The Two Mannose 6-Phosphate Receptors Transport Distinct Complements of Lysosomal Proteins*

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Mammalian cells express two different mannose 6-phosphate receptors (MPRs) which both mediate targeting of Man-6-P-containing lysosomal proteins to lysosomes. To assess the contribution of either and both MPRs to the transport of lysosomal proteins, fibroblasts were established from mouse embryos that were homozygous for disrupted alleles of either MPR 46 or MPR 300 or both MPRs. Fibroblasts missing both MPRs secreted most of the newly synthesized lysosomal proteins and were unable to maintain the catalytic function of lysosomes. The intracellular levels of lysosomal proteins decreased to <20% and undigested material accumulated in the lysosomal compartment. Fibroblasts lacking either MPR exhibited only a partial missorting and maintained, in general, half-normal to normal levels of lysosomal proteins. The same species of lysosomal proteins were found in secretions of double MPR-deficient fibroblasts, but at different ratios. This clearly indicates that neither MPR has an exclusive affinity for one or several lysosomal proteins. Furthermore, neither MPR can substitute in vivo for the loss of the other. It is proposed that the heterogeneity of the Man-6-P recognition marker within a lysosomal protein and among different lysosomal proteins has necessitated the evolution of two MPRs with complementary binding properties to ensure an efficient targeting of lysosomal proteins.

In mammalian cells, mannose 6-phosphate receptors (MPRs) are essential elements of the transport system that ensure the targeting of newly synthesized lysosomal enzymes. Soluble lysosomal proteins become specifically modified with mannose 6-phosphate (Man-6-P) residues during their passage through the Golgi apparatus and are targeted by MPRs to lysosomes (for review, see Kornfeld (1992) and Kornfeld and Mellman (1989)). The MPRs are type I transmembrane glycoproteins and have apparent molecular masses of 46,000 Da (MPR 46) (Hoflack and Kornfeld, 1985) and 300,000 Da (MPR 300) (Kornfeld, 1992). The extracytoplasmic domain of MPR 46 is homologous to each of the 15 repeating units that build the extracytoplasmic domain of MPR 300. MPR 300 is known to bind and endocytose additionally nonglycosylated insulin-like growth factor II (IGF-II). This property of MPR 300 is considered to be of importance for controlling the extracellular IGF-II level (Filson et al., 1993), while it is unclear whether MPR 300 has a function in transmembrane signal transduction analogous to other growth factor receptors (Okamoto et al., 1990; Körner et al., 1995).

The importance of MPRs for the biogenesis of lysosomes is illustrated by the phenotype of I-cell fibroblasts, which are unable to add Man-6-P recognition markers to their newly synthesized lysosomal proteins. As a result, excessive amounts of lysosomal proteins are secreted, and a marked intracellular deficiency of lysosomal enzymes and a defective lysosomal catabolism are observed (Nolan and Sly, 1989). As all mammalian cells analyzed express both MPRs, albeit at different levels and variable relative ratios (Wenk et al., 1991), it has been difficult to assess the role of each of the two MPRs in the biogenesis of lysosomes. Each of the two MPRs contributes to the intracellular retention of lysosomal proteins and their targeting to lysosomes as indicated by the accumulation of MPR ligands in the secretions of cells missing MPR 46 or MPR 300 (Gabel et al., 1983; Ludwig et al., 1993; Wang et al., 1994). It is, however, unclear whether the two MPRs transport the same or different lysosomal proteins or different subpopulations of a lysosomal protein, whether they feed distinct populations of target organelles with newly synthesized lysosomal proteins, and to what extent they can replace each other.

