Re-expression of the Mannose 6-Phosphate Receptors in Receptor-deficient Fibroblasts

COMPLEMENTARY FUNCTION OF THE TWO MANNOSE 6-PHOSPHATE RECEPTORS IN LYSOSOMAL ENZYME TARGETING*

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We have previously generated primary embryonic fibroblasts lacking either the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (MPR) or the cation-dependent MPR, two transmembrane proteins that bind the mannose 6-phosphate (Man-6-P) recognition marker on soluble lysosomal enzymes (Ludwig, T., Munier-Lehmann, H., Bauer, U., Hollinshead, M., Ovitt, C., Lobel, P., and Hoflack, B. (1994) EMBO J. 13, 3430-3437). These two cell types partially missort phosphorylated lysosomal enzymes. Using two-dimensional gel electrophoresis, we show here that they secrete, in a large part, different phosphorylated ligands. In order to better understand the sorting function of the MPRs, we have re-expressed each MPR in MPR-negative fibroblasts. We show that the MPRs have complementary capacities for transporting the bulk of the newly synthesized lysosomal enzymes and that they target individual ligands with various efficiencies. However, high levels of one MPR do not fully compensate for the absence of the other, demonstrating that the two MPRs have complementary targeting functions, perhaps by recognizing different features on lysosomal enzymes.

The analysis of the phosphorylated oligosaccharides shows that the ligands missorted in the absence of the cation-dependent MPR are slightly but significantly depleted in oligosaccharides with two Man-6-P residues, when compared with those missorted in the absence of the cation-independent MPR. While these results could explain some differences between the structure and the sorting function of the two MPRs, they strongly suggest that the reason why cells express two different but related MPRs is to maintain an efficient Man-6-P-dependent targeting process that could be potentially regulated by MPR expression.

In higher eukaryotes, transport of soluble lysosomal enzymes to lysosomes relies on the recognition of the common mannose 6-phosphate (Man-6-P) recognition marker present on their N-linked oligosaccharides by specific receptors (Kornfeld and Melman, 1989). The key step in the synthesis of the Man-6-P signal is catalyzed by the lysosomal enzyme, N-acetylglucosamine-1-phosphotransferase, which recognizes the lysosomal enzymes among other glycoproteins and transfers one or two N-acetylglucosamine-1-phosphate units onto selected mannose residues of their multiple high mannose oligosaccharides. A N-acetylglucosaminidase by removing the outer N-acetylglucosamine uncoverts most of the Man-6-P residues, thereby allowing their interaction with the mannose 6-phosphate receptors (MPRs). Two MPRs that bind the phosphorylated oligosaccharides of the newly synthesized lysosomal enzymes have been extensively characterized (Kornfeld, 1992; Ludwig et al., 1995). The first is the cation-independent Man-6-P receptor (CI-MPR), a ~300-kDa transmembrane glycoprotein that also binds the insulin-like growth factor II, a polypeptide involved in development (De Chiara et al., 1990). Its extracytoplasmic domain is made of fifteen homologous repeating units of 150 amino acids (Lobel et al., 1988; Oshima et al., 1988). The third and the ninth repeats provide two mannose 6-phosphate binding sites (Dahms et al., 1993; Westlund et al., 1991), whereas the eleventh repeat binds IGF II (Dahms et al., 1994). The second receptor is the cation-dependent Man-6-P receptor (CD-MPR). The extracytoplasmic domain of this ~46-kDa transmembrane glycoprotein is homologous to each of the fifteen repeats found in the extracytoplasmic domain of the other MPR (Dahms et al., 1987) and contains one Man-6-P binding site (Tong and Kornfeld, 1989). The detergent-solubilized CD-MPR is a homodimer (Stein et al., 1987a), perhaps forming tetramers in Golgi membranes (Dahms and Kornfeld, 1989; Waheed and Van Figura, 1990).

Both MPRs are essential components of the Man-6-P-dependent targeting system that transports the newly synthesized lysosomal enzymes to lysosomes. The demonstration comes from gene disruption experiments in mice. Animals with a disrupted CD-MPR gene (Köster et al., 1993; Ludwig et al., 1993) or a disrupted CI-MPR gene (Wang et al., 1994) are partially impaired in intracellular lysosomal enzyme sorting. As for the human genetic disorders associated with a mistargeting of lysosomal enzymes (Nolan and Sly, 1989; Neufeld, 1991), the phenotype is better detected in primary cultures of cells. In primary fibroblasts derived from such mouse embryos, this partial missorting of lysosomal enzymes leads to the accumulation of undigested material in the late endocytic compartments of these cells (Ludwig et al., 1993, 1994). Furthermore, primary fibroblasts from embryos lacking both MPRs are almost totally impaired in intracellular lysosomal enzyme sorting and, as a consequence, accumulate undigested material in their endosomes/lysosomes (Ludwig et al., 1994). This phenotype, similar to that of I-cell fibroblasts characterized by a lack

*PArt of this research was supported by the association “Vaincre les maladies lysosomales,” by Grants BIO2-CT93-02205 and HCM ERBC-CHRXCT940592 from the European Communities (to B. H.), by National Institutes of Health Grant RO1-DK45992 (to P. L.), and by NATO. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Recipient of a European Molecular Biology Organization fellowship.

