The Phosphorylation Pattern of Oligosaccharides in Secreted Procathepsin D Is Glycosylation Site-specific and Independent of the Expression of Mannose 6-Phosphate Receptors*

(Received for publication, August 20, 1996, and in revised form, October 15, 1996)

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Mammalian cells contain two types of mannose 6-phosphate receptors (MPR), MPRs 46 and 300, that contribute with variable efficiency to the sorting of individual lysosomal proteins. To evaluate the role of phosphorylated oligosaccharides for the sorting efficiency by either of the two receptors, the structure of phosphorylated oligosaccharides on lysosomal proteins escaping sorting in cells lacking MPR 46 and/or MPR 300 was analyzed. Procathepsin D was chosen as a model because it is sorted efficiently via MPR 300 and poorly via MPR 46 and contains a distinct and highly heterogeneous mixture of phosphorylated oligosaccharides at either of its two N-glycosylation sites. Both MPRs 46 and 300 were found to have a minor but distinct preference for forms of procathepsin D and other lysosomal proteins containing oligosaccharides with two phosphomonoesters. However, the phosphorylation of oligosaccharides in procathepsin D and other lysosomal proteins that escape sorting in control cells or in cells lacking MPR 46 and/or MPR 300 was strikingly similar, and oligosaccharides with two phosphomonoesters represented the major oligosaccharide species. We conclude from these results that the position of the phosphate groups, the structure of the underlying oligosaccharide, and/or the polypeptide backbone of lysosomal proteins have major roles in determining the affinity to MPRs.

Mammalian cells express two different mannose 6-phosphate receptors (MPR) with apparent molecular masses of 46,000 Da (MPR 46) and 300,000 Da (MPR 300). The two receptors mediate the targeting of mannose 6-phosphate (Man-6-P)-containing lysosomal proteins to lysosomes (1, 2). A variable fraction, generally less than 20% of the newly synthesized lysosomal proteins, escapes targeting to lysosomes and is secreted. This is generally thought to reflect a failure of binding to MPRs in the secretory route, but there is also evidence that at least part of the secretion is mediated by MPR 46 (3). Loss of either MPR is associated with partial missorting of newly synthesized lysosomal proteins, which then are secreted (4–7). In addition to sorting, MPR 300 mediates internalization of extraacellular lysosomal proteins and of the non-phosphorylated insulin-like growth factor II. It is not clear why cells express two types of MPRs. One possibility is that the two receptors interact with different lysosomal proteins or different isoforms of a lysosomal protein. The analysis of the lysosomal proteins that escape sorting in cells lacking either MPR 46 or MPR 300 has indicated that most of the lysosomal proteins are partially missorted if either type of MPR is missing (8, 9). The extent of missorting, however, depends on the type of receptor, suggesting that the majority of lysosomal proteins interacts with both MPRs although with different affinities.

It has been suggested that structural differences in the Man-6-P-containing oligosaccharides in lysosomal proteins contribute to their different sorting efficiencies by MPRs (9). Indeed, phosphorylated oligosaccharides in lysosomal proteins are structurally a highly heterogenous population containing one or two phosphate groups. The phosphate groups may be present as diesters (interspaced between C1 of a covering N-acetylglucosamine and C6 of mannose) or as monoesters. In monophosphorylated oligosaccharides, the phosphate group may be attached to 3 of the 5 mannose residues of the α-1,6 branch. In diphasphorylated oligosaccharides, the second phosphate may be attached to 2 of the 3 mannose residues of the α-1,3 branch. Furthermore, the number and linkages of non-phosphorylated mannose residues can vary, and in monophosphorylated oligosaccharides, the α-1,3 branch may be replaced by a branch containing sialic acid residues (for review, see Ref. 10). Both MPRs have the highest affinity for oligosaccharides with two phosphomonoester groups (11–13).

