Is Movement of Mannose 6-Phosphate-specific Receptor Triggered by Binding of Lysosomal Enzymes?

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Abstract. Mannose 6-phosphate-specific receptors with an apparent molecular mass of 215,000 are present in fibroblasts at the cell surface and in intracellular membranes. The cell surface receptors mediate endocytosis of exogenous lysosomal enzymes and exchange with the intracellular receptors, which function in the sorting of endogenous lysosomal enzymes. In the present study, several methods independent of receptor ligands were designed in order to examine the exchange of receptors under conditions where receptor-ligand complexes do not dissociate (weak bases and monensin) or where receptor-ligand complexes are not formed due to absence of endogenous ligands as a result of inhibition of protein synthesis. Weak bases and monensin reduce the concentration of receptors at the cell surface by 20-30% and free cell surface receptors were replaced by occupied receptors. The latter continued to be exchanged with internal ligand-occupied receptors and the rates of the exchange were similar to the control values. The exchange of receptors between the cell surface and internal membranes was also not affected when the receptor ligands were depleted from the transport compartments by treating the cells with cycloheximide for up to 10 h. We conclude from these results that movement of mannose 6-phosphate-specific receptors along the endocytosis and sorting pathways is constitutive and not triggered by binding or dissociation of ligands.

IN many cell types targeting of lysosomal enzymes to lysosomes depends on mannose 6-phosphate-specific receptors. Two types of receptors with this specificity have been isolated to date (13). They are receptors with M_rs of 215,000 and 46,000 respectively. The smaller receptor requires divalent cations for binding. Most of the data currently available refer to the larger receptor (referred to as MPR)[1], which is known to bind newly synthesized lysosomal enzymes in the Golgi complex, to mediate their transport into a prelysosomal compartment, and to participate in endocytosis of exogenous lysosomal enzymes. Thus, MPR is present at the cell surface and in intracellular membranes. The two pools are in equilibrium, indicating that the sorting and the endocytosis pathways share at least one compartment (5, 25).

Several observations suggest that the movement of MPR is affected by occupancy with lysosomal enzymes. In cells exposed to weak bases the acid pH-dependent dissociation of lysosomal enzyme-receptor complexes is inhibited. The inability of these cells to sort endogenous and to internalize exogenous lysosomal enzymes led to the hypothesis that ligand-occupied receptors are trapped at intracellular site, where the receptors and ligands normally separate (11). In hepatocytes treated with weak bases, accumulation of receptors in structures resembling endosomes or lysosomes was reported in a morphological study; whereas, in fibroblasts deficient in mannose 6-phosphate-containing ligands, the receptors were enriched in the Golgi complex (2-4). Therefore it has been proposed that the binding of lysosomal enzymes triggers receptor movement from the binding site (Golgi complex) to the site of dissociation (endosomes/lysosomes), and that dissociation of ligands promotes the movement of free receptors back to sites of ligand binding (3, 4).

In the present study, the exchange of MPR between intracellular membranes and the cell surface was monitored in fibroblasts mostly by methods that were independent of receptor ligands. Little, if any, effect on exchange was observed when drugs that inhibit the formation or dissociation of receptor-lysosomal enzyme complexes were used. Although some of these drugs reduced the number of receptors at the cell surface.

Materials and Methods

Cells and Treatments

Normal human fibroblasts were grown in 35-mm dishes to confluency under conditions that were described previously (10). Drugs were added to cells

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1. *Abbreviations used in this paper:* MPR, mannose 6-phosphate-specific receptor, M_r 215,000; PMP-BSA, pentamannose 6-phosphate-substituted bovine serum albumin.

from stock solutions of 1 mM monensin in (Me)₂SO, 6 mM chloroquine or 1 M NH₄Cl in H₂O to final concentrations of 60 μM chloroquine, 10 mM NH₄Cl, and 10 μM monensin. Cycloheximide was used at a concentration of 0.5 mM.

Antibodies

The affinity-purified polyclonal rabbit antibodies against human liver MPR and human placental cathepsin D were those described (12, 25). The monoclonal antibody 2C2 against human liver MPR was prepared according to (8) using spleen cells from immunized BALB/c mice and X63-Ag 8.653 myeloma cells. The 2C2 antibody belonged to the IgG class and was purified on a protein A-agarose column (Pharmacia Fine Chemicals, Piscataway, NJ). Fab fragments of 2C2 were prepared by papain (16) and purified on a protein A-agarose column. Iodination with Bolton-Hunter reagent (81.4 TBq/mmol; New England Nuclear, Boston, MA) yielded Ig and Fab preparations with specific activities of 2,000–3,600 cpm/ng protein.

Pentamannose 6-phosphate–Substituted Bovine Serum Albumin (PMP-BSA)

Pentamannose 6-phosphate was prepared from phosphomannan (1), converted into the sodium salt by passage over DOWEX 50 (H⁺) (Serva, Heidelberg, FRG), and neutralized with NaOH. Coupling of pentamannose 6-phosphate (0.2 M) to bovine serum albumin (15 mg/ml) in 50 mM N,N-bis(2-hydroxyethyl)glycine, pH 9.0, and 160 mM NaCNBH₃ (21) yielded PMP-BSA with an apparent M_r of 100,000 in SDS PAGE and a carbohydrate content of 30% as determined with anthrone (24). Iodination with the aid of iodogen (Pierce Chemical Co., Rockford, IL) according to Parker et al. (18) yielded [¹²⁵I]PMP-BSA with a specific activity of 2,600 cpm/ng protein.

