, cells were chilled on ice, washed twice with 2 ml ice-cold PBS, scraped into 2 ml ice-cold PBS, and centrifuged for 10 min at 140 g. The pellets were resuspended in buffer II (10 mM sodium phosphate, pH 8.0, containing 1% Triton X-100 and a 1:500 dilution of a protease inhibitor cocktail (5 mg/ml benzamide, and 1 μ g/ml each of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethyl sulfoxide-60% ethanol)) and passed five times through a 25-gauge needle connected to a 1-ml syringe. After incubating for 1 h on ice, the cell lysates were centrifuged for 60 min at 40,000 rpm in a Ti 50 rotor. The resulting supernatants were collected and used for immunoprecipitation. Wild-type and mutant CD-MPR proteins, as well as chimeric proteins containing the CD-MPR luminal domain, were immunoprecipitated with mAb 22D4, while the rabbit antiserum against lamp1 was used for the immunoprecipitation of lamp1 and chimeric proteins with a lamp1 luminal domain. For immunoprecipitation with anti-CD-MPR mAb 22D4, 25 μ l of protein A-Sepharose was washed once

with 1 ml 100 mM sodium phosphate-0.2% BSA, pH 8.0 (buffer I), and then incubated with 2 μ l rabbit anti-mouse antibody (1 mg/ml) in 500 μ l buffer I for 2 h at room temperature. After two washes with buffer I, the beads were incubated with 20 μ l of mAb 22D4 in 500 μ l buffer I overnight at 4°C. For immunoprecipitation with anti-lamp1 polyclonal antibodies, the washed protein A-Sepharose beads were directly incubated with 2 μ l antiserum overnight at 4°C. The beads were then washed twice with 1 ml buffer I and once with 1 ml buffer II. The washed beads and radiolabeled cell lysates were combined and incubated overnight at 4°C with constant mixing. The protein A-Sepharose beads were pelleted, washed four times with buffer II, and then once with 100 mM sodium phosphate, pH 8.0, followed by a final wash step with 10 mM sodium phosphate, pH 8.0. The immunocomplexes were released from the beads by boiling for 3 min in electrophoresis sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 0.1 M DTT.

Cell Surface Biotinylation

Confluent cells grown in six-well plates were labeled for 30 min with Expre^{35S} and chased for 0 min, 30 min, 1 h, or 8 h as described above. Cell monolayers were then washed twice with ice-cold PBS followed by five wash steps with PBS supplemented with 0.7 mM CaCl₂ and 0.25 mM MgSO₂ (PBS++) over a period of 30 min before NHS-SS-biotin was added at a concentration of 3 mg/ml in PBS²⁺ for 45 min on ice. Biotinylation was stopped by washing four times with 50 mM glycine in PBS²⁺. The cells were lysed in buffer II and centrifuged for 1 h at 100,000 g in a Ti 50 rotor. The resulting supernatants were immunoprecipitated as described above.

Results

Cytoplasmic Tail of the CD-MPR Contains a Signal that Prevents Delivery of the Receptor to Dense Lysosomes

To test for a possible endosomal sorting signal in the cytoplasmic tail of the CD-MPR, we analyzed a truncated form of the receptor (Asp28Stop) that has amino acids 28–67 of the cytoplasmic tail deleted (Johnson et al., 1990). Although Asp28Stop lacks a Golgi sorting signal, it efficiently enters the endosomal system via the plasma membrane by endocytosis (Johnson et al., 1990; Johnson and Kornfeld, 1992). We first determined the half-life of Asp28Stop in mouse L cells stably expressing the truncated receptor. In these experiments, the cells were labeled with [³⁵S]methionine/cysteine for 1 h and chased for various time periods either in the absence or presence of the lysosomal protease inhibitors pepstatin A and leupeptin. Cells expressing the wild-type bovine CD-MPR served as the control. The CD-MPRs were immunoprecipitated from cell lysates with mAb 22D4, analyzed by SDS-PAGE and fluorography, and quantitated by scanning densitometry. The gels from a typical experiment are shown in Fig. 1, A and B, with the data from multiple experiments summarized in C. The CD-MPR and Asp28Stop were synthesized as 40- and 35-kD precursor forms, respectively, which were converted to 46- and 41-kD mature forms by 4 h. This indicates that these polypeptides were transported to the Golgi apparatus and underwent N-linked oligosaccharide processing with similar kinetics (Gasa and Kornfeld, 1987). The half-life of the CD-MPR was >40 h. In contrast, the half-life of Asp28Stop was reduced by more than fourfold ($t_{1/2} = 10$ h). The degradation of the truncated receptor was completely blocked by pepstatin A and leupeptin. This protection is consistent with the receptor being degraded in lysosomes.

