Neither Type of Mannose 6-Phosphate Receptor Is Sufficient for Targeting of Lysosomal Enzymes along Intracellular Routes

Dagmar Kasper, Frank Dittmer, Kurt von Figura, and Regina Pohlmann
Georg-August-University, Abt. Biochemie II, 37073 Göttingen, Germany

Abstract. Mouse embryonic fibroblasts that are deficient in the two mannose 6-phosphate receptors (MPRs) MPR 46 and MPR 300 missort the majority (>85%) of soluble lysosomal proteins into the medium. Human MPR 46 and MPR 300 were expressed in these cells to test whether overexpression of a single type of MPR can restore transport of lysosomal proteins to lysosomes. Only a partial correction of the missorting was observed after overexpression of MPR 46. Even at MPR 46 levels that are five times higher than the wild-type level, more than one third of the newly synthesized lysosomal proteins accumulates in the secretions. Two-fold overexpression of MPR 300 completely corrects the missorting of lysosomal enzymes. However, at least one fourth of the lysosomal enzymes are transported along a secretion-recapture pathway that is sensitive to mannose 6-phosphate in medium. In control fibroblasts that express both types of MPR, the secretion-recapture pathway is of minor importance. These results imply that neither overexpression of MPR 46 nor MPR 300 is sufficient for targeting of lysosomal proteins along intracellular routes.

Abbreviations used in this paper: MEF, mouse embryonic fibroblast; Man6P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; mpr-MEF, MPR-deficient cells; TGN, trans-Golgi network.

Address all correspondence to Regina Pohlmann, Georg-August-University, Abt. Biochemie II, Gosslerstr. 12d, 37073 Göttingen, Germany. Tel.: (49) 551 395983. Fax: (49) 551 395979.
pression of MPR 46, while it can be fully restored by expression of MPR 300. However, at least one fourth of the lysosomal proteins that are sorted to the lysosomes reach them via a pathway, which is barely used in wild-type cells. This pathway is sensitive to the presence of Man6P in the medium and is likely to involve secretion followed by MPR 300–mediated endocytosis. These results imply that neither MPR is sufficient to mediate transport of lysosomal proteins to lysosomes along intracellular routes.

Materials and Methods

Cells and Cell Culture

Mouse embryonic fibroblasts (MEF) from day 12.5 embryos were grown in DME supplemented with Glutamax-I (GIBCO BRL, Gaithersburg, MD) and 10% FCS (Pohlmann et al., 1995). The genetic background of the mice embryos used to establish MEF represents a mixture of C57BL/6J and 129/SvJ (Pohlmann et al., 1995; Köster et al., 1993; Wang et al., 1994). It should be noted that control MEF and MPR-deficient cells (mpr-MEF) are genetically heterogenous, representing different mixtures of the C57BL/6J and 129/Sv genomes.

Immortalization

MEF (3 × 10^5) on a 6-cm dish were incubated with 1 × 10^6 SV-40 t' virions (Feunteun et al., 1988) in 1 ml of medium for 1 h at 37°C. After addition of 4 ml of medium, the cells were grown overnight. The next morning the medium was changed. Cells were grown for ~10 d; foci were isolated and grown to confluency.

Transfections

2 × 10^5 immortalized MEF deficient in MPR 46 and MPR 300 (mpr-MEF; Pohlmann et al., 1995) were seeded on 6-cm dishes and grown to confluency. The human and mouse MPR 46 were quantitatively immunoisolated using antibodies raised against the human or mouse MPR 46 (von Figura, 1978). The transfected human MPR 46 was immunoisolated using antibodies raised against the human MPR 46 (Mortensen et al., 1991) as selection marker were transfected with the calcium phosphate technique (Chen and Okayama, 1987). The medium was changed. Cells were grown for ~10 d; foci were isolated and grown to confluency.

Expression Levels

For determination of the relative amounts of human MPR 46 and MPR 300, the iodinated mAbs 21D3 and 2C2 recognizing luminal epitopes of MPR 46 and MPR 300, respectively, were used (Chao et al., 1990). For determination of the surface-associated MPRs, the incubation with the mAbs was performed in the absence of saponin.