Binding and Uptake of Anti-MPR ¹²⁵I-Antibodies

Fibroblasts were incubated for 1 h at 0°C (placed on ice water) or at 37°C in 0.5 ml Eagle's minimum essential medium containing 7.5% fetal calf serum and 160–350 ng of the iodinated antibodies (9). After the incubation, the cells were washed six times with Hank's balanced salt solution and solubilized in 1 ml 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl, 1% NP-40, and 1% sodium deoxycholate. Cell protein was determined according to Peterson (19) with bovine serum albumin as standard. In some experiments, cell surface–associated ¹²⁵I-antibodies were dissociated from the cells by incubation for 1 h at 0°C in 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl and 0.1% pronase. All values represent the mean of duplicates and are not corrected for nonspecific binding and uptake, which was <5% as assessed in the presence of an excess of unlabeled antibodies.

Binding and Uptake of [¹²⁵I]PMP-BSA

Fibroblasts were incubated for 1 h at 0 or 37°C with 9 nM [¹²⁵I]PMP-BSA in 0.5 ml of Eagle's minimum essential medium supplemented with 7.5% fetal calf serum. For determination of cell surface–bound [¹²⁵I]PMP-BSA, cells were incubated two times at 0°C for 15 min with either Hank's balanced salt solution adjusted to pH 3.0 with 1 M citric acid or Hank's balanced salt solution containing 5 mM mannose 6-phosphate. Both procedures solubilized 85–95% of the cell surface–associated radioactivity. Unless otherwise stated all values represent the mean of duplicates. Nonspecific uptake was <5% as determined in the presence of 5 mM mannose 6-phosphate or an excess of PMP-BSA. The apparent equilibrium binding constant at 0 and 37°C and the uptake constant at 37°C for [¹²⁵I]PMP-BSA were 0.5, 3, and 7 nM, respectively. For the determination of the apparent equilibrium binding constant, maximal binding, uptake constant, and maximal uptake, the cells were incubated in the presence of 0.01–20 nM ligand and double reciprocal plots were constructed.

Determination of [³⁵S]MPR

Metabolic labeling of fibroblasts with [³⁵S]methionine (42 TBq/mmol, Amersham Corp., Arlington Heights, IL), harvesting, preparation, and solubilization of the membrane fraction, and quantitative immunoprecipitation of [³⁵S]MPR from the membrane extracts was performed as described (9, 25). Where indicated the cells were exposed after labeling and before harvesting to antireceptor Ig, drugs, and cycloheximide for the time indicated. For determination of cell surface–associated MPR, fibroblasts were

labeled for 16 h with [³⁵S]methionine, chased for 30 min at 37°C in the presence or absence of the indicated drugs, and then placed on ice water. The cells were then incubated for 90 min at 0°C with 0.6 ml medium containing 10 μl anti-MPR antiserum and the drugs. After incubation, the cells were washed five times with Hank's balanced salt solution, harvested by scraping, and suspended in 0.8 ml, 0.1 M sodium acetate, pH 6.0, containing 0.2 M NaCl and 1 mM EDTA and unlabeled fibroblasts (2 mg protein). The cells were sonicated and subjected to centrifugation for 25 min at 50,000 g. The pellet was solubilized by ultrasonication in a buffer containing 1% Triton X-100 and 0.5% sodium deoxycholate (26). After centrifugation for 6 min at 12,000 g, immune complexes in the supernatant were collected with 0.5 mg Immuno-Precipitin (Bethesda Research Laboratories, Karlsruhe, FRG) as described (26).

Other Procedures

Treatment of labeled fibroblasts with 0.1% trypsin and subsequent isolation of cell-associated [³⁵S]MPR was performed as described (26). Electrophoretic separation of immunoprecipitated [³⁵S]MPR before and after reduction in the presence of sodium dodecyl sulfate was carried out in 12.5% polyacrylamide gel according to Laemmli (15). Radioactivity in MPR was quantified by densitometry of the fluorograms. Immunoprecipitation and quantification of [³⁵S]cathepsin D was done as described (10). The activity of β-hexosaminidase was as determined previously (25).