To confirm morphologically that the mutant receptor

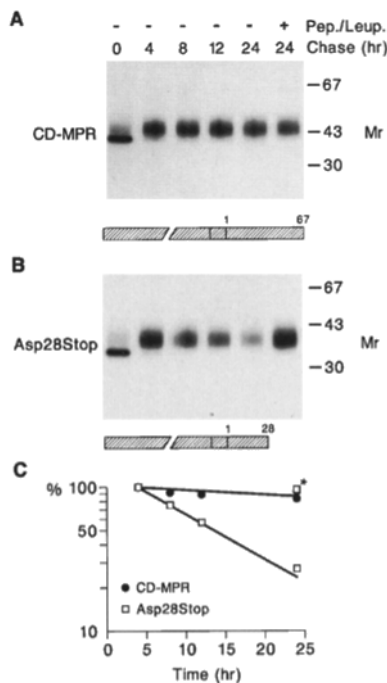


Figure 1. Pulse-chase analysis of wild-type CD-MPR and mutant Asp28Stop receptors. Mouse L cells stably expressing CD-MPR (A) or Asp28Stop (B) were metabolically labeled with [³⁵S]methionine/cysteine and chased for the indicated time intervals. The 24-h time point was performed with or without the lysosomal protease inhibitors pepstatin A and leupeptin (Pep./Leup.). Wild-type and mutant receptors were immunoprecipitated with mAb22D4 and analyzed by SDS-PAGE (10% gels). The numbers on the right margin of the gel indicate the migration of molecular mass standards in kilodaltons. Schematic illustrations of the constructs are shown underneath each autoradiograph. (C) The autoradiographs shown in A and B as well as those from additional experiments, were quantitated by scanning densitometry. At each time point the amount of wild-type receptor (●) or Asp28Stop (□) detected is plotted as the percentage of the value obtained at the 4-h chase point. The 24-h time point in the presence of pepstatin A and leupeptin is indicated by ●* (CD-MPR) and □* (Asp28Stop), respectively.

was being transported to lysosomes, we analyzed the localization of the Asp28Stop receptor by confocal immunofluorescence microscopy. For this purpose, stable cell lines expressing either CD-MPR, Asp28Stop, or the lysosomal membrane protein Lamp1 were preincubated for 12 h in the presence or absence of pepstatin A and leupeptin, fixed with paraformaldehyde, permeabilized with saponin, and stained with appropriate antibodies. As expected, the CD-MPR showed a predominant perinuclear staining, representing localization in the TGN and late endosome/prelysosomal compartments, as well as staining of vesicles (early endosomes) and, to a lesser extent, the cell surface (Fig. 2, A and B). The fluorescence pattern of Lamp1 with its ringlike structures was characteristic for lysosomal staining (Fig. 2, E and F). The staining pattern of Asp28Stop in the mock-treated cells was very similar to that observed for CD-MPR (Fig. 2 C). However, the distribution of Asp28Stop in cells pretreated with pepstatin A and leupeptin was similar to Lamp1, indicating that the truncated receptor was being degraded in lysosomes (Fig. 2 D).

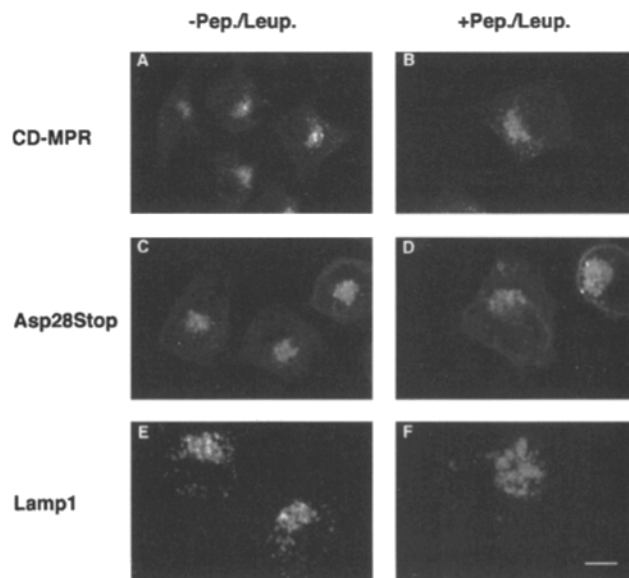


Figure 2. Confocal immunofluorescence localization of CD-MPR, Asp28Stop, and Lamp1. Cell lines stably expressing CD-MPR (A and B), Asp28Stop (C and D), and Lamp1 (E and F) were either preincubated for 12 h with pepstatin A and leupeptin (B, D, and F) or mock-treated (A, C, and E) before fixation with paraformaldehyde and permeabilization with saponin. For detection mAb 22D4 (A–D) or mAb G1/139 (E and F) followed by goat anti-mouse FITC were used. The pictures represent linear projections of several optical sections. Bar, 7.5 μ m.

The lysosomal distribution of the mutant Asp28Stop receptor was further documented using isoosmotic Percoll density gradients that separate dense lysosomes from other organelles (Green et al., 1987). Cell monolayers were grown in culture media supplemented with pepstatin A and leupeptin for either 12 or 24 h. Fig. 3 A shows the typical distribution of the lysosomal enzyme marker β -hexosaminidase on Percoll gradients using cells stably expressing CD-MPR or Lamp1. The majority of the β -hexosaminidase activity was recovered at the bottom of the gradient, well-separated from lighter membranes that accumulated in fractions 7–9 (data not shown, Green et al., 1987). Based on this distribution, the fractions were analyzed in three pools in all subsequent experiments: pool I (fractions 1–3) containing the bulk of lysosomal enzyme activity (70–80%); pool II (fractions 4–6) containing intermediate density membranes; and pool III (fractions 7–9) containing low density membranes including endosomes, Golgi complex, plasma membrane, and the ER (Green et al., 1987).