Secretion of Lysosomal Enzymes

Lysosomal acid phosphatase was measured as described using p-nitrophenylphosphate as substrate (Gieselmann et al., 1984). Other lysosomal enzymes were detected using fluorimetric assays as described (Köster et al., 1994).

Metabolic Labeling and Immunoprecipitation

MEF on 35-mm dishes were incubated in methionine-free medium for 1 h, and then labeled for 1 h (cathespin D) or 6 h (β-glucuronidase) with [35S]methionine (Amersham Buchler GmbH, Braunschweig, Germany) in the same medium containing 4% dialyzed FCS. During the following chase for 6 h (cathespin D) or 16 h (β-glucuronidase), the medium was supplemented with 0.25 mg/tel methionine. Immunoprecipitation was performed with cells and media as described (Wahed et al., 1988) with antisera specific for mouse cathespin D (Pohlmann et al., 1995) or for mouse β-glucuronidase. The antisera specific for mouse β-glucuronidase was kindly provided by R. Swank (Roswell Cancer Institute, Buffalo, NY). Radioactivity incorporated into the enzyme polyepitopes was quantified by densitometry using a Hewlett-Packard Scan Jet 4c/T (Palo Alto, CA) and the program WinCam 2.2.

Chromatography on MPR 46 and MPR 300 Affinity Columns

The media obtained after labeling were subjected to chromatography on MPR 46 and MPR 300 affinity columns as described (Pohlmann et al., 1995).

Comparison of Level of Recombinant and Wild-type MPR

To compare the level of recombinant MPR 46 with the level of MPR 46 in control MEF, Western blots of cell extracts (30 mg protein) of control MEF and mpr-/MPR 46 clone III and V were probed with an antisera against the cytoplasmic tail of MPR 46 (MSC1; Klumperman et al., 1993) and an ECL light-based immunodetection system (Amersham Buchler GmbH) were used. The films were analyzed by densitometry as described under metabolic labeling and immunoprecipitation. A comparison of the binding of [125I]21D3 and of the tail antibody indicated that binding of 13,400 cpm [125I]21D3/ng cell protein is equivalent to the level of endogenous MPR 46 in control MEF.

For MPR 300, an antisera reacting with the mouse and the human receptor at comparable affinity was not available. To correlate the levels of recombinant MPR 300 with that of endogenous MPR 300 in MEF, cells were metabolically labeled for 16 h in the presence of [35S]methionine. The human and mouse MPR 300 were quantitatively immunosolated using antiserum raised against the human (von Figura et al., 1984) and the rat (Geuze et al., 1985) MPR 300. After separation by SDS-PAGE, the radioactivity incorporated into MPR 300 was quantified by densitometry and referred to that incorporated into MPR 300 of control MEF.

Endocytosis of Arylsulfatase A

[125I]Arylsulfatase A was used as a tracer of MPR-dependent endocytosis as described (Bresciani et al., 1992).

Immunofluorescence

MEF were grown on glass coverslips for 1 d. The cells were fixed with parafomaldehyde and permeabilized with 0.5% saponin. Cathespin D was immunostained using a rabbit antisera (Pohlmann et al., 1995), lamp 1 using a monoclonal anti-mouse rat hybridoma medium (1D4B; Developmental Studies Hybridoma Bank, Iowa City, IA), mouse MPR using a rabbit antisera against the cytoplasmic MPR 46 tail (Klumperman et al., 1993), and an affinity-purified anti-rat MPR 300 antibody from rabbit (Geuze et al., 1985). The transfected human MPR 46 was immunostained with a rabbit affinity antibody directed against human MPR 46, and the human MPR 300 with a goat antisera against human MPR 300.

The primary antibodies were detected with goat anti-rabbit Texas red, donkey anti-rabbit 5-(4,6-dichlorotiazin-2-yl) amino fluorescein and goat anti-rat 5-(4,6-dichlorotiazin-2-yl) amino fluorescein (Dianova GmbH, Hamburg, Germany). After embedding in Mowiol (Calbiochem-Novabiochem GmbH), fluorescence was examined using a confocal laser scanning microscope (LSM 2: Zeiss, Oberkochen, Germany) with the filter combinations described (Schulze-Garg et al., 1993).