Results

Distribution of MPR between the Cell Surface and Internal Membranes

Under saturating conditions, cultured human fibroblasts bound (at 0°C) 8.3 ng × (10⁶ cells)⁻¹ and internalized (at 37°C) 177 ng × (10⁶ cells)⁻¹ × h⁻¹ [¹²⁵I]PMP-BSA. The binding was determined in cells that were treated with mannose 6-phosphate at 0°C in order to uncover occupied receptors (see Materials and Methods). From these data we calculated that an average cell contains 19,000 binding sites at the surface and that this number of binding sites is internalized and replaced every 2.8 min. The binding sites at the cell surface correspond to a minor proportion of the total MPR. This
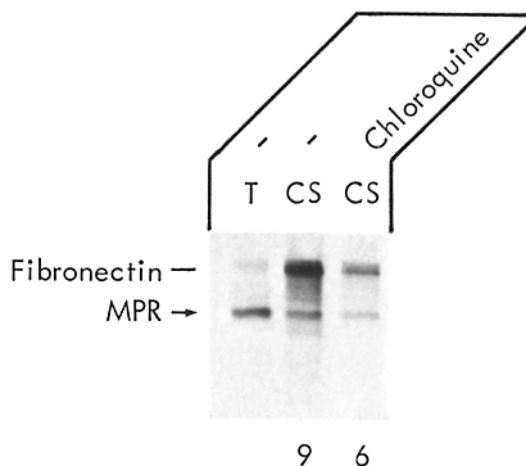

Figure 1. Cell surface–associated MPR. Fibroblasts that had been labeled for 16 h with [³⁵S]methionine were chased for 30 min in absence or presence of chloroquine. The cells were analyzed for total MPR (T) or cell surface–associated MPR (CS). Total MPR was immunoprecipitated from the extract corresponding to one-fifth of a dish. The amount of cell surface–associated MPR in percent of total MPR is given below the fluorogram. Fibronectin binds to Immuno-Precipitin and it cannot be eliminated by preadsorption in the isolation of the cell surface MPR.
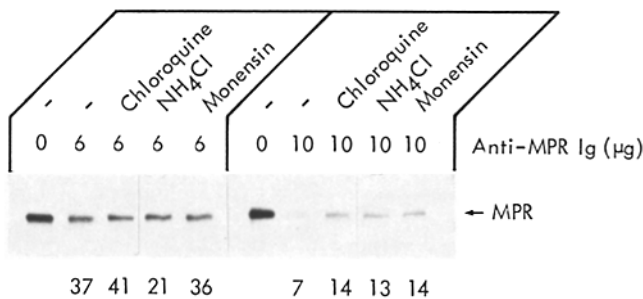
Figure 2. Recovery of [^{35}S]MPR after exposure of fibroblasts to anti-receptor Ig. Fibroblasts that had been labeled for 16 h with [^{35}S]methionine were chased for 2.5 h in the presence of chloroquine, NH$_4$Cl, or monensin. Anti-receptor Ig was added to the medium 30 min after the beginning of the chase period. The radioactivity recovered in MPR after immunoprecipitation and SDS-PAGE under reducing conditions was quantified and is given below the fluorograms as percent of MPR in cells not exposed to anti-receptor Ig.

is shown in an experiment in which, after metabolic labeling, the cell surface MPR was tagged by incubating the cells with polyclonal anti–MPR antibody at 0°C, and collected as immune complexes with *Staphylococcus* cell wall protein A in the presence of an excess of unlabeled receptor. The fraction of [^{35}S]MPR accessible to antibodies at 0°C accounted for 9% of the total [^{35}S]MPR in the cell lysate (Fig. 1).

Weak Bases and Monensin Reduce the Number of Receptors at the Cell Surface

By immunoprecipitation of [^{35}S]MPR accessible to antibodies at 0°C we could show that the cell surface MPR decreased by 35%, when the cells were incubated for 30 min at 37°C with chloroquine (Fig. 1). In a control experiment (not shown) no difference in the amount of total MPR was observed between cells that were incubated for 2 h at 37°C without or with the drug.

Weak bases and monensin also decreased the binding of ^{125}I-labeled anti–MPR Ig and Fab (Table I). After a preincubation with the drugs at 37°C, the binding was decreased by 13–29%. The binding of the antibody to the cell surface MPR was affected neither by the drugs nor by endogenous ligands (not shown). To demonstrate the latter, 5 mM mannose 6-phosphate was added to the medium to displace endogenous ligands from surface MPR. The decrease in the amount of the MPR antigen at the cell surface in the presence

of the drugs was paralleled by a similar decrease in total PMP-BSA binding sites (Table II). Our data show that fibroblasts treated with chloroquine, NH$_4$Cl, and monensin have decreased amounts of MPR on the cell surface. This change must be due to a redistribution of MPR to an intracellular pool, because these drugs affect neither the binding of PMP-BSA to MPR (not shown) nor the total amount of MPR (see above). A comparison of the decrease in the binding sites at the cell surface after incubation periods of 0.5 and 5 h (Tables I and II) indicated that most of the MPR was redistributed within the first half hour. In contrast to the slight decrease in the number of total surface binding sites, the number of free binding sites at the cell surface was decreased by incubation with chloroquine, NH$_4$Cl, and monensin for 5 h to 11–27% of control (Table II). The difference between free and total binding sites suggests that receptors occupied with ligands accumulate at the cell surface of treated cells. This could result from transport of ligand–receptor complexes from internal compartments to the cell surface or from formation of the complexes at the cell surface. The latter possibility appears to be unlikely. In spite of the increased secretion of lysosomal enzymes in the presence of weak bases, the concentration of MPR ligands in the medium is too low to account for the occupation of more than 70% of the cell surface MPR.