The distribution of the CD-MPR, Asp28Stop, and Lamp1 was determined by electrophoresis of the Percoll density fractions followed by Western blotting (Fig. 3, B and C, for quantitation). The CD-MPR was excluded almost completely from the dense lysosome fraction (4% recovered in pool I) whereas 62–68% of Lamp1 was recovered in pool I. After the 12-h incubation with pepstatin A and leupeptin, $24 \pm 5\%$ of Asp28Stop was recovered in the dense lysosome fraction and this value increased to $44 \pm 6\%$ after 24 h of incubation with the inhibitors. These data further corroborate the lysosomal distribution of the Asp28Stop receptor.

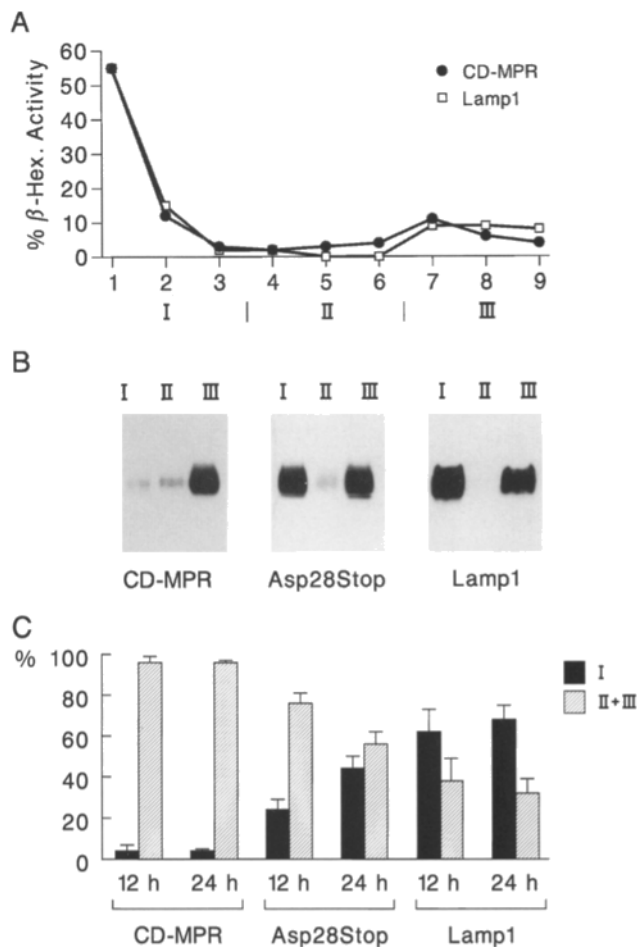


Figure 3. Subcellular distribution of CD-MPR, Asp28Stop, and Lamp1 on Percoll density gradients. Cells were homogenized with a ball bearing homogenizer and postnuclear supernatants were subjected to Percoll density gradient centrifugation (18% Percoll). (A) Percoll gradient fractions of mouse L cells stably expressing either CD-MPR (●) or Lamp1 (□) were assayed for β -hexosaminidase activity. The value for each fraction was expressed as its percentage of the total activity recovered from the gradient. For further experiments the fractions were combined into pool I, II, and III as indicated below the marker profile. (B) Mouse L cells stably expressing CD-MPR, Asp28Stop, or Lamp1 were preincubated for 24 h with pepstatin A and leupeptin, and subjected to fractionation as described above. Proteins of pool I, II, and III were analyzed by SDS-PAGE and immunoblotting with mAb 22D4 (CD-MPR, Asp28Stop) or mAb G1/139 (Lamp1). (C) The immunoblots shown in B and those from additional experiments were quantitated. Preincubation with pepstatin A and leupeptin was for 12 or 24 h as indicated. For each construct (x-axis) the value of pool I (dense lysosomes, filled bars) and the values of pools II and III combined (stippled bars) were expressed as their percentage of the total value of all three pools (y-axis).

Upon examining the sequence of the cytoplasmic tail of Asp28Stop, we noted that the truncation exposed a Leu-ValAla sequence at the carboxyl terminus. Since a Leu-Val sequence in the cytoplasmic tail of LimpII has been shown to direct this membrane protein to lysosomes (Ogata and Fukuda, 1994; Sandoval et al., 1994), we considered the possibility that a lysosomal targeting signal was inadvertently

unmasked in the deletion mutant. Therefore a construct was prepared (Leu25Ala Asp28Stop) in which the leucine at position 25 was mutated to an alanine. This mutation in LimpII prevents targeting to lysosomes (Ogata and Fukuda, 1994; Sandoval et al., 1994). When L cells expressing Leu25Ala Asp28Stop were analyzed by Percoll gradient fractionation after 24 h of incubation with protease inhibitors, $56 \pm 5\%$ of the mutant receptor was recovered in dense lysosomes (Table I). This result excludes the possibility that the exposed LeuValAla sequence was serving as a positive lysosomal targeting signal.

