Cysteine^{34} of the Cytoplasmic Tail of the Cation-dependent Mannose 6-Phosphate Receptor Is Reversibly Palmitoylated and Required for Normal Trafficking and Lysosomal Enzyme Sorting

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Abstract. We have examined whether the two cysteine residues (Cys^{30} and Cys^{34}) in the cytoplasmic tail of the cation-dependent mannose 6-phosphate receptor are palmitoylated via thioesters and whether these residues influence the biologic function of the receptor. To do this, mouse L cells expressing wild-type and mutant receptors were analyzed by metabolic labeling with [^{3}H]palmitate, immunoprecipitation, and SDS-PAGE. Both Cys^{30} and Cys^{34} were found to be sites of palmitoylation and together they accounted for the total palmitoylation of the receptor. The palmitate rapidly turned over with a half-life of ~2 h compared to a half-life of greater than 40 h for the protein. Mutation of Cys^{34} to Ala resulted in the gradual accumulation of the receptor in dense lysosomes and the total loss of cathepsin D sorting function in the Golgi. A Cys^{30} to Ala mutation had no biologic consequences, showing the importance of Cys^{34}. Mutation of amino acids 35-39 to alanines impaired palmitoylation of Cys^{30} and Cys^{34} and resulted in abnormal receptor trafficking to lysosomes and loss of cathepsin D sorting.

These data suggest that palmitoylation of Cys^{30} and Cys^{34} leads to anchoring of this region of the cytoplasmic tail to the lipid bilayer. Anchoring via Cys^{34} is essential for the normal trafficking and lysosomal enzyme sorting function of the receptor.

THE cation-dependent mannose 6-phosphate receptor (CD-MPR)[1] is a type I integral membrane protein that functions to transport newly synthesized acid hydrolases from the *trans*-Golgi network (TGN) to an acidified endosomal (prelysosomal) compartment (Kornfeld and Mellman, 1989; Ludwig et al., 1995; Hille-Rehfeld, 1995). After discharging its ligand, the receptor either returns to the Golgi to repeat the process or moves to the plasma membrane where it is rapidly internalized via clathrin-coated vesicles. This trafficking between the TGN, endosomes, and the plasma membrane is directed by signals located in the receptor's 67 amino acid cytoplasmic tail. A di-leucine containing sequence near the carboxyl terminus of the cytoplasmic tail is required for efficient entry into Golgi clathrin-coated pits while two signals mediate the rapid internalization at the plasma membrane (Johnson et al., 1990; Johnson and Kornfeld, 1992). One of these signals includes Phe 13 and Phe 18 while the second signal involves Tyr 45.

Recently, we reported that the cytoplasmic tail of the CD-MPR contains a third signal which functions to prevent the receptor from trafficking from endosomal compartments to lysosomes (Rohrer et al., 1995). Analysis of a series of truncation and alanine scanning mutants implicated amino acids 34-39 of the cytoplasmic tail (CysArgSerLysProArg) as being necessary for avoidance of lysosomal degradation. In addition, the transmembrane domain of the CD-MPR contributed to this function. Our data did not allow us to distinguish whether amino acids 34-39 constituted part or all of this signal or if these amino acids determined a critical conformation of the cytoplasmic tail that is required for the expression of a sorting signal located elsewhere in the cytoplasmic tail.

The cytoplasmic tail of the CD-MPR contains two cysteine residues which are located at positions 30 and 34. Thus Cys^{34} is part of the amino acid sequence that is necessary to prevent receptor trafficking to lysosomes while Cys^{30} is located close to this critical region. When a construct (MPR C30C34A) containing alanine residues in place of Cys^{30} and Cys^{34} was expressed in mouse L cells, the mutant receptor was found to accumulate in dense lysosomes to the same extent as a receptor with amino acids 34-39 changed to alanines (MPR 34-39A) (Rohrer et al.,

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

1995). This result indicated that Cys^{34} and perhaps Cys^{30} were involved in preventing the receptor from trafficking to lysosomes.

In the present study, we demonstrate that both Cys^{30} and Cys^{34} are reversibly palmitoylated and that the extent of palmitoylation is influenced by amino acids 35-39 of the cytoplasmic tail. A mutant receptor with Cys^{34} changed to Ala (MPR C34A) has the same phenotype as the MPR 34-39A construct whereas a receptor with Cys^{30} mutated to Ala (MPR C30A) behaves like the wild-type receptor. Further, we show that MPR C34A, MPR 34-39A and MPR 35-39A, are greatly impaired in their ability to sort cathepsin D in the Golgi, implying that mutations in this region of the cytoplasmic tail have generalized effects on receptor function.

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, FCS, and lipofectin were from GIBCO BRL (Gaithersburg, MD); Percoll from Pharmacia Diagnostics AB (Uppsala, Sweden); [^{3}H]Palmitate, Amplify, and enhanced chemiluminescence Western blotting reagents from Amersham Corp. (Arlington Heights, IL); Expre^{35}S^{35}S label from New England Nuclear (DuPont Co., Wilmington, DE); protein A–Sepharose beads from Repligen Corp. (Cambridge, MA); rabbit anti-mouse IgG from Zymed Laboratories, Inc. (San Francisco, CA); protease inhibitors, hydroxylamine, and palmitate from Sigma Chem. Co. (St. Louis, MO), nitrocellulose from Schleicher and Schuell, Inc. (Keene, NH); and cell culture dishes from Falcon Labware (Becton Dickinson 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).