Table II. Effect of a Preincubation with Weak Bases and Monensin on the Apparent and Total Binding Capacity for [^{125}I]PMP-BSA

| | Binding sites (percent of control) | | | |
| | Free preincubation | | Total | |
Addition	0.5 h	5 h	0.5 h	5 h
Chloroquine	66	11	80	63
NH$_4$Cl	83	12	82	56
Monensin	80	27	93	95

Fibroblasts were incubated at 37°C for the indicated time in the absence or presence of the drugs and chilled on ice. Before the incubation with [^{125}I]PMP-BSA, the cells were subjected to two incubations for 15 min each with ice-cold Hank's buffered solution containing the drugs (mannose 6-phosphate, 5 mM, was added to the Hank's solution, when total binding sites were determined) and four washes with unsubstituted Hank's solution. The free and total binding capacities of the control cells in the experiments with the 0.5-h preincubation were 3.2 and 4.9 ng ligand per mg protein. In experiments with a 5-h preincubation the average binding capacities were 4.0 and 5.1 ng ligand per mg protein. All values represent means from two (5-h preincubation) or three (0.5-h preincubation) experiments.

Table I. Binding of Anti–Receptor ^{125}I-Ig and ^{125}I-Fab-2C2 in Cells Treated with Weak Bases and Monensin

| | Binding (percent of control) | |
	^{125}I-Ig	^{125}I-Fab-2C2
Chloroquine	71.5 ± 9.2	73.8 ± 9.8 (82)
NH$_4$Cl	75.8 ± 20.7	73.5 ± 10.3 (72)
Monensin	76.3 ± 10.0	87.5 ± 17.7 (76)

Cells were pretreated for 0.5 h at 37°C with the drugs. Controls bound 5.9 ± 2.9 ng ^{125}I-Ig and 5.1 ± 2.5 ng ^{125}I-Fab-2C2 per mg cell protein, respectively. The mean and the standard deviation of five independent experiments using two different cell lines are given. The numbers in brackets give the binding of ^{125}I-Fab-2C2 by cells pretreated for 5 h at 37°C with the drugs.

Table III. Uptake of [^{125}I]PMP-BSA in the Presence of Weak Bases and Monensin

| | Uptake (percent of control) [^{125}I]PMP-BSA* | |
| | Preincubation | |
Addition	0.5 h	5 h
Chloroquine	21	3
NH$_4$Cl	26	4
Monensin	34	12

* Cells were pretreated for 0.5 or 5 h with the drugs. Controls internalized 68.3 ng [^{125}I]PMP-BSA/mg cell protein in 1 h at 37°C.

Weak Bases and Monensin Do Not Prevent the Exchange of MPR between the Intracellular Pool and the Cell Surface

The uptake of PMP-BSA in cells treated for 0.5 or 5 h with weak bases or monensin was greatly reduced (Table III). From a comparison of Table II and Table III it is evident that the uptake of PMP-BSA was decreased more profoundly than its binding, irrespective of the length of preincubation with the drugs. The ratio of internalized versus bound ligand was 17:21 in controls and 7:13 in treated cells. This may suggest that the drugs increase the cycling time of receptors engaged in endocytosis of PMP-BSA. Alternatively, the presence of ligands during the uptake experiment (PMP-BSA was present in the medium at a concentration threefold higher than the apparent equilibrium binding constant) may accelerate the decrease of free receptors at the surface of treated cells.

Since weak bases and monensin affected the occupation of receptors with ligands, it was not possible to use ligands for measuring the exchange of surface receptors with internal receptors in treated cells. For the assessment of the exchange we used several ligand-independent approaches. In the first approach uptake of anti-MPR antibodies was measured. The second approach was based on the observation that exposure of fibroblasts to divalent anti-MPR antibodies results in formation of antibody-receptor complexes that cannot be recovered in soluble extracts and thereby mimics a loss of receptors (9). In the third approach, fibroblasts were exposed to trypsin at 37°C. Such incubation results in fragmentation of MPR, and the kinetics of the loss of receptor are related to the rate of exchange of the receptors between intracellular membranes and the cell surface (9, 25). Finally, we measured the reappearance of internalized ligand-MPR complexes at the cell surface.

Previously, to study the binding and internalization of anti-MPR antibodies, we characterized the interaction of the latter with the ligand binding site in MPR. The data presented in Table IV show that the polyclonal antibody blocked the binding of β-hexosaminidase to MPR. In contrast, the binding was not affected by the monoclonal antibody. Unlike the polyclonal antibody, the monoclonal antibody 2C2, when added to the medium of cells, inhibited neither targeting of endogenous lysosomal enzymes to lysosomes nor endocytosis of lysosomal enzymes (for experimental details and the effects of the polyclonal antibody see references 9 and

Table IV. Binding of β-Hexosaminidase to MPR-Antibody Complexes

	MPR form	Enzyme bound
		mU
I	MPR free	0.85
II	MPR-Ig (polyclonal)	0.09
III	MPR-2C2 (monoclonal)	0.84

MPR, 1 µg, was incubated overnight with buffer alone (I) or with 10 µg of polyclonal (II) or monoclonal (III) anti-receptor antibody. Then 6 mU of β-hexosaminidase were added. After 2 h at 4°C, 10 µg of monoclonal antibody (I) or buffer (II and III) were added and another 2 h later the immune complexes were collected with the aid of 3 mg Immuno-Precipitin. All incubations were performed at 4°C. The precipitates were washed twice with buffer and assayed for β-hexosaminidase activity. All values were corrected for the β-hexosaminidase activity bound in the presence of 5 mM mannose 6-phosphate. The buffer used for incubation and washing contained 10 mM sodium phosphate, pH 6.5, 0.15 M NaCl, and 0.1% Triton X-100.