Results

Sorting of Lysosomal Enzymes in Immortalized MEF

To obtain MEF cell lines that stably express different levels of MPR 46 or MPR 300, MPR-deficient cells (mpr-MEF) were immortalized before transfection. The cells were immortalized by infection with an SV-40 virus deficient in...
the small t-protein (Feunteun et al., 1978). To examine the effect of immortalization on transport of lysosomal enzymes, control MEF and mpr-MEF were analyzed before and after immortalization for various parameters.

These included morphology of lysosomes and late endosomes by staining for the lysosomal membrane glycoprotein lamp 1. Furthermore, the distribution between cells and medium for lysosomal enzymes known to be transported via MPR was determined either by measuring the activity in cells and medium (β-galactosidase, β-glucuronidase, β-glucosidase, α-L-fucosidase) or in the medium for lysosomal enzymes known to be transported via MPR by staining for the lysosomal membrane glycoprotein lamp 1. The results indicated that immortalization neither affects the morphology of lysosomes and late endosomes nor the sorting of the lysosomal enzymes in control and mpr-MEF (for values of immortalized cells, see below). All further experiments were performed with immortalized MEF.

**Expression and Distribution of Transfected MPR 46 and MPR 300 in mpr-MEF**

mpr-MEF were transfected with cDNAs encoding human MPR 46 or human MPR 300. Stably expressing clones were selected, subcloned, and analyzed for the expression of MPRs by the binding of 125I-labeled mAb directed against human MPR 46 (mAb 21D3) or human MPR 300 (mAb 2C2). For further analysis, clones were selected that expressed receptor levels varying by about one order of magnitude (see Tables I and II). To compare the level of the recombinant MPR 46 with that of the endogenous mouse MPR, Western blots were probed with an antiserum against the cytoplasmic tail of MPR 46, which is identical in mouse and man. The level of recombinant MPR 46 in the clones I-V was 0.4- to 5.1-fold of that of endogenous MPR 46 (Table I).

For MPR 300, an antiserum reacting with the mouse and the human receptor at comparable affinity was not available. MEF cells were therefore metabolically labeled for 16 h with [35S]methionine, and cell lysates were prepared for quantitative immunoprecipitation of human and mouse MPR 300. Assuming that mouse and human MPR 300 have a similar stability and content of methionine, the radioactive incorporation into MPR 300 is a relative measure for its concentration. The data suggested that the level of recombinant MPR 300 in clones A-D varies between 0.2- and 2.2-fold of the endogenous MPR 300 (Table II).

The fraction of recombinant MPR accessible at the cell surface to iodinated antibodies was ~10% for MPR 46 and 10–20% for MPR 300. In general the fraction at the cell surface was higher when the expression level was lower (Tables I and II). The subcellular distribution of the receptors as visualized by indirect immunofluorescence was comparable to that of the endogenous MPR in control MEF (not shown). The effect of MPR expression on the morphology of organelles containing lamp 1, which serves as a marker for late endosomes and lysosomes, was dramatic. In mpr-MEF, the lamp 1–positive structures are grossly enlarged vesicles occupying most of the cytoplasm (Fig. 1a). After expression of MPR 46 (Fig. 1c) or MPR 300 (Fig. 1e), the number of lamp 1–positive vesicles increased and their size decreased, the smaller vesicles being preferentially located in the cell's periphery, similar to lamp 1–positive vesicles in control MEF (Fig. 1g). Furthermore, expression of MPR 46 or MPR 300 resulted in an accumulation of cathepsin D in the lamp 1–positive vesicles (see Fig. 1, right).

**Sorting of Lysosomal Enzymes in mpr-MEF Expressing Human MPR 46 or MPR 300**

To assess the efficiency of the sorting of soluble lysosomal enzymes, cells were incubated for 24 h in a defined medium, and the activity of five lysosomal enzymes known to be transported via MPR was determined in the cells and the medium (Fig. 2). In mpr-MEF, the activity recovered in the medium accounted for 85–95% of the total activity in cells and medium. With increasing levels of MPR 46 or MPR 300, the fraction of secreted enzyme decreased. MPR 300 levels 1.5- and 2.2-fold higher than those in control MEF were sufficient to reduce the fraction of secreted enzyme to values close to those in control MEF. In contrast, MPR 46 levels even five times higher than those in control MEF corrected secretion only partially by reducing the excessive secretion to about half (Fig. 2, left).