In the present study, we analyzed the phosphorylated oligosaccharides of the lysosomal proteinase procathepsin D, accumulating in the secretions of mouse embryonic fibroblasts (MEF), that express physiological levels of either MPR 46 or MPR 300. The oligosaccharides were compared with those of procathepsin D secreted by MEF lacking or expressing both MPRs. Procathepsin was chosen because its sorting efficiency greatly depends on the type of receptor that is expressed. It is sorted poorly by cells expressing only MPR 46 and efficiently by cells expressing MPR 300 (8, 9). Secretions of MEF expressing MPR 300 and/or MPR 46 should be depleted in procathepsin D forms that are targeted by the receptors that are expressed. Analysis of the oligosaccharides should, therefore, reveal whether MPRs 46 and 300 interact with procathepsin D forms, which differ in their phosphorylation. Oligosaccharides from the two sites were analyzed separately since the bilobar procathepsin D contains in each of its two lobes one N-glycosylation site that might differ in its phosphorylation pattern.

The analysis of newly synthesized procathepsin D revealed that a heterogeneous mixture of phosphorylated oligosaccharides is attached to either of the two N-glycosylation sites of procathepsin D and that the phosphorylated oligosaccharides at the two sites are clearly distinct. Unexpectedly, the pattern of phosphorylated oligosaccharides in procathepsin D secreted...
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by MEF expressing MPR 46 and/or MPR 300, or neither MPR, was strikingly similar. This holds true also for the phosphorylated oligosaccharides of lysosomal proteins secreted by immortalized MEF overexpressing either MPR 46 or MPR 300. We conclude from these results that the presence of oligosaccharides with two phosphomonoesters is not sufficient for efficient sorting and that other factors, e.g., the polypeptide backbone of lysosomal proteins, determine the sorting efficiency via MPRs.

MATERIALS AND METHODS

Cells—MEF expressing both MPRs, either MPR 46 or MPR 300, and no MPR were obtained as described previously (9). Immortalized mouse embryonic fibroblasts stably transfected with MPRs 46 and/or 300, respectively, are described elsewhere (14). All cells were maintained in Dulbecco's minimal essential medium supplemented with Glutamax I (Life Technologies, Inc.) and 10% fetal calf serum.

Purification of [2-3H]Mannose-labeled Procathepsin D—MEF on 10-cm dishes were incubated in minimal essential medium containing 0.5 mM glucose and 5% dialyzed fetal calf serum for 1 h and then labeled for 16 h with n-[2-3H]mannose (Amersham Life Science, Inc., 666 GBq/mm mol) in the same medium. Proteins of the conditioned media were concentrated by amonium sulfate precipitation (50% w/v). Subsequent purification of labeled procathepsin D was performed according to Conner (15) with slight modifications. Briefly, the ammonium sulfate precipitate was dialyzed against buffer A (0.1% Triton X-100, 0.4 mM NaCl in 0.1 mM sodium formate, pH 3.5) and then applied to a 0.5-ml preparative Acarose column (Sigma). The material was mixed by rotating over night at 4 °C. The column was then washed with 4 volumes of buffer A, with 6 volumes of buffer A containing 6 mM urea, and again with 4 volumes of buffer A. Finally, bound [2-3H]procathepsin D was eluted by 0.2% Triton X-100, 0.4 mM NaCl in 20 mM Tris-HCl, pH 8.3. Purity of procathepsin D preparation was confirmed by SDS-PAGE and fluorography.

Reductive Carboxymethylation, Tryptic Digestion, and Separation of Tryptic Peptides—Labeled procathepsin D was mixed with 80 μg of mouse liver cathepsin D purified according to Claussen et al. and concentrated in ultra microfuge. The samples were adjusted to 6 mM guanidine hydrochloride, 10 mM EDTA, and 50 mM dithiothreitol in 400 mM Tris-HCl, pH 8.6. Reduction was carried out under an oxygen-resistant reaction in darkness. To complete the reaction, a second cycle with 65 mM Tris-base for subsequent QAE-Sephadex fractionation (19). Samples were immediately frozen, lyophilized, and resuspended in 6 M NaCl in 0.1 M sodium formate, pH 3.5. Fractions of 1-ml aliquots were collected and monitored for radioactivity. For further analysis, peak fractions were pooled, lyophilized, and desalted on a Sephadex G-25 column.