Taken together, these results indicate that the deleted portion of the cytoplasmic tail of the CD-MPR contains a signal to prevent the receptor from entering lysosomes and being degraded.

Effect of Different Cytoplasmic Domain Deletions on Receptor Degradation

We next analyzed the effect of truncating increasing portions of the COOH-terminal region of the CD-MPR cytoplasmic tail on the accumulation of the receptor in dense lysosomes. Stable clones of L cells that had been transfected with cDNAs encoding mutant CD-MPRs containing 62 amino acids (His63Stop), 57 amino acids (Glu58Stop) 53 amino acids (Gly54Stop), and 50 amino acids (Asp51Stop) of the 67-amino acid cytoplasmic tail were studied (Fig. 4 A). The cells were incubated with pepstatin A and leupeptin for 24 h and then cell lysates were analyzed on Percoll gradients. As summarized in Fig. 4 B and Table I, the deletion of 5 (His63Stop) or 10 (Glu58 Stop) amino acids from the carboxyl terminus of the cytoplasmic tail did not result

Table I. Summary of Results of Percoll Density Gradients

Mutant	Percentage in dense lysosomes
CD-MPR	4 ± 1 $4 \pm 3^*$
Asp28Stop	44 ± 6 $24 \pm 5^*$
Leu25Ala Asp28Stop	56 ± 5
Asp51Stop	26 ± 5
Gly54Stop	14 ± 3
Glu58Stop	6 ± 1
His63Stop	4 ± 3
MPR 28-33A	5 ± 1
MPR 34-39A	30 ± 3
MPR 40-45A	6 ± 2
MPR 46-50A	4 ± 2
MPR 35-39A	27 ± 3
MPR 34-36A	16 ± 4
MPR 37-39A	16 ± 3
MPR C30C34A	30 ± 2
LLM	$33 \pm 7^*$
LMM	$11 \pm 11^*$
LML	$50 \pm 10^*$
Lamp1	68 ± 7 $62 \pm 11^*$

All experiments were performed after a 24-h preincubation with pepstatin A and leupeptin, except for those indicated by the *, where the preincubation was for 12 h. Values are the average of 3 to 10 separate experiments.

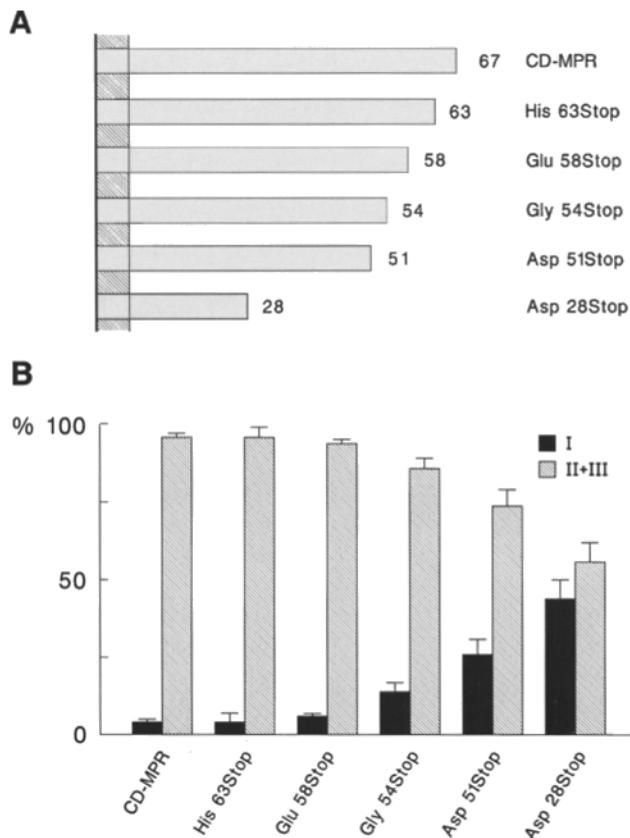


Figure 4. Effect of tail length on receptor targeting. (A) Schematic illustration of the cytoplasmic tails of CD-MPR and truncation mutants. Bars represent wild-type and mutant cytoplasmic tails. The numbers at the right margin of truncated tails indicate the position of the stop codons. (B) Mouse L cells stably expressing His63Stop, Glu58Stop, Gly54Stop, and Asp51Stop were preincubated with pepstatin A and leupeptin for 24 h and then fractionated as described in Fig. 3. Proteins of pool I, II, and III were subjected to SDS-PAGE and immunoblotting with mAb 22D4. The quantitation of several experiments is shown. For each construct the value of pool I (dense lysosomes, filled bars) and the values of pools II and III combined (striated bars) were expressed as their percentage of the total value of all three pools. The distribution of CD-MPR and Asp28Stop from Fig. 3 is given for comparison.

in significant accumulation of receptor in dense lysosomes. Deletion of 14 amino acids (Gly54Stop) resulted in a modest accumulation of receptors in dense lysosomes ($14 \pm 3\%$) whereas the deletion of 17 amino acids (Asp51Stop) had a more profound effect ($26 \pm 5\%$ accumulation in dense lysosomes). However, this accumulation was less than that observed with Asp28Stop after 24 h of pepstatin A and leupeptin treatment ($44 \pm 6\%$ accumulation). These experiments indicated that amino acid residues 28–57 are required to prevent targeting of the receptor to dense lysosomes and within this sequence, residues 28–50 appear to be most important.

