Differential sorting of lysosomal enzymes in mannose 6-phosphate receptor-deficient fibroblasts

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In higher eukaryotes, the transport of soluble lysosomal enzymes involves the recognition of their mannose 6-phosphate signal by two receptors: the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (CI-MPR) and the cation-dependent mannose 6-phosphate receptor (CD-MPR). It is not known why these two different proteins are present in most cell types. To investigate their relative function in lysosomal enzyme targeting, we created cell lines that lack either or both MPRs. This was accomplished by mating CD-MPR-deficient mice with T^{hp} mice that carry a CI-MPR deleted allele. Fibroblasts prepared from embryos that lack the two receptors exhibit a massive missorting of multiple lysosomal enzymes and accumulate undigested material in their endocytic compartments. Fibroblasts that lack the CI-MPR, like those lacking the CD-MPR, exhibit a milder phenotype and are only partially impaired in sorting. This demonstrates that both receptors are required for efficient intracellular targeting of lysosomal enzymes. More importantly, comparison of the phosphorylated proteins secreted by the different cell types indicates that the two receptors may interact *in vivo* with different subgroups of hydrolases. This observation may provide a rational explanation for the existence of two distinct mannose 6-phosphate binding proteins in mammalian cells.

Key words: lysosomal enzymes/mannose 6-phosphate receptors/T-associated maternal effect

Introduction

In mammalian cells, two distinct proteins are able to interact with the mannose 6-phosphate recognition marker on soluble lysosomal enzymes (for reviews, see Kornfeld and Mellman, 1989; von Figura, 1991; Kornfeld, 1992; Hoflack and Lobel, 1993). The first protein is the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (CI-MPR). Its biological function is first to divert soluble lysosomal enzymes from the secretory pathway for efficient delivery to lysosomes and, second, to endocytose extracellular phosphorylated ligands and IGF II. Beside this receptor, there is a second mannose 6-phosphate binding protein, referred to here as the cation-dependent mannose 6-phosphate receptor (CD-MPR). Although the CD-MPR is endocytosed, it fails to bind ligand at the cell surface. Gene disruption experiments in mice demonstrate, however, that this protein functions in intracellular transport of hydrolases to lysosomes. Thus, CD-MPR-negative fibroblasts missort multiple lysosomal enzymes (Köster *et al.*, 1993; Ludwig *et al.*, 1993) and, as a consequence, accumulate undigested substrates in their lysosomes (Ludwig *et al.*, 1993). A similar phenotype is observed in the human diseases mucolipidoses II and III, characterized by an inefficient synthesis of the mannose 6-phosphate recognition marker on soluble lysosomal enzymes (Neufeld and McKusik, 1983; Nolan and Sly, 1989; Neufeld, 1991, and references therein). Thus far, it has remained unclear why high eukaryotic cells express two different mannose 6-phosphate binding proteins. If the two MPRs are both required for intracellular transport of newly synthesized lysosomal enzymes to lysosomes, do they carry out specific functions in this process or are they redundant proteins?

In mice, the CI-MPR gene (or *Igf 2r* gene) is imprinted and exclusively expressed from the maternally inherited chromosome. The evidence comes from studies showing that the CI-MPR gene is closely linked to the T-associated maternal effect (*Tme*) locus (Barlow *et al.*, 1991). The *Tme* phenotype is characterized by the death of embryos at day 15 of gestation due to the inheritance of the maternal T^{hp} (or T^{lub2}) deleted chromosome 17 (Johnson, 1974). Thus, the description of the exclusive maternal expression of CI-MPR gene in mice and the availability of viable, fertile mice homozygous for a disrupted, non-functional CD-MPR gene (Köster *et al.* 1993; Ludwig *et al.*, 1993) made it possible to prepare cells that do not express either or both of the MPRs. This was achieved through a series of matings of the CD-MPR-deficient and T^{hp} mice. To answer questions concerning the precise role of the MPRs in lysosomal enzyme transport, we performed a detailed analysis of these matched cells. Cells lacking the CI-MPR hypersecrete multiple lysosomal enzymes, store undigested material in lysosomes and therefore resemble those lacking the CD-MPR. Cells lacking both MPRs resemble mucolipidosis II fibroblasts in that they are almost completely impaired in targeting of multiple lysosomal enzymes and accumulate undigested substrate in their lysosomes. This demonstrates that the two MPRs are required in concert for efficient intracellular targeting of lysosomal enzymes. More importantly, analysis of the hydrolases secreted by cells lacking the CI-MPR or the

CD-MPR indicates that the two receptors may interact *in vivo* with distinct but overlapping subsets of phosphorylated ligands. Thus, the differences in binding specificity may provide an explanation for the requirement of two distinct but related mannose 6-phosphate binding proteins to transport soluble hydrolases to lysosomes in mammalian cells.

Results

### Genotypes of the embryonic fibroblasts used in this study

CD-MPR ($-/-$) females were first crossed with T^{hp} ($+/-$) males in order to produce CD-MPR ($+/-$) T^{hp} ($+/-$) females. These latter were then crossed to CD-MPR ($-/-$) and wild-type males. At day 13.5 of gestation, the mice were killed and the embryos were used to establish primary cultures of fibroblasts. The genotype of these cells was determined by Southern blotting analysis using a CD-MPR genomic fragment as a probe and by immunoprecipitation using a specific anti-CI-MPR antibody. Among these different cells, two independent cultures of wild-type fibroblasts, fibroblasts lacking either CD-MPR or CI-MPR expression, and fibroblasts lacking both MPRs were selected for subsequent studies (Figure 1).