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of synthesis of the Man-6-P signal on lysosomal enzymes, clearly illustrates the importance of the two MPRs as major components of the Man-6-P-dependent targeting system in fibroblasts.

The MPRs are distributed over several intracellular compartments, the trans-Golgi network, the plasma membrane and the endosomes, where they are predominantly found at steady state (for review see Kornfeld and Mellman (1989)). In the trans-Golgi network, they divert the newly synthesized lysosomal enzymes from the secretory pathway to the endocytic pathway where they unload their bound ligands. The MPRs are also present at the plasma membrane where they undergo endocytosis. Although the CD-MPR does not bind ligand at the cell surface under physiological conditions (Stein et al., 1987b), the CI-MPR mediates the internalization of phosphorylated ligands and more importantly that of the extracellular IGF II. Its function in IGF II binding is probably to control the level of circulating IGF II (for review see Ludwig et al. (1995)). The lethality associated with the T-maternal effect as seen in Thp cellsurface under physiological conditions (Stein et al.). Although the CD-MPR does not bind ligand at the pathway where the yunload their bound ligands. The MPRs are lysosomal enzymes from the secretory pathway to the endocytic steady state (for review see Kornfeld and Mellman (1989)). In compartments, the trans-Golgi network, the plasma membrane fibroblasts.

Collectively, these results demonstrate that the two distinct but related MPRs are required to efficiently transport the bulk of the lysosomal enzymes.

EXPERIMENTAL PROCEDURES

Materials—Bovine liver acetone powder, glucose 6-phosphate, and mannose 6-phosphate were purchased from Sigma. Affi-gel 15 was from Bio-Rad, and ampholines were from Pharmacia Biotech Inc. Hygromycin B was purchased from Boehringer Mannheim. The ECL detection kit was from Amersham Corp. L-[35S]Methionine (1175 Ci/mmol) was from DuPont NEN. Media and reagents for cell culture were purchased from Life Technologies, Inc. and Sigma.

Cells—The wild type and MPR-negative mouse embryonic fibroblasts and mouse embryonic fibroblasts heterozygous or homozygous for a CD-MPR-disrupted gene were obtained as described previously (Ludwig et al., 1994). Wild type or MPR-negative embryonic fibroblasts were immortalized by subsequent culture using a 3T3 schedule or by transfection with cDNAs encoding the large and middle T antigen of SV40. All cellswere maintained in Dulbecco’s modified Eagle’s medium containing one-tenth of the normal methionine concentration of the 3T3 Hepes (pH 7) to 10% dialyzed fetal calf serum and then chased for 4 h. Cells were scraped, centrifuged, and lysed in detergent. Catepsin D was immunoprecipitated from both cell’s extracts and the corresponding culture media with a rabbit polyclonal anti-bovine catepsin D antibody. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. The secreted catespin D (52-kDa precursor form) and the intracellular catespin D (48-kDa mature form) were then quantitated using a PhosphorImager. Alternately, the catespin D isoforms were analyzed by two-dimen- sional gels as described below.

Purification of Secreted Lysosomal Enzymes—Cells were labeled overnight with [35S]Methionine (0.2 mc/ml) in Dulbecco’s modified Eagle’s medium containing one-tenth of the normal methionine concentration of the 3T3 Hepes (pH 7) to 10% dialyzed fetal calf serum and then chased for 3-h chase, the medium was collected. The secreted lysosomal enzymes were purified on affinity columns containing a mixture of two MPRs as described previously (Ludwig et al., 1994). The amount of secreted lysosomal enzymes was normalized according to the total amount of secreted proteins. The different cell types secreted similar amounts of proteins.

Two-dimensional Gel Electrophoresis—[35S]Methionine-labeled lysosomal enzymes were separated by two-dimensional PAGE essentially as described previously (Bravo, 1984). For isoelectric focusing, samples were solubilized in 9.8 mM urea, 4% (w/v) Nonidet P-40, 2% (v/v) ampho- lhelines (pH 7–9), and 100 mM diethiothreitol. Tube gels used for the first dimension were 25 cm long and had an internal diameter of 2.5 mm. The gels were run at 1,200 V for 17 h. For the second dimension, 8.5% SDS-PAGE was used. Labeled proteins were visualized by autoradiography. For [35S]Methionine-labeled secreted precursor of catespin D, samples were solubilized in the same solution described above. Isoelec- tric focusing gels were run at 750 V for 3 h and 30 min. For the second dimension, 10% SDS-PAGE was used.