The PCR procedure of Ho et al. (1989) was used to generate the MPR C30A and MPR C34A constructs with pBSK-MPR$^{TMD/tail}$ (Rohrer et al., 1995) serving as a 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 replacement 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-) (Rohrer et al., 1995) and the EcoRI–MluI fragment of pSFFVneo in a three part ligation.

The generation of all other constructs used in this study has been described previously (Rohrer et al., 1995).

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 FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% humidified CO_2 atmosphere (Gabel et al., 1983). The cells were transfected with 20 μg of XbaI-linearized DNA using lipofectin (GIBCO BRL) according to the manufacturer's directions. Selection for resistance to neomycin (G418) was as described previously (Rohrer et al., 1995). Resistant colonies were screened for expression by immunoblotting. Selected clones were expanded for further study and maintained in selective medium.

Antibodies

mAb 22D4 specific for the bovine CD-MPR has been described (Messner, 1993). For the detection of cathepsin D, rabbit anti-human cathepsin D antiserum (provided by Walter Gregory of this laboratory) (Johnson and Kornfeld, 1992; Faust et al., 1987) was used.

Metabolic Labeling with [^{3}H]Palmitate and [^{35}S]Methionine/Cysteine

Cells were grown in 60-mm dishes. For labeling with [^{3}H]palmitate, the cells were washed twice with serum-free α-MEM and labeled in 1.5 ml α-MEM containing 5% FCS, 20 mM Hepes, pH 7.4, and 600 μCi of [^{3}H]palmitate for 3 h at 37°C. For labeling with [^{35}S]methionine/cysteine, the cells were rinsed twice with PBS, preincubated in 1.5 ml of methionine- and cysteine-free growth medium containing 10% dialyzed FCS and 20 mM Hepes, pH 7.4, for 20 min, and pulsed for 3 h with 150 μCi of Expre^{35}S^{35}S protein labeling mixture in 1.5 ml of preincubation medium. For pulse chase experiments cells were labeled with [^{3}H]palmitate for 90 min at 37°C as described above, and then chased in 2 ml of normal culture medium supplemented with 20 mM Hepes, pH 7.4, and 100 μM unlabeled palmitic acid for 0–12 h. Proteins were immunoprecipitated from Triton X-100–solubilized cells as described in Rohrer et al. (1995). 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, and 0.001% bromophenol blue.

Hydroxylamine Treatment

CD-MPR protein labeled with [^{3}H]palmitate or [^{35}S]methionine/cysteine was electrophoresed in duplicate lanes on 10% SDS-polyacrylamide gels. The gels were fixed with 25% methanol, 10% acidic acid for 90 min, and subsequently rinsed in water three times for 10 min to remove the acidic acid. One gel was treated for 14 h with 1.0 M Tris, pH 7.0, as a control while the other gel was soaked for 14 h in 1.0 M hydroxylamine, pH 7.0. The gels were then rinsed in water and prepared for fluorography.

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. 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 μl of HB, and passed 12 times through a ball-bearing homogenizer (Balch and Rothman, 1985) with a clearance of 51.2 μm. The homogenate was diluted with an additional 850 μl 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 × HB and 8.5 ml of an 18% Percoll solution in 1 × 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 fractions of 1.2 ml were collected from the bottom of the tube. The gradient fractions were then combined as follows: fractions 1–3 (pool I, containing 70–80% of lysosomal enzyme activity), fractions 4–6 (pool II, containing intermediate density membranes), and fractions 7–9 (pool III, containing low density membranes including endosomes, the Golgi complex, plasma membranes, and the endoplasmic reticulum). 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 volume 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 volume of 300 μl. The samples were adjusted to 0.5% TX-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 μl of 3× nonreducing electrophoresis sample buffer was added to the remaining sample and the mixture was boiled for 3 min. Aliquots corresponding to 1:15 of the final sample volume were analyzed by SDS-PAGE and immunoblotting.

Cathepsin D Sorting Assay

Confluent cell monolayers in 6-well tissue culture dishes were labeled for 30 min at 37°C with 300 μCi of Expre^{35}S^{35}S protein labeling mixture in a total volume of 1 ml as described above, and then chased in 1 ml of normal culture medium in the presence of 10 mM unlabeled methionine and 20 mM Hepes, pH 7.4, for 4 h. At the end of the chase, the efficiency of sorting was determined by immunoprecipitating the cellular and secreted cathepsin D. The media were clarified by centrifugation at 20,000 rpm for 30 min in a JA-20 rotor. The 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 resulting pellets were resuspended in 1 ml of homogenization buffer (PBS, containing 10 mM EDTA, 0.5% Triton X-100, and a 1:500 dilution of a protease inhibitor cocktail [5 mg/ml benzamidine, and 1 μg/ml of pepstatin A, leupeptin, antipain, and chymostatin in 40% dimethylsulfoxide-60% ethanol]), passed five times through a 25-gauge needle connected to a 1-ml syringe, and then sonicated on ice for 10 s at 120 W with a probe sonicator. After incubating for 30 min on ice, the cell lysates were centrifuged for 60 min at 39,000 rpm in a Ti50 rotor. The cell and media high speed supernatants were then adjusted to 0.1 M Tris-HCl, pH 8.0, 0.1 M KCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% Na deoxycholate, 0.3% SDS, 40 μg/ml phenylmethylsulfonyl fluoride and 1× of the above described protein inhibitor cocktail. Rabbit antiserum to cathepsin D was added, and the samples were incubated overnight at 4°C with constant mixing. 25 μl of protein A–Sepharose beads was added, and the incubation continued for another 2 h at 4°C. The protein A–Sepharose beads were pelleted, washed four times with 1 ml 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 1% Triton X-100, and twice with 1 ml 0.1 M Tris-HCl, pH 8.0. Immunoprecipitates were eluted by boiling for 3 min in nonreducing SDS sample buffer as described above.