Answers to these questions would be greatly facilitated if cells could be studied that lack both MPRs and that allow the expression of either or both of the receptors at various levels. In this study, we report on the generation of mouse embryonic fibroblasts that lack MPR 46 and MPR 300. Mice strains carrying disrupted MPR 46 or disrupted MPR 300 alleles were crossed to obtain offspring homozygous for each of the disrupted MPR alleles. As deficiency of MPR 300 leads to death before birth (Wang et al., 1994; Lau et al., 1994), fibroblasts were established from embryos at day 12.5. The phenotype of these fibroblasts was compared with that of fibroblasts from embryos lacking either of the two MPRs and with the phenotype of control fibroblasts. Compared with fibroblasts with a deficiency of one of the MPRs, the missorting of soluble lysosomal proteins was much more pronounced in double MPR-deficient fibroblasts. Analysis of individual lysosomal enzymes and of the Man-6-P polypeptides accumulating in secretions indicated that lysosomal proteins bind to both MPRs, although with different affinities. As a result, the two MPRs transport distinct complements of lysosomal proteins that consist of the same components, but at different ratios.

MATERIALS AND METHODS

Cell Culture—Mouse embryonic fibroblasts were prepared from day 12.5 embryos as described (Hogan et al., 1986) and grown in Dulbecco's minimal essential medium (Life Technologies, Inc.) and 10% fetal calf
Transport of Distinct Complements of Lysosomal Proteins by MPR

**RESULTS**

Generation of Embryonic Fibroblasts Lacking MPR 46 and MPR 300—MPR 300−/− males were crossed with MPR 46−/− females. Double MPR heterozygous males of the offspring were mated with MPR 46−/− females to obtain MPR 46−/−/MPR 300−/− males and females, which were interbred. Embryos were prepared at day 12.5 of gestation. The fibroblast cultures established thereof were analyzed for their genotype and expression of MPRs. In Fig. 1, the genotype and Western blot analysis of embryonic fibroblasts homozygous for the targeted MPR 46 and MPR 300 alleles are shown and compared with control and fibroblasts homozygous for either of the two targeted MPR alleles. It is apparent that the MPR 46−/−/MPR 300−/− fibroblasts lack both MPR and that fibroblasts missing either type of MPR have a normal complement of the other MPR.

Intracellular Retention of Lysosomal Enzymes in MPR-deficient Fibroblasts—Intracellular and extracellular activities of five soluble lysosomal enzymes known to be transported to lysosomes via MPRs (β-hexosaminidase, β-glucuronidase, α-fucosidase, mannosidase, and α-galactosidase) were measured after a 24-h culture period. As a control, the activity of a lysosomal enzyme known to be transported as an integral membrane protein independent of MPR (lysosomal acid phosphatase) was determined.

In fibroblasts lacking both MPRs (MPR 46−/−/MPR 300−/−), the activities of the five soluble lysosomal enzymes were reduced to 7–21% of the control (Fig. 2). In single MPR-deficient fibroblasts, this decrease was less pronounced (39–81% in MPR 46−/− fibroblasts and 42–80% in MPR 300−/− fibroblasts). In each of the MPR-deficient fibroblast lines, the intracellular activity of acid phosphatase was in the range of controls (95–107%).

For the five soluble lysosomal enzymes, the fraction of activity that accumulated in the secretions during a 24-h incubation period varied between 5 and 33% (expressed as percentage of enzyme activity in cells and medium). This fraction increased to 72–93% in MPR 46−/−/MPR 300−/− fibroblasts (Fig. 3). A deficiency of MPR 46 increased the fraction of enzyme in the secretions only to a minor extent (10–41%), while a deficiency of MPR 300 produced an intermediate level of accumulation (35–70%). Lysosomal acid phosphatase was not detectable in secretions of embryonic fibroblasts.

In summary, these results suggest that lysosomal enzymes
that are normally transported via a MPR-dependent mechanism are only poorly retained by MPR 46 fibroblasts and are mostly released into the secretions. In single MPR-deficient fibroblasts, the missorting is less pronounced. It should be noted that the loss of either MPR 46 or MPR 300 produces a similar decrease in the intracellular level of the lysosomal enzymes tested, while the accumulation in their secretions is much more pronounced in MPR 300 fibroblasts.