Fibroblasts lacking CI-MPR or both MPRs missort their lysosomal enzymes

The fibroblasts lacking either the CI-MPR or both MPRs were first tested for their ability to transport lysosomal enzymes to lysosomes. The different cells were pulsed with [^{35}S]methionine, chased and the labeled phosphorylated ligands secreted in the culture medium were purified on MPR affinity columns and quantitated. On average, CI-MPR-negative fibroblasts secreted about five times more phosphorylated ligands than control fibroblasts, but about half as much as fibroblasts lacking the two MPRs (Figure 2A). As shown in our previous study (Ludwig *et al.*, 1993), the matched fibroblasts lacking the CD-MPR also missorted their newly synthesized lysosomal enzymes (~3–4 times more than control fibroblasts, not shown). Conversely, the CI-MPR-negative and the CI-MPR-, CD-MPR-double-negative fibroblasts contained less intracellular lysosomal enzyme activities than the matched control fibroblasts. Lysosomal enzyme assays for three different glycosidases (β-hexosaminidase, β-galactosidase, β-glucuronidase) revealed that CI-MPR-negative and CI-MPR-, CD-MPR-double-negative fibroblasts contained ~50% and 20% of the normal level of intracellular enzyme activities, respectively (Figure 2B). From these results, we draw two conclusions: (i) fibroblasts lacking CI-MPR expression missort a significant fraction of their newly synthesized lysosomal enzymes; (ii) fibroblasts lacking the two MPRs missort their hydrolases almost completely, indicating that in embryonic fibroblasts the two MPRs are responsible for most of the intracellular transport of soluble lysosomal enzymes.

Fibroblasts lacking the CI-MPR or both MPRs are impaired in lysosomal function

We showed earlier that the lack of the CD-MPR causes the accumulation of undigested material inside the late
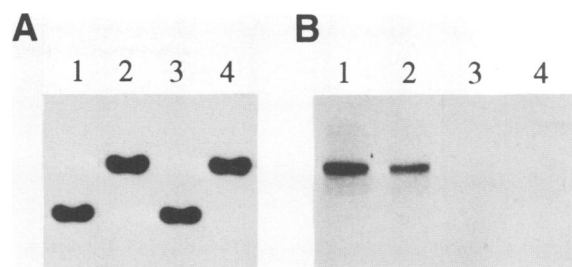

Fig. 1. Genotype of the different primary fibroblasts used in this study. (**A**) Southern blot analysis of the CD-MPR gene (see Materials and methods). The lower band (2.7 kb) represents the wild-type allele, the upper band (3.5 kb) represents the disrupted allele. (**B**) Immunoprecipitation of the CI-MPR. Lane 1: wild type; lane 2: CD-MPR ($-$) CI-MPR ($+$); lane 3: CD-MPR ($+$) CI-MPR ($-$); lane 4: CD-MPR ($-$) CI-MPR ($-$).
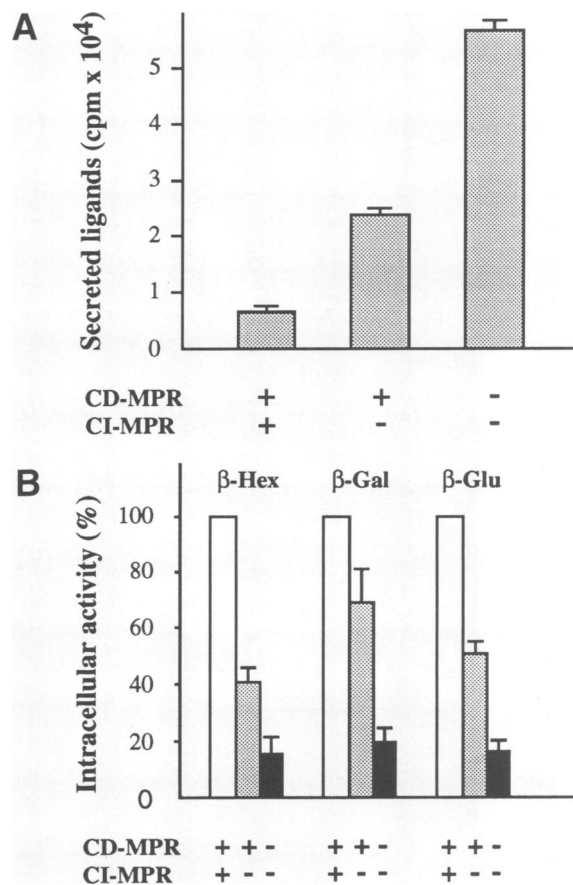
Fig. 2. Lysosomal enzyme sorting in primary fibroblasts lacking the MPRs. (**A**) The secreted lysosomal enzymes were purified on MPR affinity columns as described in Materials and methods. The indicated numbers represent the amount of secreted phosphorylated ligands normalized to 1×10^6 TCA precipitable c.p.m. of secreted proteins. The amount of proteins secreted by each cell type was similar and represented ~10% of the total newly synthesized proteins. (**B**) Intracellular activities of β-hexosaminidase (β-Hex), β-galactosidase (β-Gal) and β-glucuronidase (β-Glu). The enzyme activities were measured as described in Materials and methods.