SDS-PAGE, Fluorography, and Immunoblotting

Proteins were separated on 10% SDS-polyacrylamide minigels (BioRad 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) (metabolic labeling experiments; cathepsin D sorting assays) 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 (Schnuck Markets, Inc., St. Louis, MO) in PBS. The blot was subsequently incubated with mAb 22D4 (diluted 1:500 in PBS-3% powdered milk) followed by 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 fluoro- and autoradiographs were quantitated using a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Assays and Miscellaneous Methods

β-Hexosaminidase activity was determined as described (Rohrer et al., 1995). Protein was determined with the BioRad (Richmond, CA) protein assay kit using protein standard I, or with the Micro BCA protein assay (Pierce, Rockford, IL).

Results

CD-MPR Is Reversibly Palmitoylated

Since a mutant CD-MPR containing alanines at positions 30 and 34 of the cytoplasmic tail instead of the normal cysteines accumulated in dense lysosomes, we were interested in determining whether the cysteines were palmitoylated under normal conditions. To do this, a mouse L cell line (ML4) stably expressing the wild-type bovine CD-MPR was labeled with either [^{3}H]palmitate or [^{35}S]methionine/cysteine for 3 h. The expressed CD-MPR was then immunoprecipitated and subjected to SDS-PAGE. As shown in Fig. 1, the receptor incorporated both labels. As expected for a thioester linkage (Schlessinger et al., 1980; Kaufman et al., 1984), the ^{3}H label bound to the CD-MPR was mostly released by treatment with hydroxylamine at neutral pH (lane *2*). This treatment had no effect on the amount of [^{35}S]methionine/cysteine incorporated into the protein (lane *4*). It is also apparent from Fig. 1 that there is no [^{3}H]palmitate incorporated in the "ER-form" of the CD-MPR (the lower molecular weight band in the [^{35}S]methionine/cysteine lane). This is an indication that the palmitate is added in a late-Golgi or post-Golgi compartment.

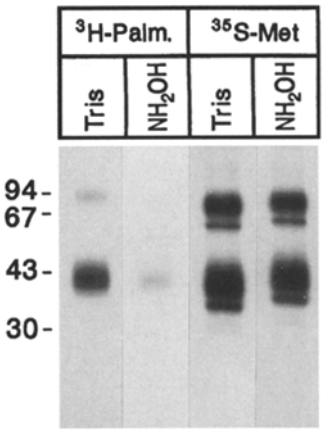

Figure 1. [^{3}H]Palmitate labeling of CD-MPR. Mouse L cells stably expressing wt CD-MPR were labeled with [^{3}H]palmitate or [^{35}S]methionine/cysteine for 3 h. Duplicate samples of CD-MPR immunoprecipitates were separated on 10% SDS polyacrylamide gels. One gel was subsequently soaked for 14 h in 1 M Tris, pH 7.0, as a control, whereas the other gel was treated for 14 h with 1 M NH_2OH, pH 7.0, followed by fluorography. The upper band at ~90 kD is the dimeric form of the receptor. The numbers at the left margin of the gel indicate the migration of molecular mass standards in kilodaltons.

When the ML4 cells were labeled with [^{3}H]palmitate for 90 min and chased for up to 12 h followed by immunoprecipitation of the CD-MPR and SDS-PAGE, it was apparent that the covalently bound [^{3}H]palmitate was rapidly turning over (Fig. 2). A plot of these data gave rise to a biphasic curve, with the initial $\tau_{1/2}$ being ~2 h followed by a second, slower decay ($\tau_{1/2}$ of ~20 h). The second phase of the curve is most likely the result of reutilization of the [^{3}H]palmitate (Magee et al., 1987; Staufenbiel, 1987). The $\tau_{1/2}$ of 2 h is much shorter than the $\tau_{1/2}$ of the protein (>40 h) as determined by [^{35}S]methionine/cysteine labeling (Fig. 2 *B*). These data establish that the CD-MPR is reversibly palmitoylated.

Cys^{30} and Cys^{34} Are the Sites of Palmitoylation in CD-MPR

Cys^{30} and Cys^{34} are the only cysteines in the 67–amino acid cytoplasmic tail of the CD-MPR and none are present in the transmembrane domain (Fig. 3 *A*). Thus, Cys^{30} and Cys^{34} were the likely candidates to undergo palmitoylation via thioester linkages (Sefton and Buss, 1987). To pursue this, three constructs were analyzed. One construct (MPR C30C34A) had both cysteines changed to alanines while the other two constructs (MPR C30A and MPR C34A) had either one or the other cysteine mutated to alanine. Cells expressing these mutant receptors were labeled with [^{3}H]palmitate and analyzed as before. As shown in Fig. 3 *B*, there was no detectable incorporation of [^{3}H]palmitate into MPR C30C34A while both MPR C30A and MPR C34A were labeled. When the [^{3}H]palmitate incorporation was expressed as a function of receptor content, as determined by quantitative Western blotting (Fig. 3 *C*), it could be calculated that MPR C30C34A, MPR C30A, and MPR C34A contained 0, 90, and 56% as much [^{3}H]palmitate as the wild-type receptor (Fig. 3 *D*).