The cell-associated activity of lysosomal enzymes increased with increasing levels of MPR 46 or MPR 300. Al-

| Cell line | Bound [35S]methionine | Cell surface receptor | MPR 46 level |
|-----------|------------------------|-----------------------|--------------|
| mpr-MEF | cpm/mg* | % of total* | fold endogenous |
| clone I | 5,400 ± 750 | 29.9 ± 1.3 | 0.40 |
| clone II | 10,400 ± 1,900 | 12.9 ± 4.0 | 0.78 |
| clone III | 13,900 ± 2,100 | 12.9 ± 4.0 | 1.04 |
| clone IV | 32,100 ± 9,300 | 13.3 ± 2.8 | 2.40 |
| clone V | 68,200 ± 5,400 | 13.5 ± 5.1 | 5.09 |

*All values were corrected for the binding of [35S]methionine and represent the means of 3 to 4 independent determinations.

The amount of MPR 46 in control MEF and mpr-MEF clones III and V was determined by Western blot analysis using a tail-specific antiserum against MPR 46. Correlation of the binding of the tail-specific antiserum with that of [35S]methionine showed that binding of 13,400 cpm [35S]methionine per mg cell protein is equivalent to the endogenous level of MPR 46 in control MEF. For clones I to V, a high correlation between the binding of the polyclonal antiserum and the monoclonal 21D3 antibody was observed (r = 0.99).
Figure 1. Morphology of late endosomes/lysosomes in mpr and MPR-expressing MEF. (Left) Distribution of lamp 1, a marker of late endosomes and lysosomes. (Right) Distribution of cathepsin D, a lysosomal enzyme transported via MPR, in the same cells detected by indirect immunofluorescence. (a and b) mpr-MEF; (c and d) mpr-/MPR 46 (clone V); (e and f) mpr-/MPR 300 (clone D); (g and h) control MEF. Bar, 10 μm.
Figure 2. Distribution of lysosomal enzymes between medium and cells in MEF expressing different levels of MPR 46 or MPR 300. After an incubation for 24 h, the activities of β-hexosaminidase, β-glucuronidase, β-mannosidase, β-galactosidase, and α-L-fucosidase were measured. (Left) Activity in media as the percentage of the total activity in cells and media; (right) intracellular enzyme activity in reference to cell protein. The abscissa gives the level of MPR 46 or MPR 300 in reference to the level of endogenous MPRs in control MEF (see Tables I and II). Shown are the values for the mpr−/MPR 46 clones I, III, IV, and V (filled circles); the mpr−/MPR 300 clones B, C, and D (open circles); and mpr− MEF (filled triangles). Each value represents the mean of three to five independent determinations. (Lower right) Intracellular activity of lysosomal acid phosphatase, which is transported independently of MPR and not found in secretions. The fraction of secreted and intracellular enzyme activity in control MEF is indicated by a horizontal dashed line. SD is indicated by vertical bars, and for control MEF, by a horizontal gray bar.

though overexpression of MPR 46 corrected the missorting only partially, the level of intracellular activity reached values close to those in mpr− MEF overexpressing MPR 300, in which missorting was fully corrected (Fig. 2, right). The lack of a correlation between correction of secretion and increase of intracellular activity may be in part ascribed to prolonged half-lives of lysosomal proteins in cells such as mpr−/MPR 46 that secrete part of their lysosomal proteinases. It should be noted that the genotype of mpr− MEF expressing different levels of MPR is different from that of the control MEF (see Materials and Methods). This may explain why for some lysosomal enzymes (e.g., β-mannosidase), the intracellular activities were different in control MEF and mpr− MEF with a fully corrected secretion as in mpr−/MPR 300 clone D.

As a control, the activity of lysosomal acid phosphatase, which is targeted to lysosomes via an MPR-independent mechanism, was determined and found to be similar in transfected and nontransfected mpr− MEF and in control MEF (Fig. 2, right).