Missorting of Newly Synthesized Cathepsin D and β-Glucuronidase in MPR-deficient Fibroblasts—To determine the sorting of newly synthesized lysosomal enzymes, fibroblasts were metabolically labeled in the presence of [35S]methionine for 2 h. Cells were then chased for 4 h to allow transport of labeled lysosomal enzymes to their final destination. Cathepsin D was immunoprecipitated from the cells and secretions. A minor fraction of the intracellular cathepsin D polypeptides was recovered as a 51-kDa precursor form. The bulk of [35S]-labeled cathepsin D polypeptides was recovered as 38-45-kDa forms, which represent processing intermediates that can be further processed by endoproteolytic cleavage into a heavy (30 kDa) and a light (14–16 kDa) chain representing mature cathepsin D (Hasilik, 1992). In the secretions, only the precursor forms were detectable (Fig. 4). In controls, 20% of the labeled cathepsin D was recovered in the secretions and 80% intracellularly, while in MPR 46/MPR 300 fibroblasts, the percentages were 96% and 5%, respectively. In secretions of MPR 46 and MPR 300 fibroblasts, 30 and 94%, respectively, of the newly synthesized cathepsin D were recovered.

This clearly demonstrates that the bulk of newly synthesized cathepsin D is secreted in MPR 46/MPR 300 fibroblasts, but also in MPR 300 fibroblasts. Moreover, in cells lacking MPR 300 alone or in combination with MPR 46, the relative level of newly synthesized cathepsin D was 2.3–2.4 higher than in control and MPR 46 fibroblasts.

Parallel to cathepsin D, β-glucuronidase was immunoprecipitated from cells and media. β-Glucuronidase is synthesized and secreted as a 72-kDa precursor, while the intracellularly retained polypeptides are processed to a 69-kDa mature form. In MPR 46/MPR 300 fibroblasts, 86% of the labeled β-glucuronidase was recovered in the secretions in contrast to 6% in controls. MPR 46 and MPR 300 fibroblasts accumulated in their secretions 10 and 69%, respectively, of the labeled β-glucuronidase (Fig. 4), indicating that missorting of β-glucuronidase and cathepsin D in MPR-deficient fibroblasts is similar.

Enlarged Lysosomes in MPR 46/MPR 300 Fibroblasts—The deficiency of a lysosomal enzyme is generally associated with the storage of the material that is the substrate of this enzyme. This may lead to the enlargement of lysosomes and the development of a lysosomal storage disease. In fact, one of the light microscopic hallmarks of MPR 46/MPR 300 cells distinguishing them from other cells was the presence of numerous phase-dense granular structures throughout their cytoplasm (Fig. 5A). Immunofluorescent staining for lamp-1, a transmembrane protein enriched in dense lysosomes, revealed that these granular structures are surrounded by lamp-1-positive membranes (Fig. 5B), thus characterizing them as lysosomes/endosomes. For comparison, the distribution and frequency of lamp-1-positive organelles in control fibroblasts are shown in Fig. 5C.

Cathepsin D in Lysosomes of MPR 46/MPR 300 Fibro-
The steady-state concentration of cathepsin D in MPR
2
2/MPR 300
2 cells varied between 10 and 20% of controls as
analyzed by Western blotting (Fig. 6). In normal fibroblasts,
the precursor and 30-kDa mature forms of cathepsin D are
minor species, with the bulk (85%) being represented by the
38–45-kDa intermediate forms. In double receptor-deficient
fibroblasts, the intermediate forms appear to be 15–20% of
the precursor form, and the mature form is barely detectable.
Furthermore, the intermediate form is 2 kDa larger in size
than in controls. This resembles findings in I-cell disease fibro-
blasts, in which the processed forms of cathepsin D are also
2 kDa larger in size due to defective proteolytic processing at the
carboxyl terminus (Hasilik, 1992).