Oligosaccharide-samples that had been treated with HCl, alkaline phosphatase, or neuraminidase (see below) were eluted with a stepwise gradient of NaCl (20, 60, 90, and 140 mM) in 2 mM Tris-base.

Mild Acid Hydrolysis—Lysophosphorylated oligosaccharides were resuspended in 1 ml of 0.01 M HCl and heated at 100 °C for 30 min. The samples were immediately frozen, lyophilized, and resuspended in 6 M NaCl in 0.1 M sodium formate, pH 3.5. Separation of labeled oligosaccharides was accomplished by using a QAE-Sephadex column (Pharmacia, Uppsala, Sweden). The samples were dialyzed against 50 mM Tris-HCl, pH 8.0. Oligosaccharides were resuspended therein and incubated at 37 °C for 16 h (19). For digestion with Vibrio cholerae neuraminidase (Boehringer Mannheim), oligosaccharides were resuspended in 50 mM sodium acetate, 9 mM CaCl2, 150 mM NaCl, pH 5.5, and incubated for 16 h at 37 °C.

Metallic Labeling of Cells and Immunoprecipitation of Cathepsin D—MEF on 35-mm dishes were incubated in methionine-free medium for 1 h and then labeled with [35S]methionine (Amersham Life Science, Inc.) in the same medium containing 5% dialyzed fetal calf serum. During the following chase for 6 h, the medium was supplemented with 0.25 mg/ml l-methionine. Immunoprecipitation from cells and media was carried out as described previously (20) with antiserum specific for mouse cathepsin D (9). Densitometric quantification of cathepsin D was done using a Hewlett-Packard ScanJet 4c/T (Palo Alto, CA) and the program WinCam 2.2 (Microsoft).

Preparation of Oligosaccharides Derived From All Secre ted Man-6-P-containing Proteins—For the production of Man-6-P-containing lysosomal proteins, immortalized mpr- MEF overexpressing MPR 46 or MPR 300 were labeled as described above. The secretions were concentrated by ammonium sulfate precipitation (50% w/v). To purify Man-6-P-containing lysosomal proteins on MPR-columns, the precipitates were dialyzed against binding buffer (50 mM Tris-base, pH 7.4, containing 5% dialyzed fetal calf serum). The material was solubilized by heating for 5 min at 95 °C in 1% SDS, 125 mM Tris-HCl, pH 6.8, and 20% glycerol. Citrate-phosphate, pH 5.6, was added to a final concentration of 50 mM, and the samples were incubated with 3 units of endo-H at 37 °C for 40 h with addition of the same amount of endo-H after 16 h. Separation of endo-H-released oligosaccharides from peptides were performed as described above.

RESULTS

Characterization of the Biosynthetic Pattern of Oligosaccharides in Procathepsin D—Cathepsin D contains in each of its two lobes one N-glycosylation site, which corresponds in mouse cathepsin D to Asp-134 (site I) and Asp-261 (site II). To analyze the pattern of phosphorylated oligosaccharides in cathepsin D, we chose as a source procathepsin D from secretions of immortalized mouse embryonic fibroblasts that lack MPR 46 and MPR 300 (mpmr- MEF). These cells secrete more than 90% of the newly synthesized cathepsin D as procathepsin D. The procathepsin D in these secretions is therefore representative for its oligosaccharides should reflect the biosynthetic pattern generated in the Golgi cisternae unchanged by secondary modifications due to lysosomal phosphatases and glycosidases. The oligosaccharide chains in procathepsin D were metabolically labeled by incubation of mpr- MEF with [2-3H]mannose. After purification from the secretions, the [3H]-labeled procathepsin D (see
FIG. 1. Separation by reverse phase-HPLC of tryptic peptides from [3H]procathepsin D. Shown are the absorbance at 210 nm (solid line), the gradient from 0 to 90% acetonitrile (dotted line), and radioactivity (stippled bars). The positions of the glycopeptides containing the glycosylation sites I and II of the amino and carboxyl lobes of procathepsin D are indicated by horizontal bars. The inset illustrates the radiochemical purity of the [3H]procathepsin D (arrow) analyzed by SDS-PAGE and fluorography.