These data demonstrate that both Cys^{30} and Cys^{34} are palmitoylated and together they account for all the palmitoylation that occurs in the CD-MPR. The loss of palmitoylation at Cys^{30} appears to be compensated by increased palmitoylation at Cys^{34}.

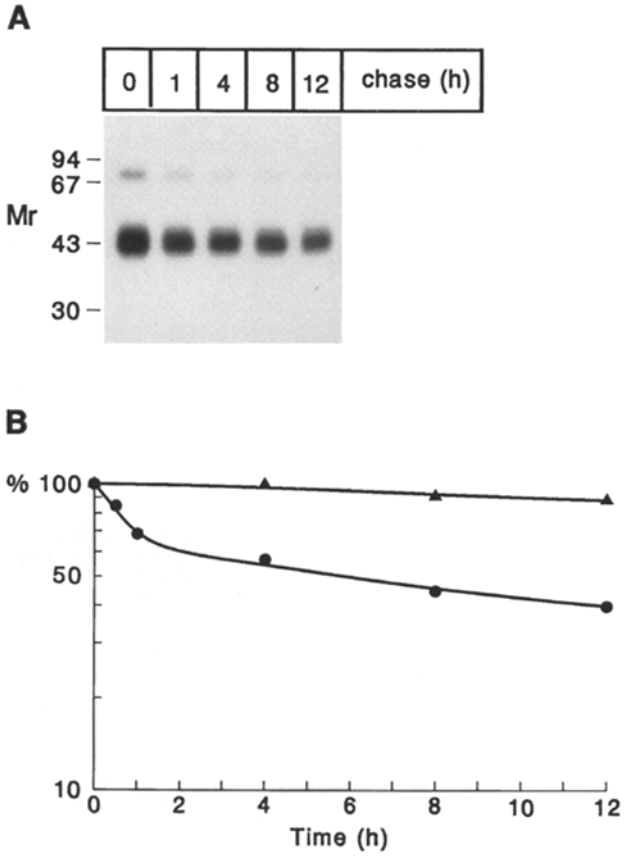

Figure 2. Kinetics of palmitate turnover on CD-MPR. (*A*) Mouse L cells stably expressing wt CD-MPR were labeled with [^{3}H]palmitate and chased for the indicated time intervals. CD-MPR was then immunoprecipitated with mAb 22D4 and analyzed by SDS-PAGE (10% gel). (*B*) The fluorograph shown in *A* and those from additional experiments were quantitiated by scanning densitometry. At each time point the amount of [^{3}H]palmitoylated receptor (●) detected is plotted as the percentage of the value obtained at the 0-h chase point. The turnover rate of [^{35}S] methionine-labeled CD-MPR (▲) is shown for comparison (Rohrer et al., 1995).