Replacement of Amino Acid Residues 34–39 of the Cytoplasmic Tail Leads to Rapid Degradation in Lysosomes

To define more precisely the determinants present within the amino acid 28–50 region of the CD-MPR cytoplasmic

tail, a series of alanine stretches were created. As indicated in Fig. 5 A, amino acids 28–33 (MPR 28–33A), 34–39 (MPR 34–39A), 40–45 (MPR 40–45A), or 46–50 (MPR 46–50A) were changed to alanines using PCR mutagenesis. When L cells stably expressing these various constructs were incubated with pepstatin A and leupeptin for 24 h and analyzed on Percoll gradients, the mutant receptor containing alanine residues in positions 34–39 of the cytoplasmic tail was found to accumulate in dense lysosomes ($30 \pm 3\%$) whereas the other three mutant receptors did not (Fig. 5, B and C, for quantitation, and Table I). It should be noted that even though constructs MPR 40–45 and 46–50 have the Tyr-based internalization signal mutated, these receptors still undergo rapid endocytosis due to the presence of the Phe-based internalization signal (Johnson et al., 1990). These results suggest that amino acids 34–39 within the cytoplasmic tail of the CD-MPR play an important role in preventing lysosomal targeting of the receptor.

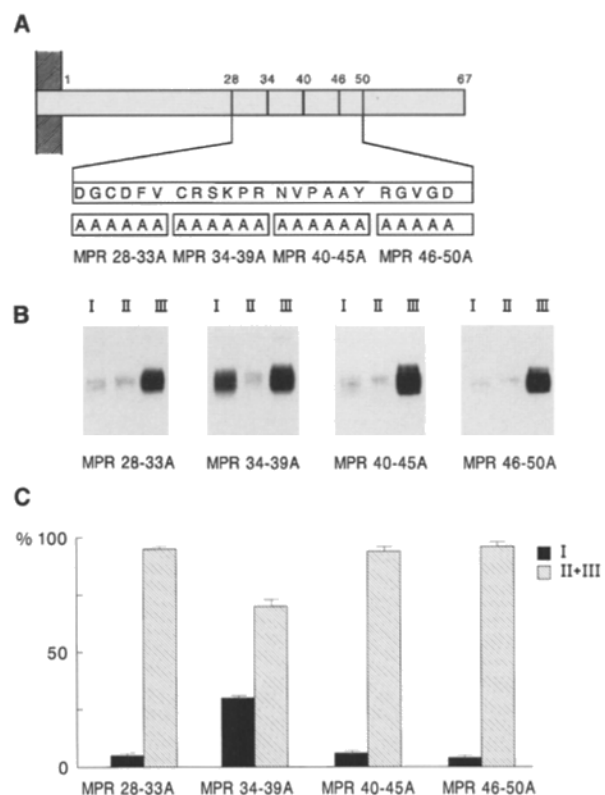


Figure 5. Amino acids 34–39 in the cytoplasmic tail of CD-MPR are critical for receptor targeting. (A) Schematic illustration of alanine scanning mutants within the cytoplasmic tail of CD-MPR. The bar represents the cytoplasmic tail of CD-MPR and the numbers indicate individual amino acid positions. The wild-type sequence of amino acids 28–50 is shown underneath the bar. Amino acids 28–33 (MPR 28–33A), 34–39 (MPR 34–39A), 40–45 (MPR 40–45A), and 46–50 (MPR 46–50A) were substituted by alanines as indicated. (B) Mouse L cells stably expressing MPR 28–33A, MPR 34–39A, MPR 40–45A, and MPR 46–50A were preincubated with pepstatin A and leupeptin for 24 h and then fractionated as described in Fig. 3. Proteins of pool I, II, and III were subjected to SDS-PAGE and immunoblotting with mAb 22D4. (C) Quantitation of the immunoblots shown in B and those from additional experiments. The values were calculated as described in Fig. 3. Filled bars, pool I (dense lysosomes); striated bars, pools II and III combined.

Three additional constructs involving alanine replacements of subsets of the residues 34–39 (CRSKPR) were prepared to dissect further the role of this region of the cytoplasmic tail. An additional construct was made in which the two cysteines present in the cytoplasmic tail at positions 30 and 34 were changed to alanines. As summarized in Table I, MPR 35–39A and MPR C30C34A accumulated in dense lysosomes to the same extent as MPR 34–39A (27 ± 3 and $30 \pm 2\%$, respectively, versus $30 \pm 3\%$). The mutant receptors MPR 34–36A and MPR 37–39A exhibited somewhat less accumulation in dense lysosomes (16 ± 4 and $16 \pm 3\%$, respectively). These findings indicate that multiple residues in the amino acid 34–39 sequence are involved in preventing the receptor from trafficking to lysosomes.