To determine the subcellular location of cathepsin D, the
postnuclear supernatants of control and double receptor-defi-
cient fibroblasts were subjected to Percoll density gradient
centrifugation (Fig. 7). This separates dense membranes en-
riched in lysosomal markers (fraction 1) from more buoyant
membranes enriched in markers for the Golgi apparatus,
plasma membrane, and endosomes (fractions 4 and 5). In controls,
60–70% of the lysosomal acid phosphatase and \( \beta \)-hex-
osaminidase was associated with the dense and 15–20% with
the light membranes (Fig. 7, A and B). In MPR 46
2/MPR 300
2 fibroblasts, the fraction of both lysosomal markers associated
with dense membranes was reduced to 40–45%. In control
fibroblasts, the dense membranes contained the bulk of cathep-
sin D (80%) and essentially all of the 30-kDa mature form.
Similarly, in MPR 46
2/MPR 300
2 fibroblasts, the dense mem-
bres were enriched in proteolytically processed forms, while
the light membranes were enriched in precursor forms. Thus,
the residual amount of cathepsin D that is retained and pro-
teolytically processed in double receptor-deficient fibroblasts
appears to be associated with lysosomes.

To obtain direct evidence for a lysosomal localization of ca-
thepsin D in MPR 46
2/MPR 300
2 fibroblasts, cryosections were
double-immunolabeled for lamp-1 and cathepsin D (Fig. 8). In
control fibroblasts, the majority of cathepsin D (small gold) and
lamp-1 (large gold) label was associated with lysosomal profiles
that were filled with rather homogeneous electron-dense ma-
terial and multiple membranes. Profiles containing only one
The unique property of MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} fractions was analyzed by Western blotting. Given as percent of total gradient activity. Cathepsin D in the gradient fractions was analyzed by Western blotting. P, I, and M denote the precursor and intermediate and mature forms of cathepsin D.

**Fig. 7.** Subcellular fractionation of control and MPR-deficient mouse embryonic fibroblasts by Percoll density centrifugation. 
Postnuclear supernatants of cell homogenates were subjected to Percoll density centrifugation. After fractionation and removal of Percoll, the activity of lysosomal \(\beta\)-hexosaminidase and acid phosphatase was determined as described under "Materials and Methods." The activity is given as percent of total gradient activity. Cathepsin D in the gradient fractions was analyzed by Western blotting. P, I, and M denote the precursor and intermediate and mature forms of cathepsin D.

type of label were rarely seen (Fig. 8A). This was also true for MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} cells, in which two types of structures contained the bulk of the label (Fig. 8B). One type resembled the lysosomal profiles seen in controls, while the other was represented by large multivesicular structures filled with floccular electron-dense material. Occasionally, mitochondria were detectable inside these structures, suggesting an autophagic origin. Striking differences were found for the relative intensities of cathepsin D and lamp-1 labeling in the various structures (Table I). In the lysosome-like structures of controls, labeling for lamp-1 was 6 times higher, while in the lysosome-like structures, labeling for cathepsin D was 6 times higher. Surprisingly, the intensity of cathepsin D labeling seen in MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} fibroblasts did not reflect the 5–10 times lower content of cathepsin D compared with controls. Irrespective of the unexplained labeling intensities, these results provide clear evidence that a small fraction of cathepsin D is targeted to lysosomes in fibroblasts lacking MPR.

Pattern of Man-6-P Polypeptides Secreted by MPR-deficient Fibroblasts—The pattern of MPR ligands recovered in secretions of MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} fibroblasts is likely to reflect the pattern of Man-6-P-containing polypeptides synthesized by the cells. Secretions from controls or cells that are missing one of the two MPRs are depleted of those Man-6-P-containing polypeptides that have undergone MPR-dependent sorting. The unique property of MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} secretions in representing an unbiased collection of MPR ligands allows us to test whether Man-6-P polypeptides have different affinities for MPR 46 and MPR 300.

For this purpose, secretions of metabolically labeled MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} fibroblasts were passed over Affi-Gel 10 columns to which human MPR 46 or MPR 300 had been coupled. After extensive washing, bound ligands were eluted with Man-6-P and characterized by SDS-PAGE and fluorography. Since the two MPRs were reported to have a distinct pH dependence of binding above pH 6.5 (Hoflack et al., 1987; Distler et al., 1991), we examined the binding over a pH range of 5.4–7.4 (Fig. 9). Three aspects became apparent. First, the pH profile for the overall binding of Man-6-P polypeptides showed for both receptors an optimal binding between pH 6.2 and 6.6. Rechromatography of the material unbound at pH 6.6 revealed that >85% of the ligands had bound to either column during the first run. Second, the pattern of polypeptides bound to the MPR 46 or MPR 300 affinity column differed, and the differences were observed over the whole pH range tested. Receptor ligands with a pH optimum for binding below pH 6.2 or above pH 6.6 were not apparent. Third, none of the Man-6-P polypeptides bound exclusively to one type of MPR. Taken together, these data indicate that the Man-6-P polypeptides differ in their affinity for the two MPRs, but not to a degree that would prevent binding to either of the two MPRs.