endocytic structures of the mutant cells (Ludwig *et al.*, 1993). Therefore, we examined in detail the morphology of the embryonic fibroblasts lacking the CI-MPR and both MPRs. The different matched cultures of embryonic fibroblasts were first labeled with an antibody against Lamp-1, an example of a transmembrane protein enriched
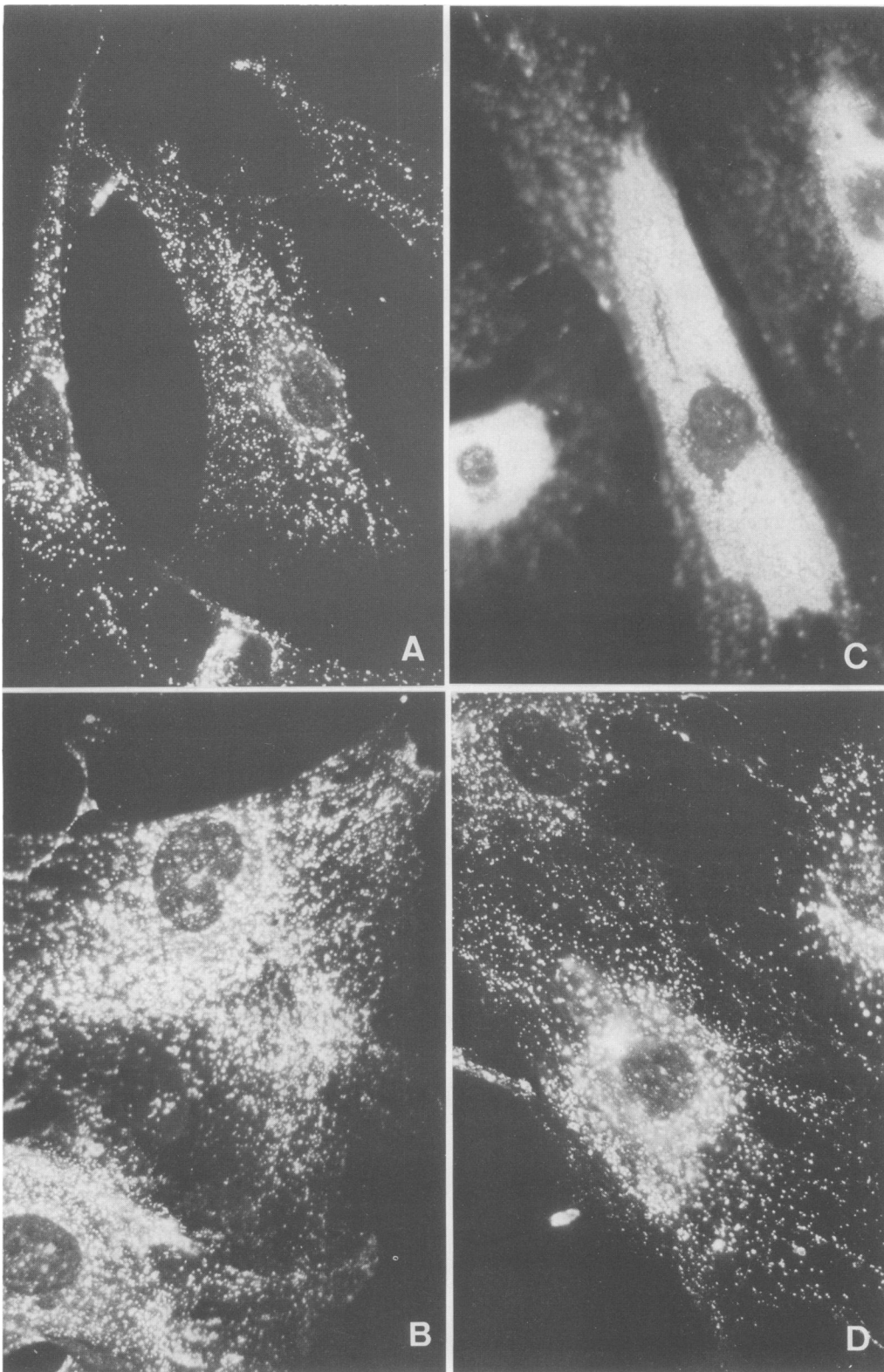
Fig. 3. Late endocytic compartments of MPR-deficient fibroblasts. The membrane glycoprotein Lamp-1, taken as a marker of late endosomes and lysosomes, was detected by immunofluorescence as indicated in Materials and methods. (**A**) shows a wild-type fibroblast, (**B**) a CI-MPR-negative fibroblast; for comparison, a CD-MPR-negative fibroblast is shown in (**D**); (**C**) shows a fibroblast lacking both the CD-MPR and the CI-MPR. The different cell types were observed at the same magnification.