FIG. 2. Separation of endo-H-sensitive [3H]-labeled oligosaccharides from glycosylation sites I and II of procathepsin D by QAE-Sephadex anion exchange chromatography. Anionic [3H]-labeled oligosaccharides were eluted with a linear gradient from 0 to 150 mM NaCl in 2 mM Tris-base, pH 9.5. The fractions were analyzed for radioactivity (filled circle) and conductivity (dotted line). Top panel, [3H]-labeled oligosaccharides released from glycosylation site I. Bottom panel, [3H]-labeled oligosaccharides released from glycosylation site II. The fractions containing uncharged oligosaccharides or oligosaccharides with 1–4 negative charges were pooled as indicated by bars. Below the bars, the percentage of radioactivity present in the fraction is given.

inset of Fig. 1) was mixed with unlabeled cathepsin D purified from mouse liver, subjected to reductive carboxymethylation, and digested with trypsin. Two major [3H]-labeled glycopeptide fractions were obtained after separation by reverse phase-HPLC (indicated by horizontal bars in Fig. 1), which were identified by amino acid sequencing as the glycopeptides carrying the N-glycosylation sites I and II, respectively.

Digestion with endo-H released, from either of the two glycopeptides, more than 95% of the radioactivity as [3H]-labeled oligosaccharides, which were separated from (glyco-)-peptides by reverse phase chromatography. The oligosaccharides were then separated according to their charge at pH 9.5 by QAE-anion-exchange chromatography.

The oligosaccharides attached to site I separated into species lacking a negative charge (I-0, 3%) or carrying 1 (I-1, 29%), 2 (I-2, 55%), or 3 (I-3, 13%) negative charges (Fig. 2, top). The percentages in the brackets refer to the radioactivity recovered in these species. 97% of the oligosaccharides attached to site II were anionic, carrying 1 (II-1, 1%), 2 (II-2, 24%), 3 (II-3, 15%), or 4 (II-4, 57%) negative charges (Fig. 2, bottom).

The anionic oligosaccharides were further characterized by subjecting them to mild acid hydrolysis, treatment with alkaline phosphatase or neuraminidase. [3H]-labeled oligosaccharides of fraction II-3 (see Fig. 2, bottom panel) were subjected to rechromatography on QAE-Sephadex either untreated (panel A), after mild acid hydrolysis (panel B), after treatment with alkaline phosphatase (panel C), or after treatment with neuraminidase (panel D). [3H]-labeled oligosaccharides with 1, 2, 3, or 4 negative charges were eluted with a stepwise gradient of 20, 60, 90, and 140 mM NaCl in 2 mM Tris-base (indicated at the top). The fractions were analyzed for radioactivity (filled circle).

FIG. 3. Analysis of [3H]-labeled oligosaccharides by mild acid hydrolysis and treatment with alkaline phosphatase or neuraminidase. [3H]-labeled oligosaccharides of fraction II-3 (see Fig. 2, bottom panel) were subjected to rechromatography on QAE-Sephadex either untreated (panel A), after mild acid hydrolysis (panel B), after treatment with alkaline phosphatase (panel C), or after treatment with neuraminidase (panel D). [3H]-labeled oligosaccharides with 1, 2, 3, or 4 negative charges were eluted with a stepwise gradient of 20, 60, 90, and 140 mM NaCl in 2 mM Tris-base (indicated at the top). The fractions were analyzed for radioactivity (filled circle).