Due to the differential affinities of Man-6-P polypeptides for either type of MPR, it was expected that their pattern would be different in secretions from either MPR 46\textsuperscript{2} or MPR 300\textsuperscript{1} fibroblasts. Secretions of MPR 46\textsuperscript{2} cells were expected to be enriched in Man-6-P polypeptides that bind preferentially to MPR 46, and conversely, secretions of MPR 300\textsuperscript{1} cells in those that bind preferentially to MPR 300.

The results shown in Fig. 10 demonstrate that this prediction was met only in part. While the frequency of Man-6-P polypeptides that bound to the receptor columns increased from ~1% in controls to 3–4.5% in single MPR-deficient fibroblasts to up to 7–8% in MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} fibroblasts, secretions from single MPR-deficient fibroblasts contained Man-6-P polypeptides that bound almost equally well to either type of receptor column.

Only few receptor ligands were markedly enriched in secretions of either MPR 46\textsuperscript{2} or MPR 300\textsuperscript{1} fibroblasts, e.g. secretions of MPR 46\textsuperscript{2} cells were enriched in a 43-kDa polypeptide. Conversely, a 68-kDa polypeptide was enriched in secretions of MPR 300\textsuperscript{1} cells. The 43- and 68-kDa polypeptides, however, apparently bound equally well to the MPR 46 and MPR 300 affinity columns (see Fig. 10). As predicted for the deficiency of both MPRs, the 43- and 68-kDa polypeptides were also present in secretions from MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} cells. It should be noted that the binding of Man-6-P polypeptides to MPR affinity columns might significantly differ from the binding to MPRs in vivo. This is indicated by the observation that the cathepsin D polypeptides present in secretions of MPR 46\textsuperscript{2}/MPR 300\textsuperscript{1} cells, only 26% were bound to the MPR 46 affinity column and only 14% to the MPR 300 affinity column (data not shown). In vivo, the bulk of cathepsin D is retained intracellularly as long as MPR 300 is expressed, indicating efficient binding to MPR 300 (see Fig. 4). Thus, the pattern of ligands identified by the affinity column approach may significantly differ from the pattern of ligands that bind in vivo to the MPRs in the Golgi/trans-Golgi network compartment.

**DISCUSSION**

Deficiency of MPRs and of Man-6-P Recognition Marker Results in a Similar Missorting of Soluble Lysosomal Proteins—In I-cell disease, the deficiency of N-acetylgalacosamine 1-phosphotransferase, the key enzyme required for the synthesis of Man-6-P residues on soluble lysosomal enzymes, is responsible for the lack of Man-6-P recognition markers on lysosomal proteins. As a result, the bulk of newly synthesized soluble lysosomal proteins is secreted. In I-cell fibroblasts, the excessive secretion leads to a marked intracellular deficiency of soluble lysosomal proteins, which results in defective lysosomal catabolism and storage of undigested material. Upon light microscopic exami-
nation, the enlarged lysosomes filled with storage material appear as phase-dense inclusions, from where I-cell disease (I = inclusion) acquired its name (for review, see Nolan and Sly (1989)).