in late endosomes and lysosomes (Chen *et al.*, 1985), and then examined by fluorescence microscopy (Figure 3). Like the CD-MPR-negative fibroblasts, the CI-MPR-negative fibroblasts contained significantly more late endocytic structures than control fibroblasts. Again, a more drastic phenotype was observed in fibroblasts lacking the
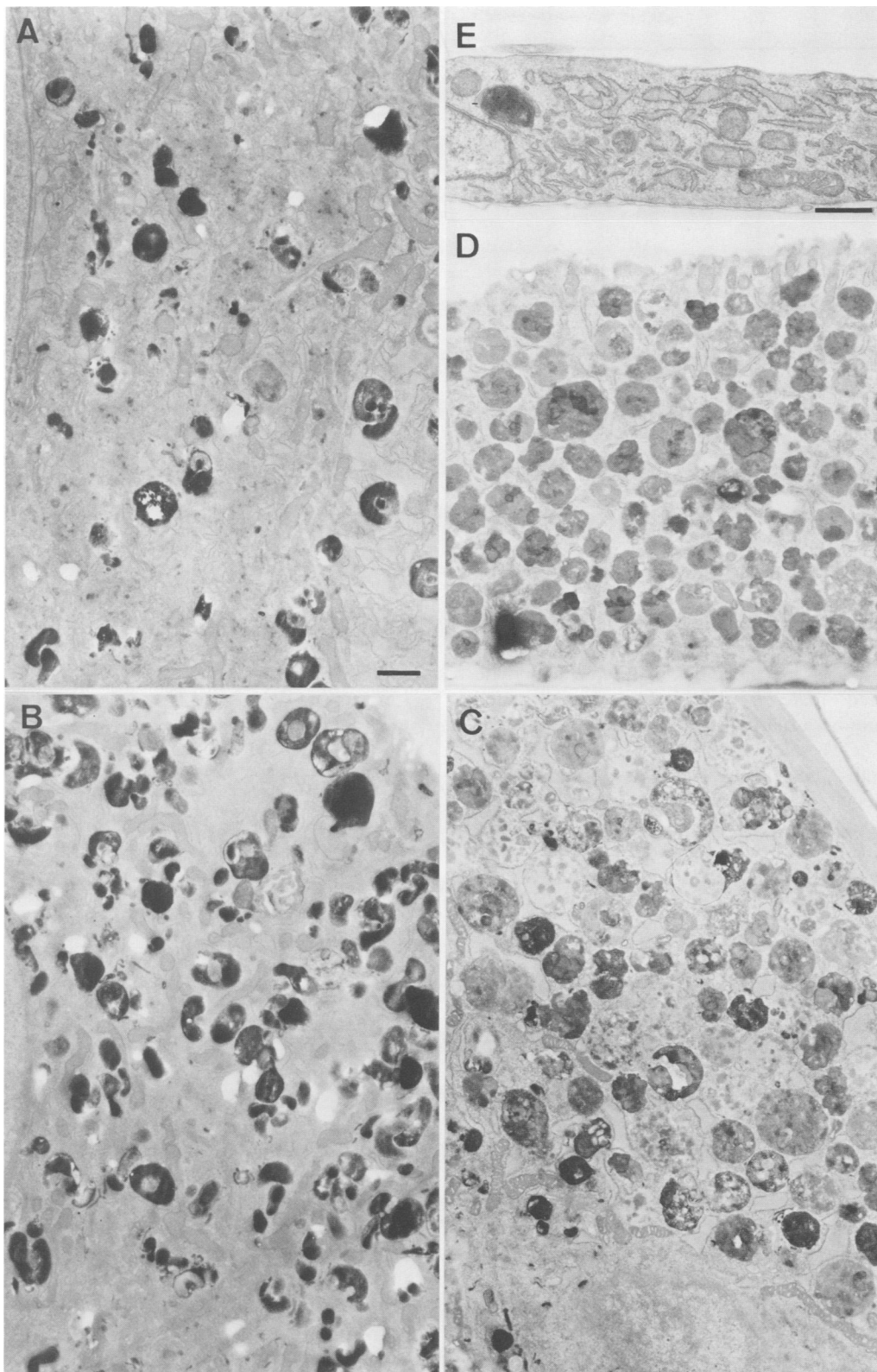
Fig. 4. Morphology of the endocytic compartments in Epon-embedded, MPR-deficient fibroblasts. (**A**) shows a representative section of a wild-type embryonic fibroblast after 60 min HRP internalization and 30 min reaction with substrate (magnification 9K, bar 1 μm). (**B**) shows a section from a CI-MPR-negative fibroblast processed similarly. Note the high number of endosome/lysosome structures (magnification 9K). (**C**) Representative section of embryonic fibroblasts lacking both the CD-MPR and the CI-MPR. Note the very high number of endosomes/lysosomes labeled with the HRP reaction product (magnification 9K). For comparison with flat embedded cells sectioned parallel to the substratum (A, B and C), sections perpendicular to the substratum are shown: (**E**) a wild-type fibroblast (magnification 22K, bar 500 nm); (**D**) a CD-MPR- and CI-MPR-negative fibroblast (magnification 9K).
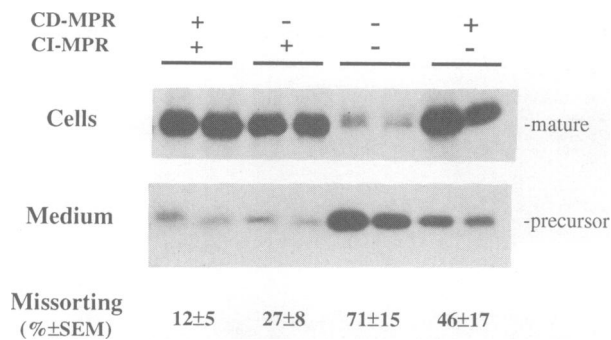
Fig. 5. Sorting of cathepsin D in MPR-deficient fibroblasts. The fibroblasts were metabolically labeled and the newly synthesized cathepsin D was immunoprecipitated from the cells (upper panel) and the medium (lower panel) as described in Materials and methods. The secreted cathepsin D corresponds to the 52 kDa precursor form, whereas the intracellular cathepsin D corresponds to the 48 kDa mature form. The extent of missorting was quantitated using a PhosphorImager. The indicated numbers (% missorting) are the means ± SD of four experiments performed in duplicate using two cultures of each genotype.

two MPRs in which Lamp-1-positive structures filled the cytoplasm completely (Figure 3). Electron microscopy performed on thin sections of glutaraldehyde-fixed, Epon-embedded embryonic fibroblasts lacking either the CI-MPR or both MPRs revealed that these late endocytic structures stored material that appeared undigested (Figure 4). A number of large vacuoles containing smaller translucent vesicles surrounded by a membrane were observed in both CI-MPR-negative and CI-MPR-, CD-MPR-double-negative fibroblasts. These structures were endocytic, as indicated by their accessibility to the internalized horseradish peroxidase (HRP) used as a fluid phase marker and visualized by cytochemistry. Structures with a similar morphological appearance could also be decorated with the anti-Lamp-1 antibody using thawed cryosections of these cells (not shown). Thus, the morphology of these cell types resembles that of the previously described CD-MPR-negative fibroblasts and fibroblasts from patients with mucolipidoses II and III. They all exhibit an extended endocytic apparatus whose volume appears to be proportional to the extent of missorting of lysosomal enzymes. Thus, the morphology of these cells shows that the lack of the receptors leads to impaired lysosomal function.