Isolation of Phosphorylated Oligosaccharides from Lysosomal Enzymes—Confluent cells were incubated in glucose/ bicarbonate-free Dulbecco’s modified Eagle’s medium containing 20% Hepes (pH 7). 10% dialyzed fetal calf serum and 2 mM/l of O-(2-H) Mannose. After a 2.5 h pulse at 37 °C, the glucose concentration was adjusted to 4.5 g/l and the cells were chased at 37 °C for 6 h. The secreted lysosomal enzymes were purified on MPR affinity columns and their phosphorylated oligo- saccharides were purified as described (Chen et al., 1983; Varki and Kornfeld, 1983; Hoffack et al., 1987). Briefly, the lysosomal enzymes were concentrated by precipitation and incubated overnight at 56 °C in 1 ml of 100 mM Tris/HCl (pH 8), 50 mM N-acetylgalactosamine, 50 mM sodium β-glycerophosphate, 20 mM CaCl2 and 1 mg pre-digested Pronase. The Pronase digestes were boiled for 10 min, diluted with 3 ml of 10% Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 0.02% azide (TBS) and clarified by centrifugation. The supernatants were applied to 1-ml columns of concanavalin A equilibrated in TBS, and the columns were eluted with 3 × 1 ml of TBS, 6 × 1 ml of 10 mM α-methylglucoside in TBS, and 8 × 1 ml of 100 mM α-methylmannoside in TBS preheated at 56 °C. The glycopolypeptides eluted with α-methyl- mannoside were concentrated by lyophilization, desalted on a Sephadex G-25, and incubated overnight at 37 °C with 2.5 milliuunits of endo-β-N-acetylglucosaminidase H in 200 μl of 0.1 mM citrate phosphate (pH 5.5) under a toluene atmosphere. The resulting oligosaccharides were diluted with 2 ml of 2 molar pyridium acetate (pH 5.3) and applied to a 1 × 11 cm QAE-Sephadex column equilibrated in 2 molar pyridium acetate (pH 5.3). The oligosaccharides were eluted with a linear gradient (2 × 100 ml) of pyridium acetate (from 2 to 500 molar, pH 5.3). 1.8-ml fractions were collected and monitored for radioactivity. The identity of each species was confirmed as described (Varki and Kornfeld, 1983).
RESULTS

Analysis of the Lysosomal Enzymes Secreted by MPR-deficient Fibroblasts—Our previous studies on mouse primary embryonic fibroblasts deficient in one or two MPRs have strongly suggested that a few lysosomal enzymes are preferentially secreted in the absence of one MPR, whereas some others were preferentially secreted in the absence of the other (Ludwig et al., 1994). To extend this analysis, the hydrolases secreted by these primary fibroblasts were first analyzed by two-dimensional gel electrophoresis, which takes into account both the molecular weight and the charge of the analyzed material. The different primary fibroblasts were labeled with [35S]methionine and then chased. The secreted lysosomal enzymes were purified on MPR affinity columns and analyzed by two-dimensional PAGE. Fig. 1 shows typical patterns of phosphorylated lysosomal enzymes secreted by these different cell types. More than 50 different Man-6-P containing proteins could be purified from the medium of fibroblasts lacking both MPRs (Fig. 1C). The fibroblasts lacking the CI-MPR secreted some of these ligands (Fig. 1A), whereas some others were recovered in the material secreted by fibroblasts lacking the CD-MPR (Fig. 1B). Thus, fibroblasts lacking either the CD-MPR or the CI-MPR predominantly secrete different phosphorylated proteins. Interestingly, several phosphorylated proteins were only found secreted by cells lacking the two MPRs but not by those lacking only one MPR. These results suggest that three different subgroups of Man-6-P containing proteins can be distinguished according to their in vivo interactions with the MPRs. The first contains those that are preferentially secreted in the absence of the CD-MPR and therefore do not interact well with the remaining CI-MPR. The second contains those that are preferentially secreted in the absence of the CI-MPR and therefore do not interact well with the remaining CD-MPR. Finally, the third group contains Man-6-P proteins that are only secreted in the absence of the two MPRs and therefore might interact equally well with either MPR.

Sorting of Phosphorylated Ligands—In order to investigate the sorting of the lysosomal enzymes as a function of MPR expression, MPR-negative fibroblasts were immortalized, and each MPR was stably re-expressed in these cells. Several clones expressing various amounts of either MPR were selected, and their capacity to carry out efficient transport of the bulk of the newly synthesized lysosomal enzymes was determined. For this, the cells were labeled with [35S]methionine and chased. The lysosomal enzymes secreted into the medium were purified on MPR affinity columns and quantitated (Fig. 2A). As for the