The deficiency of MPRs was predicted to result in a similar mis-sorting of soluble lysosomal proteins and lysosomal dysfunction as the deficiency of Man-6-P recognition markers in I-cell disease. In fact, a comparison of I-cell fibroblasts and MPR 46⁻/MPR 300⁻ fibroblasts shows that this prediction is met. In both cell types, the bulk of newly synthesized soluble

| Cells         | Type of profile            | Gold particles counted | lamp-1 | Cathepsin D |
|---------------|----------------------------|------------------------|--------|-------------|
| Control       | Lysosome-like profiles     | 2479                   | 73     | 27          |
| MPR 40⁻/MPR 300⁻ | Lysosome-like profiles     | 2123                   | 12     | 88          |
|               | Autophagosome-like profiles | 2392                   | 86     | 14          |
lysosomal proteins is secreted. Intracellularly, a profound deficiency of these proteins is found, and the lysosomal compartment is enlarged due to storage material. These observations underline the critical function of MPRs for targeting of soluble lysosomal proteins. A similar conclusion was reached in a recent study related to the present one, in which MPR-deficient lysosomal proteins. A similar conclusion was reached in a recent study related to the present one, in which MPR-deficient fibroblasts were studied that were obtained by mating MPR+/− mice. The secretions were passed over Affi-Gel 10 columns coupled with human MPR 46 (●) or MPR 300 (○) at the respective pH between pH 5.4 and 7.4. The fraction of radioactivity that was bound and then eluted with 5 mM Man-6-P was calculated (upper panel). The pattern of polypeptides in the Man-6-P eluate was analyzed by SDS-PAGE and fluorography (lower panel).

Residual Targeting of Soluble Lysosomal Proteins in MPR-deficient Fibroblasts—For cathepsin D and β-glucuronidase, the fraction of newly synthesized polypeptides retained in MPR-deficient fibroblasts was 4 and 14%, respectively. This was sufficient to maintain, for both enzymes, steady-state concentrations of ~20% of that in controls. Several mechanisms may help to maintain steady-state concentrations that are higher than expected from the residual targeting, e.g., the half-life of lysosomal proteins may be prolonged in MPR-deficient fibroblasts due to the deficiency of lysosomal proteinases. For cathepsin D, a 2–3-fold higher rate of synthesis appears to contribute to the higher residual steady-state concentration. This increase in synthesis correlated with the deficiency of MPR 300. It will be of interest to see whether expression of MPR 300 in these cells will restore the rate of synthesis of cathepsin D to a normal level.

Biochemical and morphological data demonstrated that the cathepsin D retained in MPR-deficient cells was associated with lysosome-like structures. This raises the question by which mechanism the residual amount of lysosomal proteins found in lysosomes of MPR-deficient fibroblasts is transported to lysosomes. Signal receptor-dependent and signal receptor-independent (default) pathways, such as missorting at the trans-Golgi network, fluid-phase endocytosis, or cell-cell contact-mediated transfer of lysosomal proteins, may account for this residual targeting.

MPR 46 and MPR 300 Have Distinct but Overlapping Affinities for Lysosomal Proteins—The amount of Man-6-P ligands in secretions from MPR-deficient fibroblasts was about twice as high as in single MPR-deficient fibroblasts. Surprisingly, all Man-6-P polypeptides that were detectable in secretions of MPR-deficient fibroblasts were also found in secretions of MPR 46−/+ and/or MPR 300−/+ fibroblasts. This clearly indicates that at the normal level of MPR expression, neither MPR 46 nor MPR 300 can substitute for the other MPR with respect to the targeting of lysosomal proteins. As a result, each of the Man-6-P polypeptides is found in the secretions of both single MPR-deficient fibroblasts.

Support for transport of distinct complements of lysosomal proteins by the two MPRs came from the comparison of the Man-6-P polypeptides accumulating in secretions of MPR 46−/+ and MPR 300−/+ fibroblasts. Man-6-P polypeptides preferentially accumulating in secretions of MPR 46−/+ or MPR 300−/+ fibroblasts were identifiable. This clearly demonstrates that the two MPRs have distinct affinities for Man-6-P polypeptides, which, in vivo and in vitro, results in the binding of a different pattern of lysosomal proteins. This was also supported by the distinct effect that loss of either MPR had on the sorting of cathepsin D and β-glucuronidase. It should be noted, however, that we failed to identify lysosomal proteins that bound in vitro or in vivo exclusively to either type of MPR.