Table V. Uptake of Anti-Receptor [^{125}I]-Ig and [^{125}I]Fab-2C2 in the Presence of Weak Bases and Monensin

Addition	Uptake (percent of control)	
	^{125}I-Ig	^{125}I-Fab-2C2
Chloroquine	70.0 ± 14.5	75 (66)
NH$_4$Cl	69.1 ± 15.3	72 (53)
Monensin	80.2 ± 11.3	79 (66)

Cells were pretreated for 0.5 h at 37°C with the drugs. Controls internalized 36.7 ± 13.8 ng ^{125}I-Ig and 16.2 ± 6.5 ng ^{125}I-Fab-2C2 per mg cell protein in 1 h, respectively. The mean and the standard deviation of eight independent experiments for ^{125}I-Ig and two for ^{125}I-Fab-2C2 using two different cell lines are given. The numbers in brackets indicate the uptake of ^{125}I-Fab-2C2 by cells pretreated for 5 h at 37°C with the drugs.

25). To further minimize unwanted effects of the antibodies on the receptor, such as cross-linking, Fab fragments were prepared. The following results were obtained with the polyclonal antibody and Fab fragments of the monoclonal antibody 2C2. Where examined, the same results were obtained with the Fab fragments of the polyclonal antibody and with the monoclonal antibody 2C2.

Uptake of the polyclonal anti-receptor ^{125}I-Ig and monoclonal ^{125}I-Fab-2C2 by fibroblasts was inhibited by 20-30% in the presence of either chloroquine, NH$_4$Cl, or monensin. The extent of the inhibition was the same after pretreatment for 0.5 or 5 h (Table V) and the uptake was linear with time for at least 4 h (not shown). Most of cell-associated radioactivity (80-85%) was resistant to solubilization when cells were incubated for 1 h at 0°C in the presence of 0.1% pronase, indicating an intracellular localization. It is probable that in control and in treated cells the antibodies were internalized together with MPR, and the cell surface MPR was replenished from an intracellular pool. In control cells the replenishment rate was 6.2 h^{-1} for the polyvalent antibody and 3.2 h^{-1} for the monoclonal Fab fragment. A comparison of the data given in Tables I and V showed that weak bases and monensin decreased the binding (Table I) and the uptake (Table V) of receptor antibodies to a similar extent. This indicated that the rate with which receptors at the cell surface of treated cells are replenished from internal compartments was similar to that in control cells.

For the second approach to examination of the exchange of cell surface and internal MPR in the presence of the drugs, fibroblasts were labeled for 16 h with [^{35}S]methionine and then incubated for 2 h in the presence of anti-receptor Ig. Depending on the concentration of Ig, up to 93% of the metabolically labeled MPR formed nonextractable MPR-Ig complexes. Chloroquine, NH$_4$Cl, and monensin did not affect the formation of these MPR-Ig complexes. In chloroquine-treated cells the rate of complex formation followed first order kinetics and was indistinguishable from the kinetics observed in control fibroblasts (25). These results suggested that the kinetics, with which (internal) receptors were transported to the site of complex formation with antibodies (cell surface) was not measurably affected by chloroquine, NH$_4$Cl, and monensin.