Fibroblasts lacking either CD-MPR or CI-MPR secrete different subsets of hydrolases

The different primary embryonic fibroblasts generated in this study provided the ideal tools to define the relative function of the two receptors in lysosomal enzyme transport under physiological levels of expression. In experiments designed to quantitate the extent of missorting of lysosomal enzymes, transport of cathepsin D was first investigated using typical pulse–chase experiments followed by immunoprecipitation (Figure 5). This lysosomal enzyme is usually taken as a reporter molecule to assay MPR function. The appearance of the cathepsin D precursor in the media reflects missorting, while the accumulation of the mature (proteolytically processed) cathepsin D in the cells reflects delivery to lysosomes.
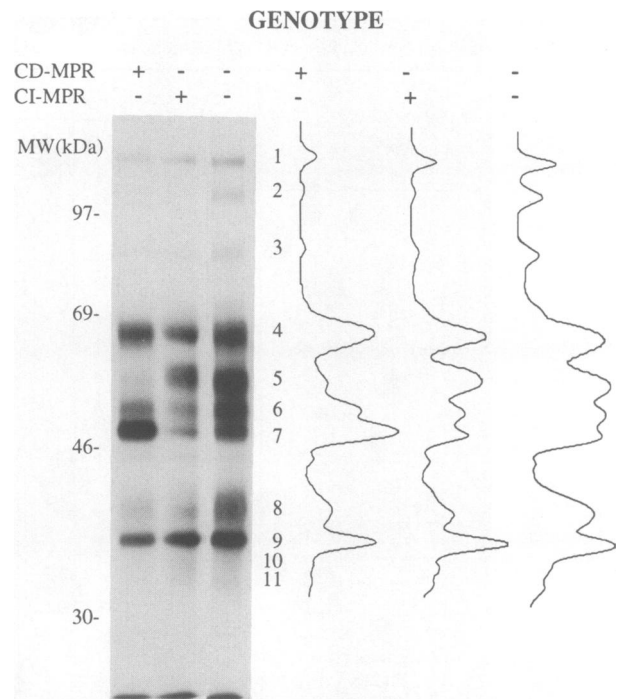
Fig. 6. Lysosomal enzymes secreted by MPR-deficient fibroblasts. The [^{35}S]methionine-labeled lysosomal enzymes, secreted by the different MPR-negative fibroblasts, were purified on MPR affinity columns and fractionated by SDS–PAGE as described in Materials and methods. A representative gel (~20 000 c.p.m. of labeled material secreted by CD-MPR-negative or CI-MPR-negative fibroblasts and ~35 000 c.p.m. of labeled material secreted by the MPR double-negative fibroblasts were loaded on this gel) and corresponding profiles are shown. Several major proteins (numbered from 1 to 11) were detected and quantitated (see Table I).

As expected, CI-MPR-negative fibroblasts missorted and secreted a large fraction of the total cathepsin D (~46%), whereas control fibroblasts secreted only a minor fraction (~12%) of this enzyme. Missorting of the cathepsin D precursor was more dramatic (~71%) in CD-MPR-, CI-MPR-double-negative fibroblasts. However, despite the lack of MPR, they could still retain a minor fraction of this enzyme (~30%) which was converted into the 48 kDa mature form. Surprisingly, sorting of cathepsin D was less affected in CD-MPR-negative fibroblasts (~27% missorted), suggesting that its transport was predominantly CI-MPR mediated. Since the glycosidase assays indicated that transport of these particular lysosomal enzymes was similarly affected in cells lacking either the CD-MPR or the CI-MPR (~50% retention in both cases), this latter result suggested that, *in vivo*, lysosomal enzymes bind to the two MPRs with different affinities. Therefore, we purified the secreted lysosomal enzymes on MPR affinity columns and analyzed by SDS–PAGE the different species secreted by each cell type (Figure 6). The fibroblasts lacking both MPRs secreted >10 major proteins that contained the mannose 6-phosphate marker. The fact that these cells exhibit a pleiotropic defect in lysosomal enzyme targeting (~80% of missorting for β-galactosidase, β-hexosaminidase, β-glucuronidase and cathepsin D) made it possible to determine the extent of missorting of individual lysosomal enzymes in CI-MPR-negative or CD-MPR-

Table I. Sorting of phosphorylated ligands in MPR-deficient fibroblasts

Genotype	Secreted ligands (c.p.m./1 $\times$ 10^6 secreted counts)		
CI-MPR	−	+	−
CD-MPR	+	−	−
Total secreted	21 527 ± 1173	17 248 ± 1356	45 304 ± 1768
Individual ligands			
1	569 ± 127 (34)	329 ± 70 (19)	1665 ± 153
2	175 ± 64 (12)	151 ± 43 (10)	1410 ± 152
3	264 ± 32 (36)	320 ± 59 (43)	734 ± 40
4 (β-hexosaminidase)	4553 ± 602 (53)	3465 ± 411 (41)	8500 ± 225
5	1393 ± 128 (21)	4172 ± 487 (63)	6600 ± 704
6	3339 ± 497 (54)	2392 ± 343 (39)	6092 ± 357
7 (cathepsin D)	5671 ± 759 (86)	1446 ± 263 (21)	6578 ± 616
8	2064 ± 368 (33)	1493 ± 77 (24)	6121 ± 1447
9	2680 ± 669 (51)	2812 ± 481 (54)	5185 ± 1595
10	<100 (0)	685 ± 189 (51)	1320 ± 479
11	<100 (0)	265 ± 120 (20)	1317 ± 604

Lysosomal enzymes secreted by the different cell types were purified and fractionated as indicated in Materials and methods. After fractionation by SDS−PAGE, the different lysosomal enzymes (numbered from 1 to 11, see Figure 6) secreted by each cell type were quantified. Some of these proteins were not detected (<100 c.p.m.) in particular cases. The amount of secreted, phosphorylated ligands was normalized to 1 $\times$ 10^6 TCA-precipitable c.p.m. of secreted proteins (the different cell types secreted similar amounts of protein: ~10% of the newly synthesized proteins). The indicated numbers represent the mean ± SD of three independent determinations for each cell type. The percent of missorting (numbers in parentheses) was calculated considering that complete missorting (100%) occurred in fibroblasts lacking both MPRs. β-hexosaminidase (band 4) and cathepsin D (band 7) were identified by immunoprecipitation (not shown).