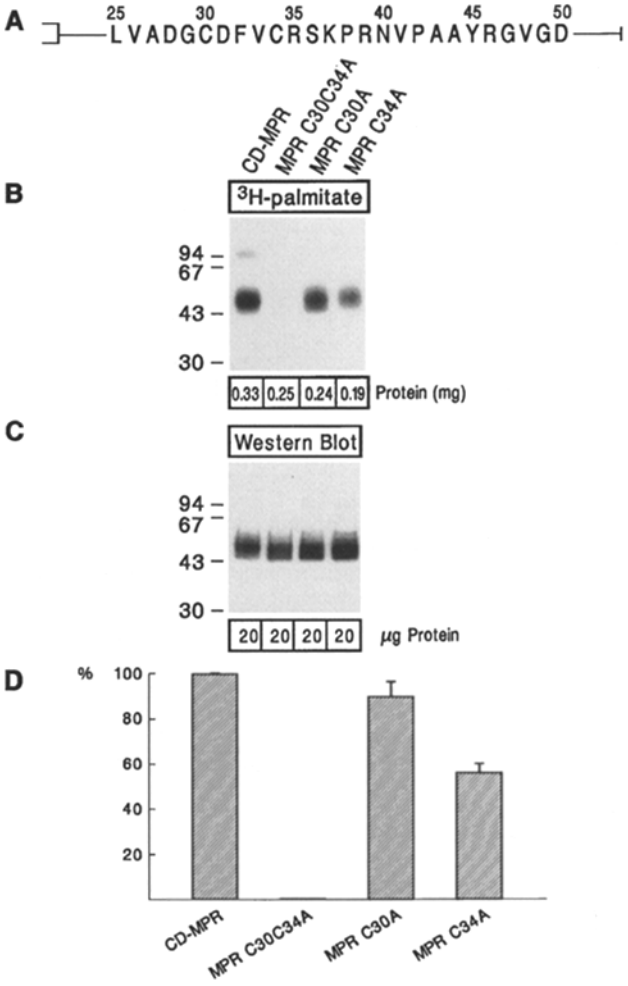

Figure 3. Cys^{30} and Cys^{34} in the cytoplasmic tail of CD-MPR are palmitoylated. (*A*) Schematic illustration of the cytoplasmic tail of CD-MPR. Selected amino acids of the tail are shown; the half box represents the terminus of the single transmembrane domain of CD-MPR. (*B*) Mouse L cells stably expressing wt CD-MPR, MPR C30C34A, MPR C30A, and MPR C34A were labeled with [^{3}H]palmitate. CD-MPR was immunoprecipitated with mAb 22D4 and analyzed by SDS-PAGE (10% gel). For each sample the total amount of protein subjected to immunoprecipitation is shown underneath the fluorograph. (*C*) Level of CD-MPR expression in the individual transfected cell lines. 20 μg of protein from the cell homogenates from duplicate plates to the ones labeled with [^{3}H]palmitate were subjected to SDS-PAGE and Western blotting with mAb 22D4. (*D*) Quantitation of [^{3}H]palmitate incorporation into CD-MPR cysteine mutants. The fluorograph and the immunoblot shown in *B* and *C*, respectively, and those from additional experiments were quantitated by densitometric scanning. In each experiment the values obtained for the [^{3}H]palmitate incorporation were corrected for the different amounts of protein used for immunoprecipitation, and for the differences in expression levels. The value obtained with the CD-MPR was set to 100%.

Effect of the Amino Acids Surrounding Cys^{30} and Cys^{34} on Palmitoylation

We next tested whether the amino acids near the palmitoylation sites influenced the extent of this modification. For this purpose, we used a series of constructs in which amino acids 28-50 of the cytoplasmic tail were substituted with stretches of alanines. The results are summarized in Fig. 4. MPR 28-33A was palmitoylated 65% as well as the wild-type CD-MPR, somewhat less than the 90% value obtained with MPR C30A. Thus the residues surrounding Cys^{30} do not influence palmitoylation in a major way. By contrast, MPR 34-39A was only palmitoylated to 10% the level of the wild-type receptor, compared to 56% for MPR C34A. This suggests that amino acid residues 35-39 of the cytoplasmic tail are required for optimal palmitoylation. Consistent with this notion was the finding that MPR 35-39A was only palmitoylated 42% as well as the wild-type receptor in spite of containing both Cys^{30} and Cys^{34}. MPR 40-45A and MPR 46-50A were palmitoylated to about the same extent as the wild-type receptor indicating that these residues do not influence palmitoylation.

MPR C34A, but Not MPR C30A, Accumulates in Dense Lysosomes

In our previous study, we demonstrated that MPR C30C34A

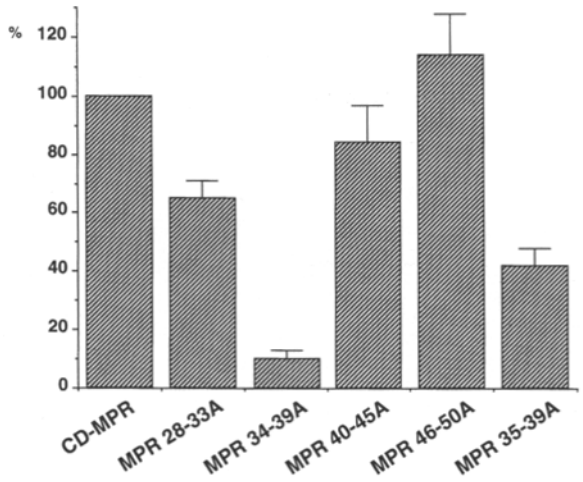

Figure 4. Determination of [^{3}H]palmitate incorporation into CD-MPR alanine scanning mutants. Mouse L cells stably expressing CD-MPR, MPR 28-33A, MPR 34-39A, MPR 40-45A, MPR 46-50A, MPR 35-39A, MPR 34-36A, and MPR 37-39A were labeled with [^{3}H]palmitate. CD-MPR was immunoprecipitated with mAb 22D4 and analyzed by SDS-PAGE. The quantitation of several experiments is shown. The values were calculated as described in Fig. 3.