Cytoplasmic Tail and Transmembrane Domain of CD-MPR Are Sufficient for Endosomal Sorting

The results with the cytoplasmic tail truncation and alanine-scanning mutants established that the cytoplasmic tail of the CD-MPR is necessary for avoiding delivery to dense lysosomes. To determine whether this domain is sufficient to exclude a lysosomal membrane protein from being targeted to lysosomes, we created a chimeric molecule, LLM, containing the luminal and transmembrane domains of Lamp1 with the cytoplasmic tail of the CD-MPR. Two additional constructs LMM (luminal domain of Lamp1, transmembrane and cytoplasmic domains of CD-MPR) and LML (luminal and cytoplasmic domains of Lamp1, transmembrane domain of CD-MPR) were generated to evaluate the role of the transmembrane domain of the CD-MPR in this sorting event. When L cells transfected with these various constructs were analyzed on Percoll gradients, only $33 \pm 7\%$ of the chimeric LLM protein was recovered in the dense lysosome fraction (pool I) compared with $62 \pm 11\%$ of Lamp1 (Fig. 6). Even less of the LMM protein was recovered in dense lysosomes ($11 \pm 11\%$). The localization of the LML protein was similar to that obtained with Lamp1, with $50 \pm 10\%$ of the protein being recovered in dense lysosomes.

To exclude the possibility that the amount of LMM and LLM chimeras present in dense lysosomes was reduced due to mislocalization to the plasma membrane, we determined the fraction of the chimeric molecules at the cell surface. Cell lines expressing the various constructs were labeled for 30 min with [35 S]methionine/cysteine and chased for up to 8 h. After the chase period, the surface appearance of the chimeric proteins was detected by biotinylation of intact cells with the membrane-impermeable probe NHS-SS-biotin on ice. The cells were solubilized and total LMM, LLM, or Lamp1 immunoprecipitated with a rabbit anti-human Lamp1 antibody. The resultant immunocomplexes were dissociated and divided into two aliquots. Biotinylated (surface) molecules were reprecipitated from three-quarters of the sample using streptavidin-agarose beads, and the remaining quarter was used to determine the total amount of labeled protein. The two samples were then analyzed by SDS-PAGE, and quantitated by scanning densitometry to give values for the total and surface molecules. The results of a typical experiment are shown in Fig. 7 A with the quantitation of multiple experiments given in Fig. 7 B. Only 3% of Lamp1 was detected at the cell surface after 1 h of chase, and by 3 h of chase Lamp1

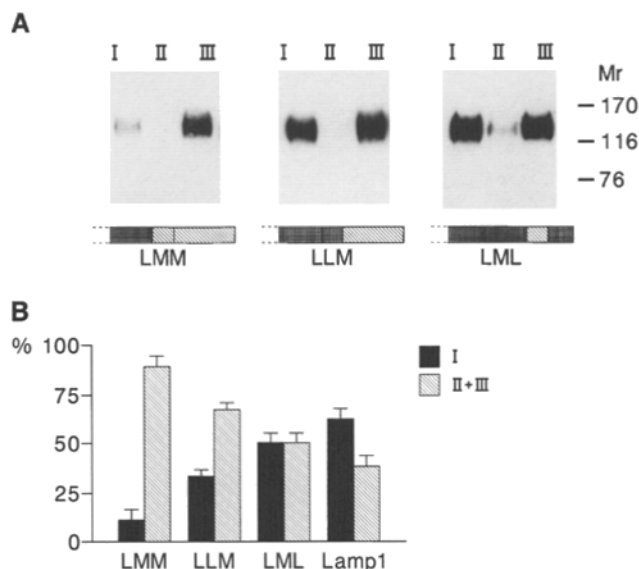


Figure 6. Distribution of LMM, LLM, and LML on Percoll gradients. Mouse L cells stably expressing LMM, LLM, and LML were analyzed as described in Fig. 3. (A) Proteins of pool I, II, and III were subjected to SDS-PAGE and immunoblotting with mAb G1/139. A schematic illustration of each construct is shown underneath each autoradiograph (luminal domain, left box; transmembrane domain, middle box; cytoplasmic tail, right box). (B) Quantitation of the immunoblots shown in A and those from additional experiments. The values were calculated as described in Fig. 3. Filled bars, pool I (dense lysosomes); striated bars, pools II and III combined. The distribution of Lamp1 of Fig. 3 is shown for comparison.

was undetectable at the surface. This is similar to the results of Harter and Mellman (1990). Surface appearance of LMM was also transient, reaching a maximum of 8% after 3 h and almost disappearing at 8 h. A greater fraction of LLM appeared at the cell surface (21% after 3 h), but after 8 h only 6% of the protein remained on the cell surface. These data show that LMM and LLM do not accumulate in the plasma membrane. These results support our contention that the cytoplasmic tail and transmembrane domain of the CD-MPR are important in preventing lysosomal targeting of the receptor.