To examine the corrective effect of MPR 46 and MPR 300 on the sorting of a larger number of lysosomal proteins, secretions of metabolically labeled MEF were subjected to affinity chromatography on columns substituted with MPR 46. These secretions contain all Man6P-containing lysosomal proteins that escaped sorting to lysosomes. The fraction of polypeptides that bound to the receptor column and eluted with Man6P was quantified and characterized by SDS-PAGE (Fig. 3). About 9% of the labeled polypeptides in secretions of mpr− MEF bound in a Man6P-dependent manner to the column, representing a mixture of >15 distinct lysosomal proteins. In secretions of control MEF, ~1% of the labeled polypeptides were represented by lysosomal proteins.

Expression of increasing levels of MPR 46 or MPR 300 decreased the frequency of Man6P-containing polypeptides in secretions to 3.8% in mpr−/MPR 46 clone V and to 1.3% in mpr−/MPR 300 clone D (Fig. 3). The same results were obtained when the secretions were passed over an affinity column substituted with MPR 300 (results not shown).
To follow directly the transport of newly synthesized lysosomal enzymes, cells were metabolically labeled with [35S]methionine, and then chased in the absence (left) or presence (right) of 5 mM manno-6-phosphate. For β-glucuronidase, a pulse of 6 h and a chase of 16 h were used; for cathepsin D, a pulse of 1 h and a chase of 6 h were used. β-glucuronidase and cathepsin D were immunoprecipitated from the cells and the medium. In the cells, the 72-kD were used. 13-glucuronidase and cathepsin D were immunoprecipitated. For 13-glucuronidase, a pulse of 6 h and a chase of 16 h were used; for cathepsin D, a pulse of 1 h and a chase of 6 h were used. β-glucuronidase and cathepsin D were immunoprecipitated from the cells and the medium. In the cells, the 72-kD mature form was recovered in the secretions, the 51-kD precursor form was recovered in the secretions of MPR 300-expressing MEF, while overexpression of recombinant MPRs leads only to a minor or partial correction of the missorting.

Targeting of Lysosomal Enzymes via a Secretion–Recapture Pathway in MPR 300–expressing MEF

MPR 300 mediates internalization of lysosomal enzymes from secretions, while MPR 46 fails to mediate internalization, despite its presence at the cell surface and its recycling between cell surface and intracellular membranes (Stein et al., 1987). In agreement with these earlier observations, we observed internalization of [3H]aryl sulfatase A in MPR 300–expressing mpr−/MEF and control MEF, but not in MPR 46–expressing mpr−/MEF (not shown). It is therefore conceivable that in MEF expressing endogenous or transfected MPR 300, a fraction of the newly synthesized lysosomal enzymes that are recovered in lysosomes was first secreted, and then internalized through MPR 300–mediated endocytosis. To examine for the contribution of this secretion–recapture pathway to the sorting of newly synthesized β-glucuronidase and cathepsin D, cells were metabolically labeled and subsequently chased in the absence or presence of 5 mM Man6P. The latter should prevent reuptake of the secreted enzymes.

Presence of Man6P in the medium of control MEF increased the fraction of secreted enzyme for β-glucuronidase from 5 to 8% and for cathepsin D from 2 to 3%. In mpr−/MPR 300 clone D, however, presence of Man6P increased the fraction of secreted β-glucuronidase from 0.9 to 24%, and that of cathepsin D from 47 to 70%. In mpr−/MEF and mpr−/MPR 46 clone V, presence of Man6P did not affect the distribution of newly synthesized β-glucuronidase and cathepsin D (Fig. 4).

These data indicate that the secretion–recapture pathway is of minor importance for the targeting of newly synthesized β-glucuronidase and cathepsin D in control MEF, and in mpr−/MPR 46 cells, while in mpr−/MPR 300 clone D cells, about one fourth of β-glucuronidase and cathepsin D is transported to lysosomes via this pathway. Furthermore, presence of Man6P increased the fraction of secreted β-hexosaminidase, β-mannosidase, and α-L-fucosidase activity in mpr−/MPR 300 clone D but not in control MEF (Fig. 5). This suggests that the secretion–recapture pathway is of general importance for transport of lysosomal proteins in mpr−/MPR 300 cells. In support of this finding, presence of 5 mM Man6P increased the frequency of Man6P-containing polypeptides in the secretions of mpr−/MPR 300 clone D from 0.7 to 3.3%, while in secretions of control MEF or mpr−/MPR 46 cells, their frequency was but marginally increased (shown for control MEF and mpr−/MPR 300 in Fig. 6).