FIG. 1. Analysis by two-dimensional gel electrophoresis of the phosphorylated ligands secreted by MPR-deficient fibroblasts. Primary fibroblasts lacking the CI-MPR (A) or the CD-MPR (B) or both MPRs (C) were pulsed with labeled methionine and then chased, and the secreted lysosomal enzymes were purified on MPR affinity columns as described under “Experimental Procedures.” The material specifically eluted with Man-6-P was fractionated by two-dimensional gel electrophoresis (50,000 cpm for each sample) and detected by autoradiography. (The amount of secreted lysosomal enzymes normalized to 10 µg trichloroacetic acid precipitable cpm of total secreted proteins: wild type, 5,000 cpm; CD-MPR , 17,000 cpm; CI-MPR , 21,000 cpm; CD-MPR /CI-MPR , 45,000 cpm). In A and B, the arrows and the arrowheads represent the different cathepsin D isoforms detected after immunoprecipitation and two-dimensional gels. The arrows point the cathepsin D isoforms that are preferentially enriched in the secretion of primary cells lacking the CI-MPR. D is a schematic drawing of the three different populations of phosphorylated ligands detected in this analysis. Open circles, phosphorylated ligands preferentially secreted by cells lacking the CD-MPR; gray circles, phosphorylated ligands preferentially secreted by fibroblasts lacking the CI-MPR; closed circles, phosphorylated ligands only secreted by MPR-negative fibroblasts.
Complementary Targeting Functions of the MPRs

The corresponding primary fibroblasts (Ludwig et al., 1994) secrete a rather low level of MPR (as judged by Northern blotting analysis and immunoprecipitation experiments) and that do or do not express physiological levels of the CI-MPR (Fig. 2B). These primary cells secreted lysosomal enzymes in an almost identical fashion to the corresponding primary fibroblasts from embryos homozygous for a functional CD-MPR gene (100% of expression). Therefore, it also appears that even in primary fibroblasts, 50% of the normal level of CD-MPR are almost sufficient for a full CD-MPR function.

For this particular lysosomal enzyme, high levels of CI-MPR expression (four to five times the physiological level) were found in CD-MPR-negative cells containing the corresponding primary fibroblasts (Ludwig et al., 1994). The cells were labeled with [35S]methionine and chased as described above for the stable cell lines. The secreted lysosomal enzymes were purified on MPR affinity columns and quantitated. The percentage of secretion was calculated considering that complete mis-sorting occurs in mock-transfected MPR-negative fibroblasts (~42,000 cpm of secreted lysosomal enzymes/10^6 cpm of trichloroacetic acid precipitable cpm of total secreted proteins). A level of expression of 1 refers to the expression of the CD-MPR or the CI-MPR in immortalized wild type fibroblasts. The values are expressed as means ± S.E. of two or three independent determinations.

B, the primary cultures of fibroblasts were generated from mouse embryos with various genotypes obtained by a series of crossings between CD-MPR negative and Thp mice as described previously (Ludwig et al., 1994). The cells were labeled with [35S]methionine and chased as described above for the stable cell lines. The secreted lysosomal enzymes were purified on MPR affinity columns and quantitated. The double MPR-negative primary cells secreted ~45,000 cpm of phosphorylated ligands/10^6 cpm of trichloroacetic acid precipitable cpm of total secreted proteins, and the wild type fibroblasts secreted ~60,000 cpm of phosphorylated ligands/10^6 cpm of trichloroacetic acid precipitable cpm of total secreted proteins.

FIG. 2. Secretion of phosphorylated ligands by MPR-negative cells re-expressing the CD- or the CI-MPR. A, immortalized wild type fibroblasts taken as positive control (●) and stable clones re-expressing various amounts of the CI-MPR (○) or the CD-MPR (●) were labeled with [35S]methionine and chased. The secreted lysosomal enzymes were purified on MPR affinity columns and quantitated. The percentage of secretion was calculated considering that complete mis-sorting occurs in mock-transfected MPR-negative fibroblasts (~42,000 cpm of secreted lysosomal enzymes/10^6 cpm of trichloroacetic acid precipitable cpm of total secreted proteins). A level of expression of 1 refers to the expression of the CD-MPR or the CI-MPR in immortalized wild type fibroblasts. The values are expressed as means ± S.E. of two or three independent determinations. B, the primary cultures of fibroblasts were generated from mouse embryos with various genotypes obtained by a series of crossings between CD-MPR negative and Thp mice as described previously (Ludwig et al., 1994). The cells were labeled with [35S]methionine and chased as described above for the stable cell lines. The secreted lysosomal enzymes were purified on MPR affinity columns and quantitated. The double MPR-negative primary cells secreted ~45,000 cpm of phosphorylated ligands/10^6 cpm of trichloroacetic acid precipitable cpm of total secreted proteins, and the wild type fibroblasts secreted ~60,000 cpm of phosphorylated ligands/10^6 cpm of trichloroacetic acid precipitable cpm of total secreted proteins.

Corresponding primary fibroblasts (Ludwig et al., 1994), the immortalized MPR-negative fibroblasts secrete most of their newly synthesized lysosomal enzymes (~75%) when compared with immortalized fibroblasts expressing physiological levels of the two MPRs (~6.5%) taken as controls. Fig. 2A first shows that expression of the CD-MPR or the CI-MPR in MPR-negative cells corrects the secretion of lysosomal enzymes in a similar manner, provided that similar levels of expression were compared. Thus, both MPRs exhibit the same capacity for transporting the bulk of the lysosomal enzymes in vivo. However, one MPR cannot fully compensate for the lost of the other. Cells expressing two to three times more than the physiological level of one MPR still secreted some lysosomal enzymes when compared with control fibroblasts expressing physiological levels of the two MPRs. Thus, even high levels of expression of one MPR are not sufficient to allow the complete transport of the bulk of the newly synthesized lysosomal enzymes. Therefore, the two MPRs are complementary in function for efficient transport of the bulk of the newly synthesized lysosomal enzymes.