Discussion

Previous studies have identified short amino acid sequences in the cytoplasmic tail of the CD-MPR that mediate rapid internalization at the plasma membrane and efficient entry into Golgi clathrin-coated pits (Johnson et al., 1990; Johnson and Kornfeld, 1992b). One internalization signal included Phe13 and Phe18, while a second, less potent internalization signal involved Tyr45. The Golgi sorting signal included the LeuLeu sequence near the carboxyl terminus of the cytoplasmic tail. The results presented here demonstrate that the cytoplasmic tail of the CD-MPR contains yet a third signal which functions to prevent the receptor from trafficking to lysosomes where it would be degraded. In addition, the transmembrane domain of the CD-MPR also contributes to this function.

The most direct evidence that this effect is mediated by a positive sorting signal within the cytoplasmic tail of the

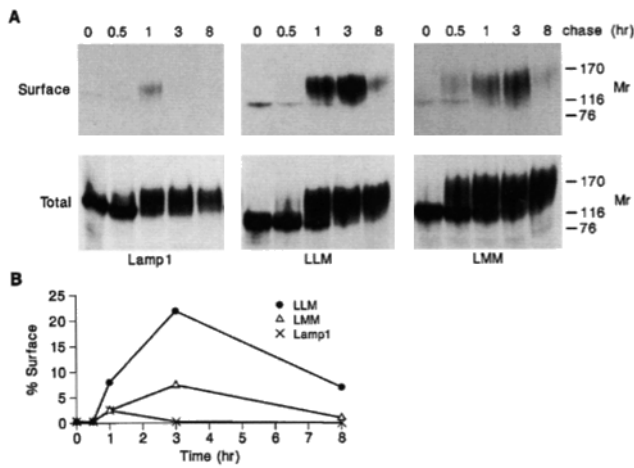


Figure 7. Surface appearance of newly synthesized Lamp1, LLM, and LMM. (A) Mouse L cells stably expressing Lamp1, LLM, and LMM were labeled with [³⁵S]methionine/cysteine for 30 min and then chased for the indicated times. At each time point, labeled cell surface proteins were derivatized using NHS-SS-biotin. The cells were lysed and surface biotinylated, and internal Lamp1 and chimeric molecules were immunoprecipitated using a polyclonal anti-Lamp1 antibody (931-A). After solubilization of the first immunoprecipitate, three-quarters of each sample was incubated with streptavidin beads to precipitate surface biotinylated molecules. All precipitates were analyzed by SDS-PAGE and autoradiography. (B) Autoradiographs shown in A and those from additional experiments were quantitated by scanning densitometry. The values presented in the graph represent the percentage of Lamp1 (x), LLM (●) and LMM (Δ) detected at the cell surface relative to the total amount detected at a given time point.

CD-MPR comes from the analysis of the truncation mutants Asp28Stop and Leu25Ala Asp28Stop that have amino acids 28–67 of the tail deleted. These mutant receptors accumulate in lysosomes as judged by three different criteria: first, a sharply reduced half-life with complete protection from degradation by the lysosomal protease inhibitors, pepstatin A and leupeptin; second, a lysosomal immunofluorescence pattern; and third, the recovery of the truncated receptors in the dense lysosomal fractions on Percoll density gradients. These results indicate that amino acids 28–67 of the cytoplasmic tail contain sorting information that is necessary to keep the receptor from being transported to lysosomes. Subsequent analysis of additional truncation mutants localized the signal to amino acid residues 28–50 and alanine scanning of this region implicated amino acids 34–39 as being most critical. Within this segment, multiple residues appear to be required. A search of the GenBank database, which includes other recycling receptors, failed to reveal any strong homologies with the sequence CysArgSerLysProArg. However, the Man-6-P/IGFII receptor does contain a CysCysArgArg-Ser sequence in its cytoplasmic tail, but its significance is currently unknown.

While the cytoplasmic tail of the CD-MPR is necessary for proper sorting of the receptor in endosomes, it is only partially effective in preventing the lysosomal membrane protein Lamp1 from entering lysosomes. Complete exclusion required the combined presence of the CD-MPR cy-

toplasmic tail and transmembrane domain, even though the transmembrane domain alone had just a minor effect on the distribution of Lamp1. The latter result also demonstrated that the transmembrane domain of Lamp1 is not absolutely required for the proper localization of that protein in lysosomes. The finding that both the cytoplasmic tail and the transmembrane domain of the CD-MPR are required for proper sorting in endosomes differs from a number of other sorting events, including sorting of receptors into plasma membrane and Golgi clathrin-coated pits, where signals on the cytoplasmic tail alone are sufficient to mediate the process. On the other hand, the luminal domain of the CD-MPR does not appear to be necessary for proper endosomal sorting as indicated by the finding that construct LMM had a distribution very similar to that of native CD-MPR. Furthermore, the luminal domain of the CD-MPR is not sufficient to prevent the delivery of the receptor to lysosomes, as shown by our data and the study of Peters et al. (1990). These investigators found that a chimera containing the luminal domain of the CD-MPR fused to the transmembrane domain and cytoplasmic tail of lysosomal acid phosphatase is rapidly transported to lysosomes. This is of interest since the luminal domain of the Man-6-P/IGF-II receptor has been reported to be essential for proper trafficking of this receptor (Dintzis and Pfeffer, 1990; Dintzis et al., 1994; Conibear and Pearse, 1994). Dintzis and her colleagues (1990; 1994) found that a chimeric molecule in which the luminal domain of the Man-6-P/IGF-II receptor was replaced by the corresponding domain of the EGF receptor localized to the plasma membrane, while the opposite chimera containing the luminal domain of the Man-6-P/IGF-II receptor colocalized with MPRs in intracellular compartments. These results are consistent with the luminal domain of the Man-6-P/IGF-II receptor having an endosomal retention function. Conibear and Pearse (1994) came to a similar conclusion with their studies of a chimera containing the cytoplasmic tail and transmembrane domain of the Man-6-P/IGF-II receptor and a luminal domain of lysozyme.