When mpr−/MPR 300 clones with different expression levels of MPR 300 were examined, it became apparent that only in the cells in which the level of recombinant MPR 300 was 1.5- and 2.2-fold higher than that of endogenous MPR 300, the secretion–recapture pathway contributed to the sorting of lysosomal proteins. In cells with lower expression levels (0.17- and 0.88-fold of endogenous
Figure 5. Effect of mannose 6-phosphate on the secretion of lysosomal enzymes. Control MEF (open bars) and mpr−/MPR 300 clone D cells (hatched bars) were incubated for 10 h in the absence or presence of 5 mM mannose 6-phosphate. Activities of three lysosomal enzymes were determined in cells and media. The activity in the secretion as percentage of total activity is given, as well as the range of values in two culture dishes processed in parallel.

Discussion

The analysis of the delivery of lysosomal enzymes to lysosomes in mpr− MEF overexpressing MPR 46 or MPR 300 clearly demonstrates that overexpression of MPR 46 cannot compensate for the loss of MPR 300, while overexpression of MPR 300 can compensate for the loss of MPR 46. However, a considerable fraction of lysosomal enzymes is delivered to lysosomes in mpr−/MPR 300 via a secretion-recapture pathway, which is barely utilized in control cells.

The inability of MPR 46 to compensate for the loss of MPR 300 was predictable from earlier studies that showed that overexpression of MPR 46 in tumor cell lines deficient in MPR 300 restored missorting of lysosomal enzymes only partially (Watanabe et al., 1991; Ma et al., 1991; Johnson and Kornfeld, 1992b). Although this would indicate that the fraction of newly synthesized lysosomal enzymes, which is secreted, did not bind to MPR 46 in the TGN, it is more likely that all ligands became bound, but dissociated from the receptor at a site from where they are secreted in part. In line with this assumption, overexpression of MPR 46 in cells that express wild-type levels of MPR 300 was found to induce the secretion of up to half of the newly synthesized lysosomal enzymes (Chao et al., 1990). The observation that MPR 46 mediates sorting of about half of newly synthesized lysosomal enzymes when overexpressed in mpr− cells (this study), while it induces secretion of about half of newly synthesized lysosomal enzymes, when overexpressed in MPR 300–containing cells (Chao et al., 1990), is best explained by the assumption that MPR 46 can bind all newly synthesized lysosomal enzymes in the TGN, but discharges ligands at a site from where they can access the medium. The dissociation may apply preferentially for low affinity ligands. Early endosomes, from where released ligands would be secreted as well as transferred to lysosomes, have been proposed as the site of dissociation (Chao et al., 1990).

In earlier studies by Lobel et al. (1989) and Johnson et al. (1992a), overexpression of bovine MPR 300 in an MPR 300−deficient clone of mouse L-cells was found to correct the sorting of cathepsin D close to normal levels. In MPR 300−deficient cells, 30−50% of newly synthesized cathepsin D was intracellularly retained. This fraction increased to 77−88% after overexpression of MPR 300, as compared with 95% in the parent L-cells expressing endogenous MPR 300. Targeting of cathepsin D was largely insensitive to the presence of Man6P in the medium (Johnson and Kornfeld, 1992a). Since the mouse L-cells contain the endogenous MPR 46, these findings are comparable to those made in this study, where sorting of newly synthesized lysosomal proteins in control MEF was found to be largely insensitive to the presence of Man6P in the medium.

The observation that the correction of missorting of lysosomal proteins in MPR-deficient MEF by overexpression of MPR 300 was in part due to transport along a route sensitive to Man6P came therefore as a surprise. It indicates that even in cells overexpressing MPR 300, part of the newly synthesized lysosomal proteins may escape binding to the receptor and become secreted (α), or that part of the MPR 300–ligand complexes is transported to a
from where they are released into the medium. The former appears to apply for MPR 300, the latter for MPR 46.

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