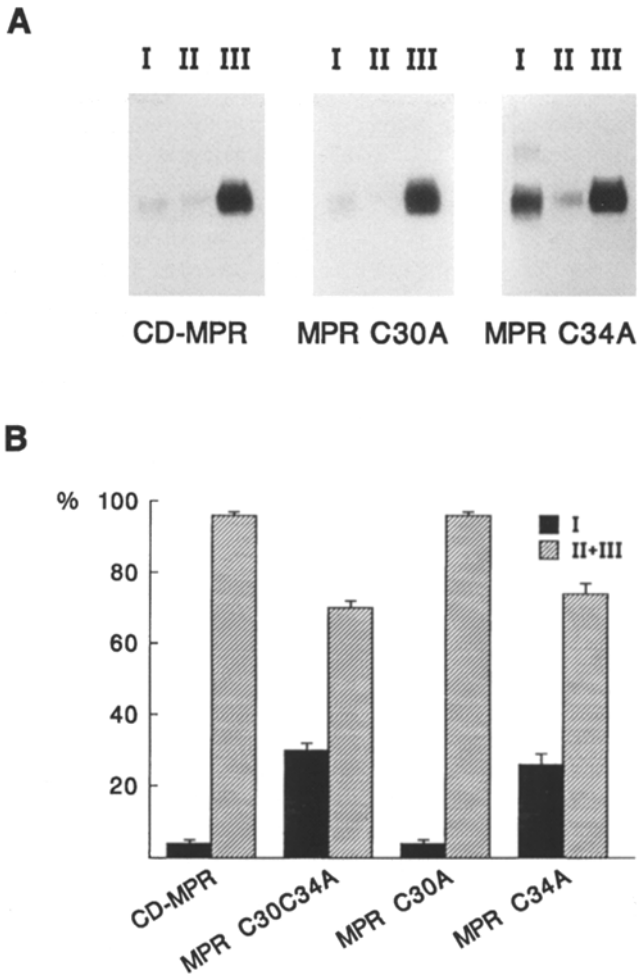

Figure 5. Subcellular distribution of MPR C30A and MPR C34A on Percoll gradients. (*A*) Mouse L cells stably expressing wt CD-MPR, MPR C30A, and MPR C34A were preincubated with pepstatin A and leupeptin for 24 h. Cells were homogenized with a ball-bearing homogenizer and postnuclear supernatants were subjected to Percoll density gradient centrifugation (18% Percoll) as described in Materials and Methods. Proteins of pool I (dense lysosomes), pool II (intermediate membranes), and pool III (low density membranes) were subjected to SDS-PAGE and immunoblotting with mAb 22D4. (*B*) Quantitation of the immunoblots shown in *A* and those from additional experiments. For each construct, the value of pool I (dense lysosomes, *filled bars*) and the values of pool II and III combined (*striated bars*) were expressed as their percentage of the total value of all three pools. The distributions of wt CD-MPR and MPR C30C34A from Rohrer et al. (1995) are shown for comparison.

accumulates in dense lysosomes whereas the wild-type receptor is excluded from that organelle (Rohrer et al., 1995). It was therefore of interest to determine whether MPR C30A and MPR C34A, which have only one cysteine mutated, retain the ability to avoid trafficking to lysosomes. For this purpose, cell lines expressing these mutant receptors were first preincubated for 24 h in the presence of pepstatin A and leupeptin in order to inhibit degradation of receptors that had entered lysosomes. The cells were then harvested, homogenized with a ball-bearing homogenizer, and subjected to Percoll density gradient centrifugation. Under these conditions, dense lysosomes are recovered at the bottom of the gradient (pool I) whereas low density membranes including endosomes, the Golgi complex, plasma membranes, and the endoplasmic reticulum are found near the top of the gradient (pool III). Intermediate density membranes are recovered in pool II (Green et al., 1987).

The distribution of the various receptors was determined by electrophoresis of the Percoll density fractions followed by Western blotting (Fig. 5, *A* and *B* for quantitation). As reported previously, the CD-MPR was almost completely excluded from dense lysosomes (4% recovered in pool I) whereas 30% of MPR C30C34A was recovered in pool I (Rohrer et al., 1995). MPR C30A behaved the same as the wild-type receptor (4% in pool I) whereas MPR C34A had a distribution similar to that of MPR C30C34A (26% in pool I). These data indicate that Cys^{34} is essential for avoiding receptor trafficking to dense lysosomes while Cys^{30} is not sufficient to prevent the receptor from entering this organelle.

Mutation of Cys^{34} of the Cytoplasmic Tail Abolishes the Cathepsin D Sorting Function

We next tested whether mutation of Cys^{30} and Cys^{34} altered the ability of the receptor to sort cathepsin D to lysosomes. This function requires the receptor to recycle to the Golgi where it must bind cathepsin D and enter Golgi clathrin-coated vesicles which transport the receptor-ligand complex to endosomal compartments. Cells expressing the various receptors were incubated with [^{35}S]methionine/cysteine for 30 min and chased for 4 h to allow the newly synthesized cathepsin D to be phosphorylated and either targeted to lysosomes or secreted. Equivalent aliquots of cell homogenates and media were immunoprecipitated and the immunoprecipitates were analyzed by

SDS-PAGE. Typical results are shown in Fig. 6. The cellular form of cathepsin D migrates faster than the secreted form because of proteolytic processing of the proform to give rise to the mature species. This processing indicates that the cathepsin D has reached the lysosomes. The results of these and additional sorting assays are summarized in Table I. The D9 cell line which expresses only endogenous mouse CD-MPR sorted 25 ± 6% of the newly synthesized cathepsin D to lysosomes ("basal level") and secreted the rest into the media. In contrast, the ML4 cell line which expresses the wild-type bovine CD-MPR, sorted 51 ± 8% of this acid protease to lysosomes whereas Cc2 cells, which express the Man-6-P/IGF-II receptor, sorted the cathepsin D very efficiently (87 ± 3%). These values are similar to those reported previously (Johnson and Kornfeld, 1992). As shown in Table I, MPR C34A and MPR C30C34A sorted cathepsin D at the basal level (29 ± 3% and 25 ± 5%, respectively) whereas MPR C30A sorted 48 ± 4% of cathepsin D, similar to the value obtained with the wild-type CD-MPR. Thus, it is apparent that mutation of Cys^{34}, but not Cys^{30}, abolishes sorting.

Table I also summarizes the results obtained with the alanine scanning mutants. Cells expressing MPR 34-39A and MPR 35-39A sorted cathepsin D at the basal level (22 ± 6% and 21 ± 6%, respectively) while MPR40-45A sorted at an intermediate level (37 ± 1%) and MPR46-50A sorted almost as well as the wild-type receptor (44 ± 4%). As shown in Table I, each of the mutant receptors that was defective in sorting cathepsin D had a steady-state level of expression comparable to that of the wild-type CD-MPR. Therefore the inability of these mutant receptors to sort cathepsin D is not due to a lack of sufficient receptor molecules. These results indicate that amino acid residues 34-39 of the cytoplasmic tail of the CD-MPR influence the lysosomal enzyme sorting function.