Why Do Mammalian Cells Possess Two Types of MPR?—The data presented here, as well as analogous observations in MPR-deficient fibroblasts due to the disruption of MPR 46
alleles and maternal inheritance of the T Hp allele (Ludwig et al., 1994), demonstrate that in vivo the two receptors are responsible for the transport of two sets of ligands that are distinctly composed, but made up of the same lysosomal proteins.

Due to the overlapping affinities, it is conceivable that the two MPRs could substitute for each other with regard to their targeting function for lysosomal proteins provided that their expression level is high enough. There is already experimental evidence that overexpression of MPR 46 cannot compensate for the targeting function of MPR 300. In cells with undetectable levels of MPR 300, overexpression of MPR 46 led only to a partial correction of the missing, while expression of MPR 300 in these cells fully corrected the missing (Watanabe et al., 1990; Johnson and Kornfeld, 1992). This can be explained by the observation that part of the ligands that are bound by MPR 46 are secreted rather than targeted to lysosomes (Chao et al., 1990). Thus, the two receptors bind different complements of lysosomal enzymes and deliver them to sites that are different in part. Whether overexpression of MPR 300 can compensate for the loss of MPR 46 needs to be investigated.

If the two MPRs transport the same lysosomal proteins, although at different ratios, what parameter determines whether a lysosomal protein binds to MPR 46 or MPR 300? Available evidence suggests that lysosomal proteins encounter and bind to MPR 46 and MPR 300 in the trans-Golgi network (Klumperman et al., 1993). If so, structural differences in the Man-6-P recognition markers on lysosomal proteins must account for the binding of distinct complements of lysosomal proteins to the two MPRs. The Man-6-P recognition markers in lysosomal proteins indeed display a high structural heterogeneity. The number of phosphate groups in a high mannose oligosaccharide can vary (up to two), as well as the position of the phosphorylated mannose residues (five different positions) and the extent to which the phosphate groups persist in their biosynthetic precursor form as diesters (for review, see von Figura and Hasilik (1986)). Earlier studies have shown that the affinities of the two MPRs for high mannose oligosaccharides containing two phosphomonoester or phosphodiester groups differ significantly (Hoflack et al., 1987; Tong and Kornfeld, 1989).

Thus, the heterogeneity of Man-6-P recognition markers in combination with the inability of a single type of MPR to bind all the Man-6-P polypeptides with sufficient affinity to ensure their retention and targeting may have been one of the reasons that necessitated the evolution of two types of MPR. Theoretically, a higher efficiency of targeting may have also been obtained by adopting the N-acetylglucosamine 1-phosphotransferase to yield products of higher homogeneity and yet sufficient affinity for a single type of MPR. The N-acetylglucosamine 1-phosphotransferase, which is the key enzyme for the synthesis of Man-6-P recognition markers, has two recognition sites. Current models propose that one recognition site of the phosphotransferase interacts with an extended surface area of a lysosomal protein. The other recognition site then has to interact with oligosaccharides at multiple sites at the surface of a lysosomal protein on which the N-acetylglucosamine 1-phosphate residue is transferred (Cantor et al., 1992; Cantor and Kornfeld, 1992). It is conceivable that evolutionary pressure has focused on establishing the specificity of the N-acetylglucosamine 1-phosphotransferase for the heterogenous group of lysosomal proteins at the expense of the homogeneity of the Man-6-P recognition marker.

After having established two types of MPRs with complementary affinities for the diverse Man-6-P recognition markers, the two MPRs may have adopted additional functions, be it the endocytosis of Man-6-P ligands or the binding of IGF-II, both of which are characteristic of MPR 300. The acquisition of IGF-II binding appears to have been a fairly recent event in evolution since MPR 300 from chicken or frog fails to bind IGF-II (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989).

Taken together, the studies on the single and double MPR-deficient fibroblasts led to the proposal that the heterogeneity of Man-6-P recognition markers necessitated more than one type of MPR to ensure sufficient retention and targeting of the multiple forms of soluble lysosomal proteins. The heterogeneity of the Man-6-P markers may in turn have been the result of compromising the needs for distinguishing lysosomal from non-lysosomal glycoproteins by the N-acetylglucosamine 1-phosphotransferase and yet allowing phosphorylation of variably located oligosaccharides.

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