The anionic oligosaccharides further characterized by subjecting them to mild acid hydrolysis, treatment with alkaline phosphatase or neuraminidase, and followed by anion exchange chromatography. This allows us to differentiate whether a negative charge is due to the presence of a phosphomonoester or a phosphodiester group. All oligosaccharides of fraction II-3 were converted into neutral species when they were first subjected to mild acid hydrolysis and then to digestion with alkaline phosphatase (not shown). Taken together, these results indicate that 70% of the oligosaccharides of fraction II-3 carry one phosphomonoester group and one sialic acid residue. The
remaining 30% contain one phosphodiester group and two acid-labile negative charges, one of which is a sialic acid residue. The other may represent a second sialic acid residue resistant to V. cholerae neuraminidase or an unknown acid-labile anionic group.

Table I summarizes the data on oligosaccharides released from sites I and II by endo-H. Of the oligosaccharides released from site I located within the amino-terminal lobe, 92% are phosphorylated. All phosphorylated oligosaccharides carry a single phosphate group that in 65% is present as a phosphomonoester and in 35% is present as a phosphodiester. About one-fifth of the phosphorylated oligosaccharides (21%) are of hybrid nature as indicated by the presence of sialic acid. Phosphodiester groups were slightly more frequent (42%) in the fraction of hybrid oligosaccharides than in the fraction of high mannose oligosaccharides (33%).

Of the oligosaccharides attached to site II located within the carboxyl lobe, 97% are phosphorylated. The major difference of the oligosaccharides attached to site I is the presence of oligosaccharides with two phosphomonoester groups, which represent the majority (57%) of the phosphorylated oligosaccharides. It should be noted that all phosphate groups in the diphasphorylated oligosaccharides are present as monoesters. Similar to site I, 65% of the monophosphorylated oligosaccharides at site II contain a phosphomonoester, and 35% contain a phosphodiester. Hybrid oligosaccharides represent about one-fifth of the phosphorylated oligosaccharides (23%). Compared with site I, this value is remarkably high since phosphorylated hybrid oligosaccharides can only be generated from monophosphorylated oligosaccharides (10). If referred to the latter, about 58% of the monophosphorylated oligosaccharides at site II are of hybrid nature. This frequency is almost 3 times as high as at site I (21%). Moreover, phosphodiesters were found exclusively in the group of hybrid oligosaccharides.

In summary, mouse cathepsin D is characterized by a glycosylation site-specific phosphorylation pattern. Oligosaccharides with two phosphomonoesters, which mediate high affinity binding to MPRs (11–13), are found only among those attached to the carboxyl lobe of cathepsin D.

Oligosaccharides in Procathepsin D Secreted by MEF Expressing MPR 46 and/or MPR 300—As a source for procathepsin D secreted by cells lacking either MPR 46 or MPR 300, we chose non-immortalized primary MEF (9). Primary MEF with endogenous MPRs 46 and 300 are targeting procathepsin D with high efficiency to lysosomes. Only 7% of the newly synthesized procathepsin D is secreted. Deficiency of MPR 46 doubles the secreted fraction to 14% (7–30%) while deficiency of MPR 300 increases the secreted fraction to 88% (86–94%). Primary MEF lacking both receptors secrete 93% (91–96%) of the 3H-labeled procathepsin D. The values given for the secretions represent the mean and the range of four determinations, one of which is shown in Fig. 4. In this figure, it is also shown that the lowering of glucose to 0.5 mM as performed during met abolic labeling with [2-3H]mannotose does not affect the sorting efficiency. The oligosaccharides in procathepsin D secreted by MEF expressing MPR 46 and/or MPR 300 should reveal whether MPR 46 and/or MPR 300 target subpopulations of procathepsin D to lysosomes, which differ in their phosphorylation. Since the oligosaccharides attached to the amino and carboxyl lobes have distinct phosphorylation patterns, the oligosaccharides attached to sites I and II were analyzed separately to also detect minor differences that would be obscured if oligosaccharides from both sites were mixed.