Exposure of cells to trypsin at 37°C was assumed to fragment mainly the MPR exposed at the cell surface. In cells incubated at 37°C for 1 h the fragmentation encompassed nearly 90% of the total MPR. As shown in Fig. 3, MPR was
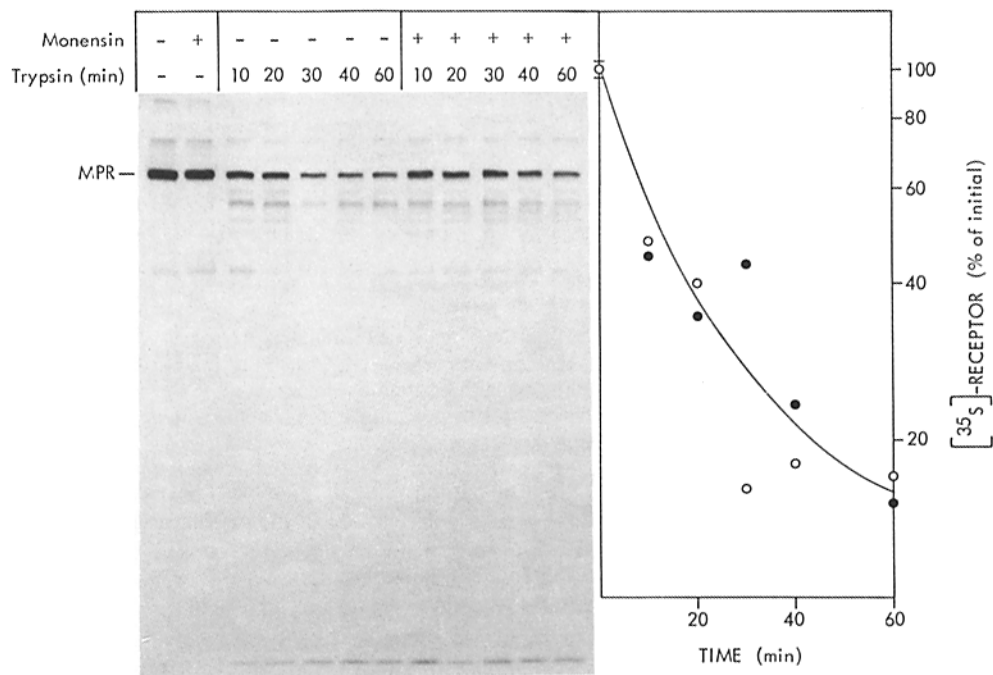
Figure 3. Fragmentation of MPR by trypsin. Fibroblasts labeled for 16 h with [^{35}S]methionine were chased for 2 h in the absence (○) or presence (●) of monensin. Fibroblasts were then incubated at 37°C for up to 1 h in the presence of 0.1% trypsin. After inactivation of trypsin, MPR was isolated (*left*) and radioactivity of the intact MPR polypeptide quantified (*right*).

not spared from degradation if monensin was included in the incubation mixture. When cells were treated for 2 h at 37°C with 10 mM NH₄Cl and then exposed for 30 min at 37°C to trypsin in the presence of NH₄Cl, 33% of MPR remained intact as compared with 25% in controls (not shown). Although we cannot exclude that trypsin becomes internalized and is active in intracellular compartments, these data support our notion that weak bases and monensin do not prevent MPR or ligand MPR complexes from moving to the cell surface.

More direct evidence for the translocation of MPR–ligand complexes from internal compartments to the cell surface in the presence of weak bases or monensin was provided by our fourth approach (Table VI). Fibroblasts were first treated for 30 min with weak bases or monensin and they were then allowed to endocytose for 1 h [^{125}I]PMP-BSA in the presence of drugs. Subsequently cell surface–bound [^{125}I]PMP-BSA was removed by an acid pH wash at 0°C (see Materials and Methods). The cells were then incubated for 40 min at 37°C in a chase medium containing the drugs. During this period controls released 4% and treated cells 14–27% of the internalized [^{125}I]PMP-BSA as TCA-insoluble material into the medium. Presence of 5 mM mannose 6-phosphate during the chase induced the secretion in controls of 8% and in treated cells of 36–59% of the cell-associated radioactivity as TCA-insoluble material. The secreted material behaved in SDS PAGE as authentic [^{125}I]PMP-BSA.

The fraction of PMP-BSA secreted in the presence of mannose 6-phosphate did not increase further in controls upon prolongation of the chase period. In cells treated with chloroquine, NH₄Cl, and monensin, between 70 and 80% of the internalized radioactivity was secreted as PMP-BSA during a chase for 3 h. This indicates that in cells treated with these drugs, more than 70% of the internalized ligand resides in compartments from which it can return to the cell surface. The effect of mannose 6-phosphate indicates that 50% or

more of the ligand returns to the cell surface as MPR–ligand complex.

Exchange of Receptors in Cells Depleted in Transport Forms of Lysosomal Enzymes

Fibroblasts were depleted of MPR ligands by inhibiting the protein synthesis with cycloheximide. The course of the depletion was determined by investigating the time necessary for conversion of the precursor of cathepsin D into the mature enzyme in cells that were subjected to a pulse labeling and a chase in the presence of cycloheximide (Fig. 4). After a 30-min pulse, most of the precursor molecules were localized proximally to the sorting organelle (9). Within 4 and 8 h chase periods the proportion of the precursor (i.e., unsorted enzyme) dropped to 22 and ≤3% in cycloheximide-treated cells (Fig. 4). Although the processing (and therefore, possi-

Table VI. Release of Internalized [^{125}I]PMP-BSA in the Absence and Presence of Mannose 6-Phosphate

Addition during uptake and chase	Internalized [^{125}I]PMP-BSA	Trichloroacetic-insoluble ^{125}I material released during chase	
		−M6P	+M6P
	cpm		
None	15,526	583	1,240
Chloroquine	3,872	526	1,681
NH₄Cl	4,284	1,175	1,536
Monensin	5,600	1,199	3,347