negative fibroblasts (Table I). The quantitative analysis shows that several groups of phosphorylated ligands could be distinguished. There were ligands whose secretion was only mildly affected by the lack of either MPRs (band 2 for example), indicating that they could bind to the two receptors with the same efficiency. In contrast, the sorting of some other phosphorylated ligands (bands 4, 6, 9) was equally affected by the lack of the CI-MPR or the CD-MPR. For example, 50% of β-hexosaminidase, identified as band 4 by immunoprecipitation (not shown), was secreted in the absence of either MPR. More interestingly, a phosphorylated protein (band 5) appeared to be predominantly secreted in the absence of the CD-MPR while another phosphorylated protein (band 7), identified as cathepsin D by immunoprecipitation (not shown), was predominantly secreted in the absence of the CI-MPR. Altogether, these results show that, in vivo, the two MPRs sort a few ligands with different efficiencies, suggesting that they exhibit different affinities for subgroups of phosphorylated lysosomal enzymes.

Discussion

Primary embryonic fibroblasts that differ by their content of mannose 6-phosphate receptors have been obtained after crossing CD-MPR-negative (Ludwig et al., 1993) and T^{hp} mice that transmit to their offspring a chromosome 17 deleted around the CI-MPR locus (Johnson, 1974; Barlow et al., 1991). Analysis of lysosomal enzyme trafficking in these cells shows that the two MPRs are the major components that function in combination to transport the bulk of the soluble hydrolases to lysosomes. Thus, MPR-deficient fibroblasts secrete the bulk of their newly synthesized hydrolases. In contrast, embryonic fibroblasts lacking either the CI-MPR (this study) or the CD-MPR (Ludwig et al., 1993, and this study) exhibit a partial missorting of multiple lysosomal enzymes. In this respect,

the CI-MPR-negative fibroblasts generated in this study behave as the previously described established cell lines (such as the P388D1 and J 774 macrophages or the mouse L-cells) characterized by a low/undetectable level of expression of this receptor (Gabel et al., 1983). However, the CI-MPR-negative fibroblasts differ from the CD-MPR-negative fibroblasts by the nature of the phosphorylated ligands they secrete, thereby supporting the notion that the MPRs may interact in vivo with different classes of hydrolases.

The MPRs are the major components of the targeting system

The importance of the two MPRs is clearly illustrated by the phenotype of the MPR-deficient cells generated in this study. These cells secrete a large proportion of their newly synthesized lysosomal enzymes and, as a consequence, store undigested material in their lysosomes. This shows that, in embryonic fibroblasts, most of the soluble hydrolases are transported to lysosomes via a mannose 6-phosphate-dependent pathway. Although the primary defects are distinct, it is remarkable to note that this phenotype resembles that of mucolipidosis II (for a review, see Nolan and Sly, 1989; Neufeld, 1991). This inherited human disease is characterized by an undetectable activity of the N-acetylglucosaminyl 1-phosphotransferase (Hasilik et al., 1981; Varki et al., 1981), the critical enzyme involved in the synthesis of the mannose 6-phosphate recognition marker on the sugar moieties of soluble hydrolases. This defect also causes a nearly complete missorting of the newly synthesized lysosomal enzymes, which results in lysosomal accumulation of undigested macromolecules. It will be of interest to examine whether the pleiotropic defect in lysosomal enzyme targeting associated with a lack of two functional MPRs triggers a pathology similar to mucolipidosis II. Although MPR-deficient embryos are expected to die at day 15, as

predicted from the *Tme* phenotype (Johnson, 1974), manifestations of the mucolipidosis II phenotype can be detected during early stages of development (Babcock *et al.*, 1986).

It is worth noting, however, that MPR-deficient fibroblasts still transport low but detectable amounts of hydrolases to lysosomes. Although we cannot formally exclude the existence of another minor mannose 6-phosphate binding protein, it is likely that this residual targeting reflects a mannose 6-phosphate-independent transport process. Similar findings in cells from mucolipidosis II patients suggest that such pathways exist (Owada and Neufeld, 1982; Waheed *et al.*, 1982). More recently, Glickman and Kornfeld (1993) identified a protein determinant on cathepsin D which could mediate its mannose 6-phosphate-independent lysosomal targeting in a B lymphoblastoid cell line issued from a mucolipidosis II patient. As in mucolipidosis II, it is unknown whether this residual targeting observed in MPR-deficient fibroblasts reflects a re-uptake of the secreted lysosomal enzymes or a direct intracellular pathway to lysosomes. This latter possibility has been proposed to occur in HepG2 cells for mannose 6-phosphate-independent targeting of cathepsin D (Rijnboutt *et al.*, 1991). The MPR-deficient fibroblasts could also provide a useful model system to investigate these alternative lysosomal enzyme targeting pathways.

Differential sorting of lysosomal enzymes by the MPRs

These matched cultures of embryonic fibroblasts with various genotypes provide a unique opportunity to gain additional insights into the biological function of the MPRs. The most significant finding is that lysosomal enzymes appear to be differently secreted by the embryonic fibroblasts devoid of either the CD-MPR or the CI-MPR, suggesting that *in vivo* these receptors exhibit various affinities for different subgroups of lysosomal enzymes. Although only 11 major lysosomal enzymes (among the 40–50 enzymes that a lysosome normally contains) could be detected in the culture medium of the different cell types, it appears that the sorting of cathepsin D is more affected in CI-MPR-negative fibroblasts than in CD-MPR-negative fibroblasts. Conversely, sorting of another major protein, also carrying the mannose 6-phosphate recognition marker, is more affected in CD-MPR-negative fibroblasts. If so, a downregulation of one MPR could result in the secretion of its preferred ligands, while the overexpression of one MPR could have milder effects on the sorting of the ligands that are preferentially transported by the other receptor. This should be testable in future transfection studies in which different amounts of each MPR are expressed in the double-negative fibroblasts. Finally, the storage of undigested material inside the late endocytic structures of fibroblasts lacking either the CD-MPR (Ludwig *et al.* 1993, and this study) or the CI-MPR could also be interpreted as being the result of a more drastic missorting of only a few hydrolases (which may not have been detected in this study) which could not be efficiently sorted by the other MPR. The lack of a single hydrolase, as seen in many lysosomal storage disorders (for a review, see Neufeld, 1991), would be sufficient to trigger the accumulation of the corresponding substrate in lysosomes.