The oligosaccharides released by endo-H from the glycosylation sites I and II were separated by QAE-Sephadex ion exchange chromatography. The distribution of the radioactivity among oligosaccharide species with 0–4 negative charges is accurately to also detect minor differences that would be obscured if oligosaccharides from both sites were mixed.

The oligosaccharides released by endo-H from the glycosylation sites I and II were separated by QAE-Sephadex ion exchange chromatography. The distribution of the radioactivity among oligosaccharide species with 0–4 negative charges is comparable with that determined for the respective fractions of procathepsin D secreted by immortalized mpr−/−MEF (see Table I).

The charge pattern of oligosaccharides from glycosylation site I, which are only monophosphorylated, was independent of

TABLE I

| Oligosaccharide fraction | Frequency of oligosaccharides carrying phosphomonoester group | Frequency of oligosaccharides carrying phosphodiester group | % of total |
|--------------------------|------------------------------------------------------------|-----------------------------------------------------------|------------|
| I-0                      | 3                                                          | ND                                                        | ND         |
| I-1                      | 5                                                          | ND                                                        | 24         |
| I-2                      | ND                                                        | 49                                                        | 6          |
| I-3                      | ND                                                        | 11                                                        | 2          |
| ∑ of I-0 to I-3 (see Fig. 2) | 8                                                          | 60                                                        | 32         |
| II-0                     | 3                                                          | ND                                                        | ND         |
| II-2                     | ND                                                        | 16                                                        | 8          |
| II-3                     | ND                                                        | 10                                                        | 5          |
| II-4                     | ND                                                        | 57                                                        | 57         |
| ∑ II-0 to II-4 (see Fig. 2) | 3                                                          | 26                                                        | 13         |

a The values refer to the radioactivity as percentage of the radioactivity in endo-H-sensitive oligosaccharides attached to site I or site II, respectively.

b Hybrid oligosaccharides carrying one sialic acid residue in addition to the phosphate group indicated.

c Hybrid oligosaccharides carrying one sialic acid residue and 1 non-identified acid-labile negative charge in addition to the phosphate group indicated.

d ND, not detectable or 0.

FIG. 4. Sorting of cathepsin D in primary MEF lacking MPR 46 and/or MPR 300 in control MEF. Primary MEF expressing the MPR indicated above the lanes were metabolically labeled with [35S]methionine in the presence of 0.5 or 5 mM glucose. Cathepsin D was immunolated from extracts of cells (C) and media (M) and separated by SDS-PAGE. The positions of procathepsin D (P) and the intermediate (I) and mature (M) forms of cathepsin D are indicated. The fraction of cathepsin D recovered in the medium is given below the lanes.
the type of MPR expressed by the MEF. For the charge pattern of oligosaccharides from glycosylation site II, which contain mostly two phosphomonoester groups, a slight but notable difference in the frequency of oligosaccharides with 4 negative charges (carrying two phosphomonoester groups) was noted. The relative frequency increased from 57% in control MEF, via 63% in MEF expressing only MPR 300, to 68% in MEF expressing only MPR 46. This increase is mainly at the expense of oligosaccharides with 2 negative charges (fraction II-2). The latter are monophosphorylated with a phosphomonoester to phosphodiester ratio of 2:1. Apparently the procathepsin D form that escape sorting via MPR 46 or MPR 300 are enriched at site II in monophosphorylated and depleted in diprophosphorylated oligosaccharides.