Discussion

The results presented in this paper demonstrate that both Cys^{30} and Cys^{34} of the cytoplasmic tail of the CD-MPR are palmitoylated in a reversible manner and that Cys^{34}, but not Cys^{30}, is required for proper trafficking and lysosomal enzyme sorting. Several aspects of the palmitoylation of the CD-MPR are of particular interest. Among the transmembrane proteins known to acquire this covalent modification, the palmitoylation sites are either localized within the transmembrane domain of the polypeptide or in the cytoplasmic tail relatively close to the transmembrane junctions. For instance, the transferrin receptor (Jing and Trowbridge, 1987, 1990) and the cell surface glycoprotein CD4 (Crise and Rose, 1992) contain palmitoylated cysteines in their transmembrane domains. CD4 has a second palmitoylation site located one amino acid from the transmembrane domain whereas this distance is two amino acids in the HLA-D–associated invariant chain (Koch and Hämmerling, 1986), six amino acids in vesicular stomatitis virus G protein (Rose et al., 1984), some subtypes of influenza virus hemagglutinin (Veit et al., 1991), and p63 (Schweizer et al., 1995), between 11 and 13 amino acids in β_2-adrenergic receptor (O'Dowd et al., 1989), bovine opsin (Karnik et al., 1993), and bovine rhodopsin (O'Brien et al., 1987; Ovchinnikov et al., 1988; Papac et al., 1992) and 15–16 amino acids in the luteinizing hormone/human choriogonadotropin receptor (Kawate and Menon, 1994). The finding of palmitoylated cysteines located 29 and 33 amino acids from the transmembrane domain of the CD-MPR expands the possibilities for this covalent modification, and has interesting implications for the structure of the cytoplasmic tail of this receptor.

Since palmitoylation has been shown to enhance membrane binding of some forms of $p21^{N\text{-}ras}$ (Hancock et al., 1989), the neuronal growth cone protein GAP (Skene and Virag, 1989; Zuber et al., 1989; Liu et al., 1993), and $G_s\alpha$ (Wedegaertner et al., 1993), it seems reasonable that palmitoylation of Cys^{30} and Cys^{34} may anchor this portion of the cytoplasmic tail of CD-MPR to the lipid bilayer. As depicted in the model shown in Fig. 7, this could have dramatic effects on the conformation of the cytoplasmic tail. For instance, one consequence would be to bring the Tyr^{45}

Table I. Sorting of Cathepsin D by L Cells Expressing Mutant CD-MRPs

Cell line	CD-MPR expression*	Cathepsin D sorted‡	
		%	
D9§		25 ± 6	(n = 8)
Cc2‖		87 ± 3	(n = 6)
ML4¶	1	51 ± 8	(n = 7)
MPR C30C34A	0.8	25 ± 5	(n = 5)
MPR C30A	1.1	48 ± 4	(n = 7)
MPR C34A	1.2	29 ± 3	(n = 8)
MPR 34–39A	1.4	22 ± 6	(n = 5)
MPR 35–39A	1.0	21 ± 6	(n = 8)
MPR 40–45A	1.1	37 ± 1	(n = 3)
MPR 46–50A	0.5	44 ± 4	(n = 3)

Cells were labeled with [^{35}S]methionine/cysteine for 30 min, chased for 4 h, and then further analyzed as described in Fig 6.
*Levels of CD-MPR expression in the individual transfected cell lines are expressed relative to the ML4 cell line. The ML4 cell line contains 0.44 ng of CD-MPR/μg of membrane protein as determined by Johnson and Kornfeld (1992).
‡Values are expressed as mean ± SE; *n* is the number of determinations.
§Parental cell line, deficient in the Man-6-P/IGF-II receptor.
‖D9 cells stably expressing wt Man-6-P/IGF-II receptor.
¶D9 cells stably expressing wt CD-MPR.

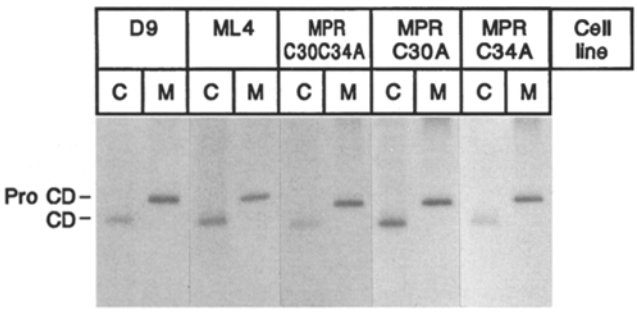

Figure 6. Sorting of Cathepsin D by L cells expressing mutant CD-MPRs. D9 cells and D9 cells stably expressing wt CD-MPR (ML4), MPR C30C34A, MPR C30A, and MPR C34A were incubated with [^{35}S]methionine/cysteine for 30 min and chased for 4 h. The amount of cathepsin D sorted was determined by immunoprecipitating cell detergent extracts (*C*) and media high speed supernatants (*M*) with antisera against cathepsin D, followed by SDS-PAGE and fluorography. The positions of the unprocessed procathepsin D (*ProCD*) and the proteolytically processed mature cathepsin D (*CD*) are indicated.