Fibroblasts were incubated for 0.5 h with the drugs and then for 1 h in the presence of [^{125}I]PMP-BSA and the drugs. After removal of cell surface-bound [^{125}I]PMP-BSA by exposure to pH 3.0, the cells were harvested for the determination of internalized [^{125}I]PMP-BSA or incubated for 40 min at 37°C in medium containing the drugs with or without 5 mM mannose 6-phosphate as indicated. The values are taken from one of three independent experiments yielding identical results.
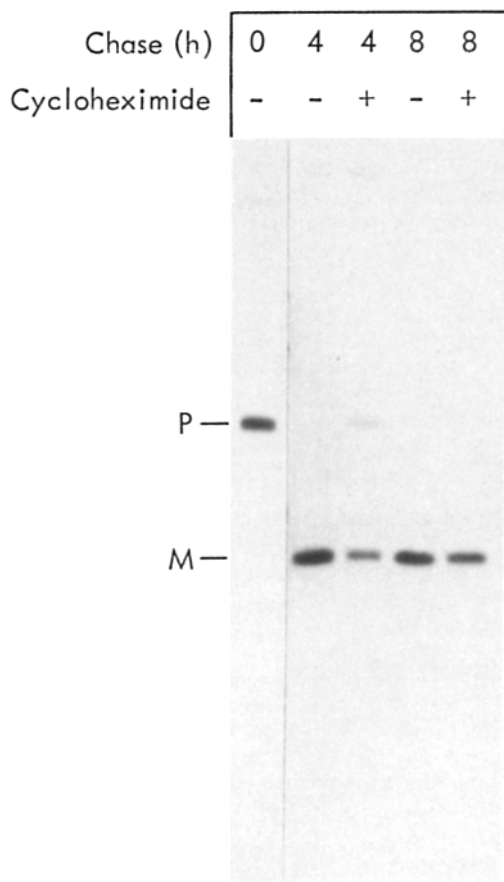
Figure 4. Proteolytic maturation of cathepsin D in the presence of cycloheximide. Fibroblasts that had been labeled for 0.5 h with [^{35}S]methionine were harvested immediately or after a chase incubation in the absence or presence of 0.5 mM cycloheximide. The precursor (*P*) and mature (*M*) forms of cathepsin D are indicated.

bly also the transport) of newly synthesized cathepsin D was slightly delayed by cycloheximide, it was clear from this experiment that treatment for 8 h with cycloheximide was sufficient to deplete the lysosomal pathway from MPR ligands. Treatment of fibroblasts for up to 8 h with cycloheximide had no effect on the binding and uptake of anti-receptor ^{125}I-Ig (Table VII). After treatment with cycloheximide, the integration of MPR into nonextractable MPR–Ig complexes was reduced from 95% in controls to 72 and 79% in treated cells (Fig. 5). Cycloheximide caused the same decrease in the integration of MPR into nonextractable MPR–Ig com-

Table VII. Binding and Uptake of Anti–Receptor ^{125}I Antibodies in Cycloheximide-treated Fibroblasts

Cycloheximide (preincubation)	^{125}I-Ig	
	Cell surface bound	Internalized
h	*ng/mg cell protein*	*ng/mg cell protein*
–	6.3	17.3
4	6.1	15.2
8	6.9	19.2

Fibroblasts were incubated for 4 or 8 h in the presence of 0.5 mM cycloheximide and then assayed for binding and uptake of anti-receptor ^{125}I-Ig in the absence or presence of cycloheximide.
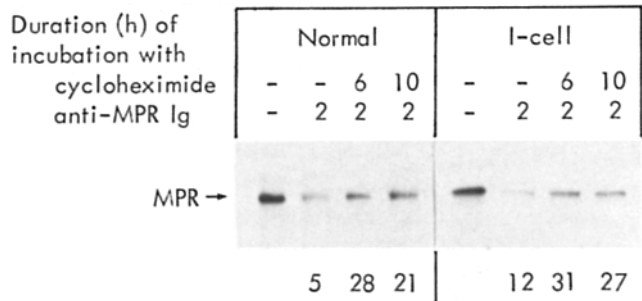

Figure 5. Effect of cycloheximide on recovery of [^{35}S]MPR in fibroblasts exposed to anti-MPR Ig. Control and I cell fibroblasts were labeled for 16 h with [^{35}S]methionine. Cells were then subjected to a chase incubation for 10 h. Cycloheximide, 0.5 mM, was added at the beginning of the chase incubation or 4 h later. At this concentration cycloheximide inhibited synthesis of trichloroacetic acid–insoluble material >97%. Anti-MPR Ig (10 µg/ml) was added 2 h before the end of the chase incubation as indicated. The amount of [^{35}S]MPR recovered in cells exposed to anti-MPR Ig is given below the fluorograms in percent of controls.

plexes in I cell fibroblasts (Fig. 5), which are deficient in ligands for MPR due to their inability to phosphorylate lysosomal enzymes. Therefore, the reduced integration of MPR into nonextractable MPR–Ig complexes in cycloheximide-treated cells was unrelated to the depletion of MPR ligands from the lysosomal pathway.