How might the cytoplasmic tail of the CD-MPR be functioning to prevent the receptor from entering lysosomes? The simplest explanation is that it contains a signal for the recruitment of the receptor into specialized subregions of the endosomes that give rise to vesicles destined for the TGN and possibly the plasma membrane. The signal could function by interacting with a coat protein(s) that is involved in vesicle formation. In the absence of this signal, the receptor would migrate to other subregions of the endosomes that either fuse directly with lysosomes or give rise to vesicles that fuse with these organelles. Either way, the receptor would enter a lysosome and be degraded. Amino acids 34–39 (CRSKPR) of the cytoplasmic tail of the CD-MPR may constitute part or all of the signal. The finding that the deletion of increasing portions of the cytoplasmic tail of the CD-MPR has an incremental effect on the accumulation of the receptor in lysosomes may indicate that the signal is less potent if positioned close to the COOH-terminal end of the cytoplasmic tail (Mallabiabarrena et al., 1995). An alternative explanation is that amino acids 34–39 determine a critical conformation of the cytoplasmic tail that is required for the expression of a sorting signal located elsewhere in the cytoplasmic tail. The effect

of the truncation mutants would also be consistent with this type of explanation. The lack of knowledge about the conformation of the cytoplasmic tail of the CD-MPR makes it difficult to evaluate this possibility. Regardless, the important point is that the cytoplasmic tail contains a sorting signal that serves to prevent trafficking to lysosomes and to facilitate transport of the receptor out of endosomes. The transmembrane domain of the receptor, which also has a role in this process, might enhance the concentration of CD-MPR molecules in these specialized subregions of endosomes by mediating protein-protein interactions, with other CD-MPRs or with specific endosomal membrane proteins. Alternatively, the transmembrane domain could serve to position the cytoplasmic tail in such a way that the sorting determinant is optimally expressed.

Pfeffer and her colleagues have developed a cell-free system for studying the transport of the Man-6-P/IGF-II receptor from late endosomes to Golgi membranes (Goda and Pfeffer, 1988, 1990; Draper et al., 1990; Lombardi et al., 1993; Riederer et al., 1994). Transport requires ATP, cytoplasmic factors, and Rab9, but does not appear to involve clathrin-coated vesicles, suggesting that a nonclathrin coat protein may participate in this process. It would be of interest to determine if the Asp28Stop and MPR34-39A mutants fail to be transported in this reconstituted system.

Based on the finding that the Man-6-P/IGF-II receptor and the low density lipoprotein receptor are transported from the cell surface to the Golgi complex at equal rates in PC12 cells, it has been suggested that receptor trafficking from late endosomes to the Golgi complex is a constitutive pathway followed by membrane proteins not actively retained in endosomes for delivery to lysosomes (Green and Kelly, 1992). However these kinetic studies do not exclude the possibility that the recycling receptors contain signals that direct transport from endosomes to the Golgi complex. Our data suggest that a specific sorting signal is required, at least for this receptor.

The finding that the CD-MPR contains a sorting determinant that prevents delivery to lysosomes may also be relevant to the trafficking of lysosomal membrane proteins, such as Lamp1. These membrane proteins contain signals that mediate sorting in the Golgi complex and/or internalization at the plasma membrane, resulting in eventual delivery to late endosomes (Sandoval and Bakke, 1994). However, the movement of these proteins from late endosomes to lysosomes is poorly understood. Based on the findings reported here, it is possible that the lysosomal membrane proteins may pass from late endosomes to lysosomes by a default mechanism. Alternatively, these proteins may contain positive sorting determinants that direct movement from late endosomes to lysosomes. Evidence for a positive signal for the lysosomal delivery of the cell adhesion molecule P-selectin has been obtained by Green et al. (1994). These investigators identified a short region in the cytoplasmic tail of P-selectin that promoted transport from endosomes to lysosomes, thereby providing a mechanism for down-regulating the surface expression of the molecule. Presumably this signal serves to retain the protein in early endosomes, thereby preventing recycling to the plasma membrane and allowing movement to late endosomes. However, whether it also directs the traffick-

ing of the protein from late endosomes to lysosomes (versus a default mechanism) remains to be shown. Interestingly, this stretch of 10 amino acids does not contain any obvious homology to the cytoplasmic tail sequences found in the known lysosomal membrane proteins, so the generality of this mechanism remains to be established.

While our experiments have focused on the CD-MPR, it seems likely that other recycling receptors will be found to contain signals in their cytoplasmic tails that prevent trafficking to lysosomes where they would be degraded. The approach taken in this study may prove useful in identifying these signals.

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