Putative specificity of the MPRs

It is worth noting that some phosphorylated ligands are similarly secreted in the absence of one MPR or the other and therefore appear to interact *in vivo* with both MPRs. The glycosidases measured in this study (β-hexosaminidase, β-galactosidase and β-glucuronidase) and a few other, still uncharacterized phosphorylated proteins would belong to this category. Their partial missorting could be interpreted as a saturation of the remaining MPR. However, the observation that at least one phosphorylated protein is almost exclusively secreted in the absence of both MPRs, while some others are predominantly missorted in fibroblasts lacking one of the two MPRs, would exclude this possibility. Analysis of various fibroblasts heterozygous for the CD-MPR also indicates that, under physiological levels of expression, this receptor is not saturated when it functions in lysosomal enzyme sorting (our unpublished data). Therefore, an alternative hypothesis is that lysosomal enzymes are heterogeneous, and that their different isoforms have preferred affinities for each MPR. Such heterogeneity on a single lysosomal enzyme could be provided by its phosphorylated oligosaccharides. Most lysosomal enzymes have multiple oligosaccharides, each containing up to two phosphomonoesters. For example, two out of the three oligosaccharides of the mouse β-glucuronidase are preferentially phosphorylated, each bearing one or two phosphate groups (Goldberg and Kornfeld, 1981). Similarly, analysis of the two oligosaccharides on cathepsin D demonstrates that each oligosaccharide is heterogeneous and contains zero, one or two phosphate groups (Cantor *et al.*, 1992), which would yield nine different isoforms. Although the detergent-solubilized MPRs bind purified high-mannose oligosaccharides bearing two phosphomonoesters with different affinities (Hoflack *et al.*, 1987; Tong and Kornfeld, 1989; Tong *et al.*, 1989), their interactions with physiological ligands are likely to be more complex, perhaps involving the number of oligosaccharides present and their pattern of phosphorylation. Thus, it remains conceivable that a single lysosomal enzyme can exhibit different isoforms that can be recognized only by the CD-MPR, only by the CI-MPR, or by both MPRs. This could easily explain why mammalian cells need to express two different mannose 6-phosphate binding proteins to transport the bulk of their lysosomal enzymes. The cell systems that we have described here can now be used to test these interesting possibilities.

Materials and methods

Crossing and genotyping

$T^{hp}(+/-)$ males were crossed with CD-MPR $(-/-)$ females to generate $T^{hp}(+/-)$, CD-MPR $(+/-)$ females. These latter were then mated to CD-MPR $(-/-)$ or CD-MPR $(+/+)$ males. At day 13.5 of gestation, fibroblasts were prepared from the different embryos and propagated in culture as described previously (Robertson, 1987). Genomic DNA was prepared from a part of the embryos and digested with *BglII* as described by Sambrook *et al.* (1989). Southern blot analysis was as described previously (Ludwig *et al.*, 1993) using a random primed, heat-denatured ^{32}P-labeled CD-MPR genomic probe (*EcoRI—SacI* fragment) added to the specific activity of 2×10^6 c.p.m./ml. To monitor expression of the CI-MPR, primary cultures of embryonic fibroblasts were labeled overnight with [^{35}S]methionine (0.1 mCi/ml) in complete α-MEM medium containing one-tenth of the normal methionine concentration and 5% fetal calf serum (FCS). After harvesting, the labeled fibroblasts were

solubilized in 50 mM Tris−HCl (pH 7), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitors. After pelleting the insoluble material, the CI-MPR was immunoprecipitated from the detergent extracts with specific antibodies. The immunoprecipitate was then analyzed by SDS−PAGE and fluorography.

Purification of secreted phosphorylated ligands and lysosomal enzyme assays

For analysis of the lysosomal enzymes secreted by the different fibroblasts, the cells were labeled overnight with [³⁵S]methionine (0.2 mCi/ml) in α-MEM containing one-tenth of the normal methionine concentration, 10 mM HEPES pH 7 and 5% dialyzed FCS. After a 3 h chase, the secreted lysosomal enzymes were purified as previously described (Hoflack et al., 1987) on MPR affinity columns equilibrated in 50 mM imidazole (pH 6.5), 150 mM NaCl, 10 mM MnCl₂, 5 mM Na β-glycerophosphate, 0.05% Triton X-100 (column buffer). Here, a mixture of CD-MPR and CI-MPR, purified on phosphomannan columns as previously described (Hoflack and Kornfeld, 1985), was coupled to Affigel 15 (0.5 mg of each receptor/ml of gel). After loading the MPR affinity columns with the culture medium, the columns were washed with column buffer and column buffer containing 5 mM glucose-6-phosphate. The bound lysosomal enzymes were eluted with 5 mM mannose 6-phosphate in column buffer and precipitated with 20% trichloroacetic acid (TCA). After centrifugation, the precipitated material was rinsed with acetone, boiled in Laemmli sample buffer and fractionated by SDS−PAGE (~20 000−40 000 c.p.m. were loaded on each lane). The counts associated with each lysosomal enzyme could then be quantitated using a PhosphorImager.