Second, Fig. 2A shows that rather low levels of MPR expression (~50% of the physiological level) can readily mediate transport of ~50% of the bulk of the newly synthesized lysosomal enzymes. For example, cells expressing physiological levels of the CI-MPR or approximately half of this level secrete nearly similar amounts (~50%) of their newly synthesized lysosomal enzymes, suggesting that low expression levels can fulfill its in vivo targeting function. The same observation could be made with primary fibroblasts from embryos heterozygous for a disrupted CD-MPR gene that express ~50% of the physiological level of this receptor (as judged by Northern blotting analysis and immunoprecipitation experiments) and that do or do not express physiological levels of the CI-MPR (Fig. 2B). These primary cells secreted lysosomal enzymes in an almost identical fashion to the corresponding primary fibroblasts from embryos homozygous for a functional CD-MPR gene (100% of expression). Therefore, it also appears that even in primary fibroblasts, 50% of the normal level of CD-MPR are almost sufficient for a full CD-MPR function.

Finally, the re-expression of the MPRs in MPR-negative fibroblasts supports the notion that each MPR recognizes lysosomal enzymes. In vivo two different pools of phosphorylated lysosomal enzymes (Fig. 2A). The first pool, representing ~50% of the bulk of the newly synthesized lysosomal enzymes, requires rather low levels of MPR expression for efficient transport to lysosomes. In contrast, the second pool of lysosomal enzymes requires rather higher levels of MPR expression for a partial transport to lysosomes, suggesting that it binds with a lower affinity to the MPRs. This interpretation could be consistent with the analysis of the lysosomal enzymes secreted by MPR-deficient fibroblasts (Fig. 1).

Transport of Individual Lysosomal Enzymes—Cells expressing different levels of each MPR were also tested for their content of lysosomal enzyme activities as a reflection of their intracellular transport. Classical lysosomal enzyme assays performed on these cells show that cells expressing high levels of CI-MPR or CD-MPR contain less β-hexosaminidase (Fig. 3A) or β-galactosidase (Fig. 3B) activities than control cells expressing the two MPRs. This result also indicates that one MPR is not sufficient for a complete transport of these individual lysosomal enzymes. In addition, cells expressing similar amount of CI-MPR or CD-MPR contained at steady state the same amount of β-hexosaminidase and β-galactosidase, suggesting that these particular lysosomal enzymes are transported by each MPR with the same efficiency. However, cells expressing each MPR at a low level of MPR (~0.5-fold the physiological level) contain more β-galactosidase than β-hexosaminidase suggesting that in vivo, β-galactosidase interacts better with each MPR than β-hexosaminidase. The cells re-expressing different levels of either CD-MPR or CI-MPR were finally tested for their ability to sort cathepsin D using pulse-chase experiments followed by immunoprecipitation. Fig. 4 shows that fibroblasts expressing physiological levels of the CI-MPR sort cathepsin D almost as efficiently as fibroblasts expressing the two MPRs. For this particular lysosomal enzyme, high levels of CI-MPR expression (four to five times the physiological level) were required for a complete sorting. In contrast, a large proportion of this lysosomal enzyme (~60%) was still secreted by fibroblasts expressing high levels of CD-MPR (two to three times the physiological level). The same differences in cathepsin D sort-
ing were obtained when mannose 6-phosphate was added to the culture medium during the pulse-chase experiments to prevent re-uptake mechanisms (not shown). These results show that in transfected fibroblasts, cathepsin D is more efficiently sorted by the CI-MPR than by the CD-MPR and support our previous observations using primary fibroblasts lacking the CI-or the CD-MPR (Ludwig et al., 1994). The cathepsin D secreted by fibroblasts expressing physiological levels of the CD-MPR or the CI-MPR was immunoprecipitated and subjected to two-dimensional gel electrophoresis. Fig. 5 shows that seven cathepsin D isoforms are secreted by MPR-negative fibroblasts. However, some cathepsin D isoforms were preferentially secreted by fibroblasts re-expressing the CD-MPR, whereas some others were preferentially secreted by fibroblasts re-expressing the CI-MPR. Thus, the two MPRs appear to interact with and to transport cathepsin D isoforms.