Discussion

In the present work, we have determined the distribution and movement of MPR between the surface and interior in cultured fibroblasts. Our data do not permit differentiation between the intracellular compartments of MPR. As in previous studies with other cells (7, 20) we found that in human fibroblasts 9% of MPR is present at the cell surface. From our data on binding of [^{125}I]PMP-BSA we calculated that these cells contain, on average, 19,000 binding sites at their surface and that these sites are replaced every 2.8 min. Using β-glucuronidase, Gonzalez-Noriega et al. (11) estimated that the pool of cell surface MPR is replaced every 5 min. Like others we found that the distribution of MPR is affected by weak bases. Weak bases have been reported to decrease the number of cell surface binding sites for β-glucuronidase (11). Using immunocytochemistry, Farquhar and associates (2–4) reported that in cells depleted of free receptors (due to treatment with chloroquine or NH$_4$Cl), or of ligands (due to I cell mutation or treatment with tunicamycin) MPR accumulates in endosome-like structures or near the Golgi complex, respectively. Since weak bases interfere with the acidification of intracellular compartments and prevent dissociation of MPR–ligand complexes (6, 11), it was postulated that the cycling of MPR between the binding (Golgi complex) and delivery (endosome/lysosome) sites is triggered by the binding (3) and dissociation (4, 11) of a ligand.

However, our data suggest a different interpretation of the effects seen in cells treated with weak bases or depleted from ligands. In the presence of chloroquine, NH$_4$Cl, and monensin, the uptake of antibodies recognizing the MPR was reduced by 20–30% (Table V). This reduction was similar to the decrease in number of cell surface MPR (Table I). There-

fore the rate at which the cell surface MPR exchanged with MPR from internal compartments was not measurably affected. The possibility that the internalization of MPR was induced by antibodies appears unlikely, since the relative rates of uptake of a polyclonal antibody blocking the mannose 6-phosphate–binding site in MPR and of the Fab fragment of a monoclonal antibody that did not block the binding site of MPR were similar in treated and untreated cells (Table IV). Furthermore, neither the integration of MPR into nonextractable MPR–Ig complexes during exposure of cells to antibodies recognizing the receptor (Fig. 2) nor the fragmentation of the receptor in cells exposed at 37°C to trypsin (Fig. 3) were measurably affected by the drugs. These results indicate that like the experiments on the uptake of receptor antibodies, the exchange of internal MPR with surface MPR was not affected by weak bases and monensin.

Weak bases and monensin induced at the cell surface the replacement of free receptors by occupied receptors (Table II). Therefore, our assumption of a continuous exchange of surface with internal receptors implies that MPR–ligand complexes are transported from internal compartments to the cell surface. This was supported by the release of internalized PMP-BSA into the medium, when cells were exposed to the drugs in the presence of mannose 6-phosphate (Table VI).

It was noted in the present study that, in cells exposed to weak bases and monensin, the uptake of ligands was more profoundly decreased than the binding of ligands. Under conditions where the dissociation of receptor–ligand complexes is inhibited, the conversion of free to occupied receptors will depend on the relative rates of synthesis for the receptors and ligands. If exogenous ligands are present, as during the uptake experiments, the conversion of free to occupied receptors will be accelerated. Therefore, the number of free binding sites, which is measured after treating the cells with the drugs in the absence of exogenous ligands, will decrease more slowly in treated cells than the uptake.

In addition to chloroquine and NH₄Cl, we have also studied the effect of primaquine on the transport of MPR and lysosomal enzymes. Primaquine directly inhibited the binding of PMP-BSA to MPR at the cell surface and appeared to impede the flow of membranes, rather than that of particular membrane constituents (Braulke, T., unpublished observation). This indicates that not all effects of weak bases can be ascribed to the perturbation of pH gradients. Another example of a pH-independent effect of a weak base is the decrease in the rate of dissociation of MPR–ligand complexes caused by chloroquine (22).

In cells depleted of ligands in the presence of cycloheximide, neither the uptake of ligands nor the integration of MPR into nonextractable MPR–Ig complexes was affected. Therefore, it is unlikely that trafficking of MPR is significantly altered in the absence of ligands. We may mention that in (ligand-deficient) I cell fibroblasts the amount of MPR per cell and the number of cell surface binding sites for PMP-BSA is severalfold higher than in normal fibroblasts and that the number of cell surface binding sites is hardly affected by weak bases or monensin (Braulke, T., unpublished observation).

In summary, our results on receptor exchange in cells accumulating ligand-occupied receptors and in cells that are deficient in ligands support the concept that MPR is constitu-

tively trafficking between the cell surface and the intracellular compartments, independent of the status of ligand occupancy. Since all receptors recycle to the cell surface (9, 25) this indirectly implies that movement of receptors between internal compartments is also constitutive. Moderate changes in the transport rates of MPR, rather than a block in movement, may cause the changes in the steady-state distribution of MPR between different compartments that have been observed by others (3, 4) and were noted for cell surface MPR in this study.

Several receptors have been thought to recycle in a ligand-independent manner. For example, in rat adipocytes, insulin receptor that was covalently labeled with a derivative of insulin was subject to internalization and recycling that was only partially sensitive to chloroquine or monensin (14). The transport of the IgA receptor from the basolateral to the apical surface of polarized cells does not depend on ligand binding (17). In K562 cells, monensin halved the number of cell surface receptors, enhanced the number of Golgi-associated transferrin receptors, and allowed the recycling of ferric transferrin complexes from at least one intracellular pool (23).

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