Transport of cathepsin D was monitored according to the following protocol. Cultured embryonic fibroblasts were labeled for 2 h in methionine-free MEM medium containing 1 mCi/ml [³⁵S]methionine, 10 mM HEPES, 5% FCS and then chased for 4 h in complete medium. The cells were harvested, centrifuged and lysed in 0.5 ml of lysis buffer. Cathepsin D was immunoprecipitated from both the cell extracts and the corresponding culture media with specific antibodies. The immunoprecipitates were analyzed by SDS−PAGE followed by autoradiography. The secreted precursor form and intracellular mature cathepsin D were then quantitated.

For lysosomal enzyme assays, primary fibroblasts from two independent cultures of the same genotype were harvested and lysed in 1 ml of lysis buffer [50 mM Tris−HCl (pH 7), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and protease inhibitors]. Duplicate samples (10 and 20 μg of protein) were assayed for the different glycosidases (β-hexosaminidase, β-galactosidase and β-glucuronidase) using as substrate the corresponding 4-methylumbelliferyl derivatives, as previously described (Ludwig et al., 1993).

Fluorescence and electron microscopy

The embryonic fibroblasts were grown on glass coverslips, methanol fixed and incubated with an anti-Lamp-1 antibody (Chen et al., 1985). After washing, the first antibody was detected with a second, fluorescein-labeled anti-rabbit IgG.

For electron microscopic studies, the fibroblasts were first incubated at 37°C for 60 min with 10 mg/ml HRP (Calbiochem, 265 U/mg) in the medium. The cells were then washed with ice-cold phosphate-buffered saline (PBS) and fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate (pH 7.4) for 30 min. After fixation, the cells were washed with cacodylate buffer and incubated for 1 min with diaminobenzidine (DAB) and then incubated with DAB plus H₂O₂. The reaction was stopped by washing with cacodylate buffer. The cells were post-fixed with osmium tetroxide, washed with water and then incubated with 0.5% Mg uranyl acetate overnight at 4°C. The samples were then embedded in Epon, sectioned and processed for electron microscopy.

References

Babcock,D.S., Bove,K.E., Hug,G., Dignan,P.S., Soukup,S. and Warren,N.S. (1986) Pediatr. Radiol., 16, 32–39.
Barlow,D., Stöger,R., Hermann,B., Saito,K. and Schweifer,N. (1991) Nature, 349, 84–87.
Cantor,A.B., Baranski,T.J. and Kornfeld,S. (1992) J. Biol. Chem., 267, 23349–23356.
Chen,J.W., Murphy,T.L., Willinghan,M.C., Pastan,I. and August,J.T. (1985) J. Cell Biol., 101, 85–95.
Gabel,C.A., Goldberg,D.E. and Kornfeld,S. (1983) Proc. Natl Acad. Sci. USA, 81, 775–779.
Glickman,J.N. and Kornfeld,S. (1993) J. Cell Biol., 123, 99–108.
Goldberg,D.E. and Kornfeld,S. (1981) J. Biol. Chem., 256, 13060–13067.
Hasilik,A., Waheed,A. and von Figura,K. (1981) Biochem. Biophys. Res. Commun., 98, 761–767.
Hoflack,B. and Kornfeld,S. (1985) J. Biol. Chem., 260, 12008–12014.
Hoflack,B. and Lobel,P. (1993) In Tartakoff,A.L., Storrie,B. and Murphy,E. (eds), Advances in Cell and Molecular Biology of Membranes—Endosomes and Lysosomes: A Dynamic Relationship. JAI Press, London, pp. 51–80.
Hoflack,B., Fujimito,K. and Kornfeld,S. (1987) J. Biol. Chem., 262, 123–129.
Johnson,D.R. (1974) Genetics, 76, 795–805.
Kornfeld,S. (1992) Annu. Rev. Biochem., 61, 307–330.
Kornfeld,S. and Mellman,I. (1989) Annu. Rev. Cell Biol., 5, 483–525.
Köster,A., Saftig,P., Matzner,U., von Figura,K., Peters,C. and Polhmann,R. (1993) EMBO J., 12, 5219–5223.
Ludwig,T., Ovitt,C., Bauer,U., Hollinshead,M., Remmler,J., Lobel,P., Rüther,U. and Hoflack,B. (1993) EMBO J., 12, 5225–5235.
Neufeld,E. (1991) Annu. Rev. Biochem., 60, 257–280.
Neufeld,E.F. and McKusik,V.A. (1983) In Stanbury,J.B., Wyngaarden,J.B., Fredrikson,D.S., Goldstein,J.L. and Brown,M.S. (eds), The Metabolic Basis of Inherited Disease. McGraw-Hill, New York, pp. 778–787.
Nolan,C.M. and Sly,W.S. (1989) In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds), The Metabolic Basis of Inherited Disease. McGraw-Hill, New York, pp. 1589–1601.
Owada,M. and Neufeld,E.K. (1982) Biochem. Biophys. Res. Commun., 105, 814–820.
Rijnboutt,S., Kal,A.J., Geuze,H.J., Aerts,H. and Strous,G.J. (1991) J. Biol. Chem., 266, 23586–23592.
Robertson,E.J. (1987). In Robertson,E.J. (ed.), Teratocarcinomas and Embryonic Stem Cells, A Practical Approach. IRL Press, Oxford, pp. 71–112.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Tong,P. and Kornfeld,S. (1989) J. Biol. Chem., 264, 7970–7975.
Tong,P., Gregory,W. and Kornfeld,S. (1989) J. Biol. Chem., 264, 7962–7969.
Varki,A., Reitman,M.L. and Kornfeld,S. (1981) Proc. Natl Acad. Sci. USA, 78, 7773–7777.
von Figura,K. (1991) Curr. Opin. Cell Biol., 3, 642–646.
Waheed,A., Pohlmann,R., Hasilik,A., von Figura,K., van Elsen,A. and Leroy,J.G. (1982) Biochem. Biophys. Res. Commun., 105, 1052–1058.