Fibroblasts Re-expressing One MPR Are Impaired in Lysosomal Degradation—We have previously observed (Ludwig et al., 1994) that primary fibroblasts lacking the two MPRs store undigested material and contain numerous late endocytic structures, whereas primary fibroblasts lacking one MPR or the other exhibited a milder phenotype. The endocytic apparatus of MPR-negative fibroblasts re-expressing physiological levels of either the CD-MPR or the CI-MPR was examined by fluorescence microscopy after antibody labeling for Lamp-1, a trans-membrane protein enriched in late endosomes and lysosomes. As shown in Fig. 6C, MPR-negative fibroblasts, as the corresponding primary cells (Ludwig et al., 1994), also contain numerous, enlarged late endocytic structures that fill the cytoplasm. Immortalized fibroblasts from embryos expressing the two MPRs (Fig. 6A) or double-transfected MPR-negative fibroblasts expressing both the CD-MPR (physiological level of expression) and the CI-MPR (4-fold the physiological level of expression) (not shown) contain only a few small, dispersed late endocytic structures. However, transfected fibroblasts expressing physiological levels of either the CD-MPR (Fig. 6B) or the CI-MPR (Fig. 6D) exhibited an intermediate phenotype and contained a significant number of enlarged endocytic structures although less abundant than in MPR-negative fibroblasts. These results show that the re-expression of physiological levels of one MPR allowing transport of 50% of the bulk of lysosomal enzymes is not sufficient to restore a normal endocytic apparatus fully functioning in intralysosomal degradation. Thus, the two MPRs must be present to restore the wild type phenotype.

Characterization of the Phosphorylated Oligosaccharides Present on Lysosomal Enzymes Secreted by MPR-deficient Fibroblasts—The results presented above suggest that mannose...
6-phosphate containing ligands that bind to one MPR differ from those binding to the other MPR. Lysosomal enzyme precursors are heterogeneous molecules, usually bearing multiple N-linked high mannose oligosaccharides, each containing one or two uncovered mannose 6-phosphate groups (Kornfeld and Mellman, 1989). Therefore, it is conceivable that the ligands showing preferred affinities for the MPRs exhibit different phosphorylation states. To test this hypothesis, MPR-negative fibroblasts stably re-expressing either the CD-MPR or the CI-MPR, as well as primary embryonic fibroblasts expressing only the CD-MPR or only the CI-MPR, were labeled with [3H]mannose. After a chase period, the lysosomal enzymes secreted into the medium were purified on MPRs affinity columns. Their oligosaccharides were released, fractionated on concanavalin A affinity columns to select the high mannose species. These latter, representing ~80% of the sugar chains in each case, were fractionated according to their phosphorylation state by ion exchange chromatography. Fig. 7 shows typical elution profiles of the phosphorylated oligosaccharides present on the lysosomal enzymes secreted by mock-transfected MPR-negative fibroblasts (Fig. 7C) or re-expressing either the CD-MPR (Fig. 7A) or the CI-MPR (Fig. 7B). Similar profiles were obtained for the primary cells (not shown). Several major species were detected: the neutral high mannose oligosaccharides (peak I), the high mannose oligosaccharides with one phosphomonoester (peak II), hybrid structures with one phosphomonoester and one sialic acid residue (peak III), and finally high mannose oligosaccharides with two phosphomonoesters (peak IV).

The oligosaccharides containing one and two phosphomonoesters were quantitated (Table I) because they are the only species interacting with the MPRs (Fischer et al., 1982; Varki and Kornfeld, 1983; Hoflack et al., 1987). The lysosomal enzymes secreted by both primary and transfected cells expressing the CD-MPR but lacking the CI-MPR contained equal amounts of oligosaccharides with one or two phosphomonoesters. However, the lysosomal enzymes secreted by both the primary and the transfected fibroblasts expressing the CI-MPR but lacking the CD-MPR contained significantly more oligosaccharides with one phosphomonoester (~65%) than oligosaccharides with two phosphomonoesters (~35%). Thus, the lysosomal enzyme isoforms interacting with the CI-MPR and secreted in its absence are slightly but significantly enriched in oligosaccharides with two phosphomonoesters when compared with those interacting with the CD-MPR and secreted in its absence. The latter are poorer in the structures with two phosphomonoesters and contain significantly more oligosaccharides with one phosphomonoester.

**DISCUSSION**

Although established cell lines naturally devoid of the CI-MPR (Gabel et al., 1983) have been instrumental for studies on this MPR, the availability of fibroblasts devoid of the two MPRs (Ludwig et al., 1994) gave us the unique opportunity to study their sorting function independently. We have immortalized MPR-negative fibroblasts and stably re-expressed either the CI-MPR or the CD-MPR back into these cells. We show here that in vivo, the MPRs have similar capacities for transporting...
Complementary Targeting Functions of the MPRs

Each MPR exhibits a high or a low affinity for incoming phosphorylated ligands, possibly due to differences in the phosphorylation of their oligosaccharides.