MEF deficient of MPR 46 secrete twice as much procathepsin D than do control MEF. Procathepsin D secreted by MPR 46-deficient MEF is therefore likely to represent a 1:1 mixture of procathepsin D that escapes sorting by either type of MPR and such that is normally sorted via MPR 46. Since the charge pattern of oligosaccharides in procathepsin D secreted by control MEF and MPR 46-deficient MEF is known, the charge pattern for oligosaccharides in the subpopulation of procathepsin D that is normally sorted via MPR 46 can be calculated. For site II, the predicted frequency of oligosaccharides with 2 and 4 negative charges is 14 and 69%, respectively. These values are very close to those (16 and 65%) observed in procathepsin D that escapes sorting in MPR 300-deficient MEF (see Table II) and normally would be sorted by this receptor.

In summary, these data show that the phosphorylation pattern of oligosaccharides released from sites I and II is strikingly similar for the forms of procathepsin D that normally would be sorted by this receptor.

Oligosaccharides in Man-6-P-containing Lyososomal Proteins Accumulating in Secretions of mpr−/− MEF Overexpressing MPR 46 or MPR 300—The analysis of procathepsin D had shown that the phosphorylation pattern of the secreted proenzyme was only marginally dependent whether the cells express MPR 46 and/or MPR 300 or lack both MPRs. To extend this analysis to other lysosomal proteins, the secretions of immortalized MEF lacking both MPRs (mpr−/− MEF) or reexpressing MPR 46 (mpr−/− MEF/MPR 46) or MPR 300 (mpr−/− MEF/MPR 300) were used as sources for lysosomal proteins. Reexpression of MPR 46 decreases the secretion of lysosomal proteins to about 40% of that in mpr−/− MEF and reexpression of MPR 300 to about 10% (14).

After metabolic labeling with [2-3H]mannose, the Man-6-P-containing lysosomal proteins were isolated from the secretions by an MPR 46/MPR 300 affinity chromatography. The oligosaccharides sensitive to endo-H were separated by anion exchange chromatography. The charge pattern of the anionic oligosaccharides was strikingly similar, irrespective of whether they were derived from MEF lacking both MPRs or overexpressing MPR 46 or MPR 300 (Fig. 5). Of the oligosaccharides, 90–91% were negatively charged carrying either 1 (2–3%), 2 (22–24%), 3 (6–9%), or 4 (56–60%) negative charges.

The ratio of di- and monophosphorylated oligosaccharides (determined by ion exchange chromatography after mild acid hydrolysis) revealed minor differences between the three genotypes. The ratio was highest in MPR-deficient cells (56:32), intermediate in MPR 46-expressing cells (54:33), and lowest in MPR 300-expressing cells (51:35). This is consistent with the assumption that both MPRs 300 and 46 have a minor preference for oligosaccharides with two phosphomonoesters. These oligosaccharides are less abundant in secretions of MEF expressing MPR 300 than in MEF expressing MPR 46 since the former retain 90% and the latter only 60% of the newly synthesized lysosomal proteins.

**DISCUSSION**

In this study, the phosphorylated oligosaccharides of procathepsin D and other lysosomal proteins that escape sorting and are secreted by cells missing MPR 46 and/or MPR 300 were analyzed. The structure of these oligosaccharides should pro-
vide information whether, in vivo, the two MPRs prefer distinct sets of phosphorylated oligosaccharides.

**Phosphorylation of Procathepsin D**—To analyze the phosphorylation of cathepsin D at its two glycosylation sites, procathepsin D from secretions of MPR-deficient MEFs was utilized. This material represents more than 90% of newly synthesized procathepsin D, and its glycosyl moieties are not modified by lysosomal hydrolases.