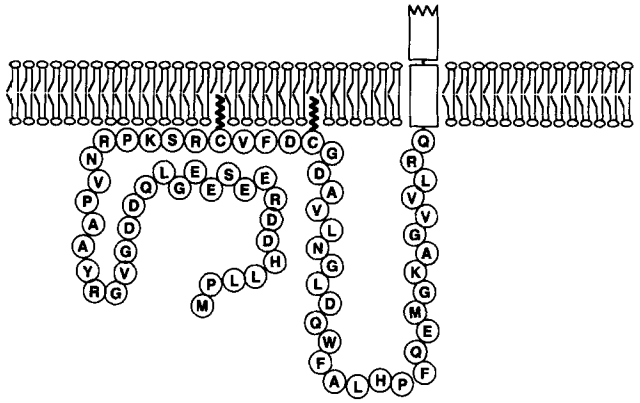

Figure 7. Model of the cytoplasmic tail of the CD-MPR. Palmitoylated Cys^{30} and Cys^{34} may be anchored to the lipid bilayer, thereby generating an intracellular loop consisting of residues 1-30 of the cytoplasmic tail. The residues on the carboxyl side of Cys^{34} would be brought closer to the membrane. The three basic residues adjacent to Cys^{34} could potentially interact with the acidic phospholipid head groups of the lipid bilayer.

containing internalization signal closer to the membrane. This signal, located 11 amino acids from Cys^{34} would now have a spacing from the membrane that is similar to that of the Phe^{13}-Phe^{18} containing internalization signal. The precise spacing of this signal from the membrane could, in turn, be an important determinant of its biologic activity. Similarly, the anchoring of the palmitoylated cysteines to the membrane could influence the presentation of the Phe-containing signal and the di-leucine signal. Three of the five residues on the carboxyl side of Cys^{34} (Arg^{35} Ser^{36} Lys^{37} Pro^{38} Arg^{39}) have positive charges and could potentially interact with the acidic phospholipid head groups of the lipid bilayer, resulting in additional conformational effects on the cytoplasmic tail. This may explain why mutation of these residues to alanines (MPR 35-39A) results in a 58% decrease in palmitoylation of Cys^{30} and Cys^{34}. The basic residues may serve to position this portion of the cytoplasmic tail in a manner that is either favorable for palmitoyltransferase to act on Cys^{30} and Cys^{34} or unfavorable for the palmitoylesterase(s) to function.

A striking finding is the fact that the palmitate is rapidly turning over in the CD-MPR, with the $\tau_{1/2}$ being on the order of 2 h whereas the protein $\tau_{1/2}$ is greater than 40 h. It is well documented that palmitoylation can be either a stable or reversible modification, although the actual $\tau_{1/2}$ for palmitate turnover has only been determined in a few instances (Omary and Trowbridge, 1981; Magee et al., 1987; Staufenbiel, 1987). Since mutation of Cys^{34} to an alanine impairs several functions of the receptor, an interesting possibility is that the reversible palmitoylation of this residue serves to modulate various signals in the cytoplasmic tail. Perhaps palmitoylation occurs at one station during the trafficking of the receptor to enhance or inhibit the activity of a particular signal while depalmitoylation occurs at another station, giving rise to the opposite effect. In this regard, it seems highly likely that the wild-type CD-MPR is incompletely palmitoylated at steady state. If palmitoylation were complete, then deletion of one of the two cysteines in the cytoplasmic tail would result in a 50% drop in palmitate incorporation rather than the 10% decrease observed with MPR C30A.

Receptor molecules carrying the Cys^{34} to Ala mutation have several altered biologic properties. One is a modification in receptor trafficking resulting in the gradual accumulation of the mutant receptor in dense lysosomes. We have suggested that the cytoplasmic tail of the receptor contains a sorting determinant that prevents delivery of the receptor to lysosomes (Rohrer et al., 1995). Cys^{34} or its palmitoylated form could be a component of this signal along with amino acids 35-39, or else 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 other alteration in the function of the receptor with the Cys^{34} to Ala mutation is the loss of ability to sort newly synthesized cathepsin D to lysosomes. This defect in the sorting function could arise in several ways. One possibility is that the mutation impairs the recycling of the receptor to the Golgi where the binding of the cathepsin D occurs. This would be consistent with the abnormal trafficking of the mutant receptor from endosomes to lysosomes. Alternatively, the mutant receptor could return to the Golgi and either fail to bind cathepsin D or not enter the Golgi clathrin-coated vesicles after binding this ligand. If the latter occurred, the CD-MPR-ligand complex would travel to the cell surface where the ligand would probably be discharged since the CD-MPR is known to bind ligands extremely poorly at the cell surface (Stein et al., 1987; Ma et al., 1991). Regardless of the particular site of the defect, it is striking that the change of this single amino acid totally abrogates this sorting function whereas mutation of Cys^{30} to Ala has no effect on either cathepsin D sorting or receptor trafficking to lysosomes.

The Man-6-P/IGF-II receptor contains a highly conserved CysCysArgArg sequence at positions 15 to 18 of its cytoplasmic tail. Westcott and Rome (1988) reported that this receptor contains covalently bound fatty acid and we have found that the receptor is palmitoylated via a thioester linkage (Schweizer, A., and J. Rohrer, unpublished data). It will be of considerable interest to determine whether palmitoylation of this receptor influences its trafficking and function in lysosomal enzyme sorting.

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