Efficient Lysosomal Enzyme Sorting Requires Both MPRs—In vivo studies on the sorting function of the MPRs have been limited thus far by the lack of cell systems devoid of the two MPRs. It has been proposed that in established cell lines expressing the two MPRs, the CI-MPR is mostly responsible for the intracellular targeting of lysosomal enzymes to lysosomes, whereas the CD-MPR plays only a minor role (Stein et al., 1987b). Over-expression of the CD-MPR in various cell systems have indicated that the CD-MPR could function in intracellular transport of lysosomal enzymes (Watanabe et al., 1990; Ma et al., 1991; Johnson and Kornfeld, 1992) or in their secretion (Chao et al., 1990). Our previous studies on mice or primary cells lacking CD-MPR expression (Ludwig et al., 1993), as well as those from Köster et al. (1993) have illustrated the functional importance of the CD-MPR in intracellular transport of lysosomal enzymes. Our present studies on the re-expression of this MPR in MPR-negative cells fully support our previous conclusions. The re-expression of the CD-MPR in MPR-negative cells that partially corrects the hypersecretion phenotype of these cells shows the critical role of this receptor in intracellular transport of lysosomal enzymes. It is also clear that the CD-MPR and CI-MPR exhibit in vivo similar capacities for transporting the bulk of the newly synthesized lysosomal enzymes to lysosomes at every level of expression examined. However, the most striking finding from both the biochemical and morphological data is that even rather high levels of expression of one MPR cannot fully compensate for the loss of the other. Thus, efficient transport of the bulk of the lysosomal enzymes requires the two MPRs.

The MPRs Bind Lysosomal Enzymes with Various Affinities—In vivo, the interactions of lysosomal enzymes with the two MPRs appear rather complex. The intracellular content in β-galactosidase and β-hexosaminidase activities of cells expressing various levels of each MPR suggests that these two lysosomal enzymes are transported by the MPRs with different efficiencies. Cathepsin D appears to interact better with the CI-MPR than with the CD-MPR. In a more general manner, each MPR appears to recognize in vivo two distinct pools of phosphorylated ligands; one requires physiological or lower levels of MPR expression for efficient transport to lysosomes, whereas the other, probably interacting with a lower affinity, requires higher levels of MPR expression for only a partial targeting. If some lysosomal enzymes bind to the two MPRs with the same efficiency, the analysis of the phosphorylated ligands or cathepsin D isoforms secreted by fibroblasts expressing one MPR or the other strongly suggest that the ligands binding with high affinity to the CD-MPR are in a large part, different from those binding with high affinity to the CI-MPR.

How could the MPRs interact differently with lysosomal enzymes? The lysosomal enzyme precursors are heterogeneous and usually contain multiple N-linked high mannose oligosaccharides with zero, one, or two phosphonomoesters (Cantor et al., 1992) as well as complex type sugars. Only one or a few of them mediate the interactions of the lysosomal enzymes with the MPRs. The analysis of the phosphorylated oligosaccharides present on lysosomal enzymes secreted by cells expressing one MPR or the other indicates that the ligands secreted in the absence of the CI-MPR are slightly but significantly enriched in high mannose oligosaccharides with two phosphonomoesters, whereas the ligands secreted in the absence of the CD-MPR contain more oligosaccharides with one phosphonomoester. Our analysis scores for every oligosaccharides present on lysosomal enzymes including those that do not interact with the

Fig. 7. Phosphorylated oligosaccharides on lysosomal enzymes secreted by MPR-deficient fibroblasts. The different cell types (primary and transfected fibroblasts) were labeled with [3H]manose and then chased, and the secreted lysosomal enzymes were purified on MPR affinity columns. The material specifically eluted with Man-6-P was treated as indicated under “Experimental Procedures,” and ~10,000 cpm of the resulting high mannose oligosaccharides were fractionated by ion exchange chromatography. Typical elution profiles obtained with the transfected cells are shown (the amount of secreted lysosomal enzymes secreted by MPR-deficient fibroblasts.

The bulk of the newly synthesized lysosomal enzymes to lysosomes and that one MPR cannot completely fulfill the targeting function of the two MPRs. Thus, this study unambiguously demonstrates the functional complementarity of the two MPRs in the targeting process, an aspect that has been difficult to address thus far. This study strongly supports the notion that
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TABLE I

Phosphorylated species present on lysosomal enzymes secreted by MPR-deficient fibroblasts

The oligosaccharides from lysosomal enzymes secreted by the different MPR-deficient cells were prepared as described under "Experimental Procedures." The high mannose oligosaccharides (~10–20,000 cpm for each sample) were fractionated by ion exchange chromatography as in Fig. 5. The species containing one or two phosphomonoesters were quantitated. The indicated values represent means ± S.E. of two or three independent determinations.

| Phosphorylated species | Phenotype |
|------------------------|-----------|
|                        | Primary fibroblasts | Transfected fibroblasts |
|                        | +CI-MPR, -CD-MPR | -CI-MPR, +CD-MPR | -CI-MPR, +CD-MPR | -CI-MPR, +CD-MPR |
| High mannose           | 30.7 ± 4.2 | 26.8 ± 2.4 | 32.8 | 23.5 ± 2.1 | 29.2 ± 4.5 | 35.1 ± 2.9 |
| Hybrid                 | 29.7 ± 3.6 | 26.5 ± 4.1 | 24.9 | 41.5 ± 2.1 | 20.7 ± 7.5 | 20.0 ± 4.3 |
| Total                  | 60.4 ± 0.8 | 53.3 ± 1.7 | 57.7 | 65.0 ± 0.5 | 49.9 ± 2.9 | 55.1 ± 1.3 |
| Two phosphomonoesters  | 39.6 ± 0.8 | 46.7 ± 1.7 | 42.3 | 35.0 ± 0.5 | 50.1 ± 2.9 | 44.9 ± 1.3 |

MPRs. For example, some of the phosphorylated oligosaccharides could interact with determinants on the protein backbone of lysosomal enzymes and not be directly available for binding to one MPR as described previously for the interaction of procathepsin L with the CI-MPR (Lazzarino and Gabel, 1990). Thus, the differences in the charged oligosaccharides that we have observed could be consistent with the simple interpretation that in vivo, the CI-MPR would preferentially bind lysosomal enzymes enriched in oligosaccharides with two phosphomonoesters, whereas the CD-MPR would preferentially bind lysosomal enzymes depleted in these phosphorylated species, possibly through interactions with multiple oligosaccharides containing one phosphomonoester.

However, it cannot be excluded at present that some other factors such as the number of oligosaccharides present and their degree of branching or the position of the phosphate groups could also be important for the selectivity of interaction with a given MPR. It is important to note that the level of MPR expression varies greatly according to the cell type or the tissue considered (Matzner et al., 1992). Whether the expression of the phosphotransferase also varies is at present unknown. Therefore, it is possible that these preferential interactions of some lysosomal enzymes with one MPR, as detected here in this system, may well be different in other tissues.

Putative Implications of the Preferential Interactions of Lysosomal Enzymes with the MPRs—The observation that cells lacking one MPR secrete a fraction of their lysosomal enzymes has usually been interpreted as indicating that the MPRs are saturated by the incoming ligands in the trans-Golgi network. However, our results showing that high expression levels of one MPR do not compensate for the loss of the other would rather argue that the secretion of cells lacking physiological levels of one MPR is due to a lack of binding of the phosphorylated ligands to the remaining MPR. Thus, it is very likely that the MPRs are not saturated by incoming ligands in the trans-Golgi network.

If lysosomal enzymes function in intralysosomal degradation in most cell types, it is also apparent that they can be secreted in a regulated fashion. A few cell types, such as macrophages recruited to tissue wounds or osteoclasts involved in bone formation/resorption, have specialized for this lysosomal enzyme secretion. Some transformed cells also secrete massive amounts of one particular lysosomal enzyme. For example, NIH 3T3 cells transformed with the Kirsten virus secrete cathepsin L precursor as a major excreted protein (Gal and Gottesman, 1986). Breast cancer cells treated with estrogens also secrete a major protein (Rochefort et al., 1987) identified as the phosphorylated cathepsin D precursor, which can be efficiently endocytosed by the CI-MPR of control cells (Capony et al., 1987). Although it is clear that the cathepsin D or cathepsin L genes are up-regulated, the secretion of these lysosomal enzymes might be explained in several ways. The secretion of one particular enzyme could first be due to the saturation of the phosphotransferase or the N-acetylgalactosaminase, which generates the Man-6-P signal. This would lead to a decrease in the phosphorylation state of the oligosaccharides of the lysosomal enzymes. Indeed, it has been proposed that the cathepsin L secreted by the virus-transformed NIH 3T3 cells is not well recognized by the CI-MPR due to the lower phosphorylation state of its oligosaccharides (Dong and Sahagian, 1990). Alternatively, the phosphorylation process could remain unaffected, but the MPRs could become saturated in the TGN by the incoming enzymes. However, our data strongly suggest that the MPRs are probably not saturated. In any case, a saturation of the Man-6-P signal synthesizing enzymes or of the MPRs should result in a general mis-sorting of the bulk of the newly synthesized lysosomal enzymes. This possibility has not been completely examined with transformed cells. Our results could lead to an alternative explanation that resides in the specificity of the MPRs for different phosphorylated ligands. A down-regulation in the expression of one MPR would most likely result in the secretion of the subset of phosphorylated lysosomal enzymes that bind to this MPR with high affinity and do not interact well with the remaining MPR. The lysosomal enzymes that bind to the remaining MPR could still be normally transported to lysosomes. Interestingly, breast cancer cells treated with oestrogens, which secrete high amounts of cathepsin D, also down-regulate the expression of the CI-MPR (Mathieu et al., 1991). The expression of the CD-MPR gene may also be regulated by use of different promoters and/or alternative splicing (Ludwig et al., 1992). Therefore, it is possible that MPR expression regulates the secretion of phosphorylated ligands.

Acknowledgments—We thank Drs. G. Griffiths, A. Alconada, and R. Le Borgne for critical reading of the manuscript.

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Re-expression of the Mannose 6-Phosphate Receptors in Receptor-deficient Fibroblasts: COMPLEMENTARY FUNCTION OF THE TWO MANNOSE 6-PHOSPHATE RECEPTORS IN LYSOSOMAL ENZYME TARGETING
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J. Biol. Chem. 1996, 271:15166-15174.
doi: 10.1074/jbc.271.25.15166

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