Analysis of endo-H-releasable oligosaccharides (more than 90% at either site) revealed an asymmetric phosphorylation pattern. More than 90% of the oligosaccharides at sites I and II were phosphorylated, but oligosaccharides with two phosphate groups were restricted to site II. About 60% of the procathepsin D polypeptides contain in their carboxyl lobe an oligosaccharide with two phosphomonoester groups in combination with monophosphorylated oligosaccharides in their amino lobe. The remaining 40% of procathepsin D contain a monophosphorylated oligosaccharide in either lobe. Phosphodiesters were recovered only in monophosphorylated oligosaccharides, suggesting that presence of the second phosphate group favors the interaction of the uncovering α-N-acetylglucoaminidase with phosphorylated oligosaccharides.

Preferred phosphorylation of specific N-glycosylation sites has been observed earlier for the α- and β-subunits of β-hexosaminidase (21, 22), β-glucuronidase (23, 24), and arylsulfatase A (25). Human cathepsin D expressed in frog oocytes was shown to be enriched at the carboxyl lobe in oligosaccharides with two phosphates and at the amino lobe in oligosaccharides with one phosphate (18). This indicates that the mechanisms controlling the transfer of either one or two N-acetylglucoamin 1-phosphate groups by the phosphotransferase onto oligosaccharides at sites I and II of procathepsin D are conserved between mouse and frog.

**MPR 46 and MPR 300 Have a Minor Preference for Procathepsin D and Other Lysosomal Proteins with Oligosaccharides Containing Two Phosphomonoesters**—To answer the question of whether MPR 46 or MPR 300 interact with subpopulations of lysosomal proteins that differ in their phosphorylation, the oligosaccharides of procathepsin D in secretions from primary MEF that lack either MPR 46 or MPR 300 or both were analyzed. Primary MEF have the advantage of expressing physiological levels of the remaining MPR but have the disadvantage of being genetically not identical. These cell lines are derived from outbred mice with a C57 BL/6J and 129 SvJ background (9).

Analysis of the procathepsin D secreted by such cells indicated that loss of either MPR leads to a minor, but notable enrichment in the secretions of cathepsin D forms with two phosphomonoesters at glycosylation site II. In consideration of the different sorting efficiencies of MPRs 300 and 46 for procathepsin D, our results indicate that both receptors share the preference for procathepsin D forms with two phosphomonoesters at site II.

A similar analysis as for procathepsin D was performed for the bulk of lysosomal proteins. The latter were isolated from secretions of immortalized MPR-deficient MEF and such cells reexpressing either MPR 46 or MPR 300 (14). This had the advantage that lysosomal proteins were compared from cells with an identical genetic background except for the type of MPR. Moreover, the level of reexpression was deliberately chosen high to minimize the likeliness that sorting of lysosomal proteins was compromised by limiting receptor levels.

Also for the bulk of lysosomal proteins, a slight, but notable, preference for forms containing oligosaccharides with two phosphomonoesters was noted for both types of MPR. Again, the intermediate effect of MPR 46 expression on the frequency of oligosaccharides with two phosphomonoesters can be explained by its intermediate effect on the sorting efficiency for lysosomal proteins.

In a similarly designed study, Munier-Lehmann et al. (26) recently observed a preference of MPR 300 for the sorting of lysosomal proteins with oligosaccharides containing two phosphomonoesters. Different from the present study, a preferred interaction of MPR 46 with lysosomal proteins containing a single phosphomonoester was noted. At present, it is not possible to explain the variance between the two studies. The differences extend also to other parameters, such as the frequency of lysosomal proteins in the secretions of MEF and of oligosaccharides with two phosphomonoesters, for both of which significantly higher values were observed in this study.

**Secretion of Lysosomal Proteins by MEF Expressing Physiological Levels of MPRs 46 and 300**—One of the unexpected observations made in this study was the similarity of the phosphorylated oligosaccharides in procathepsin D in the secretion of control MEF and MPR-deficient MEF. The minor preference of the two MPRs for procathepsin D containing oligosaccharides with two phosphomonoesters decreased the relative frequency of these oligosaccharides at glycosylation site II from 68%, in secretions that contain almost 90% of the newly synthesized procathepsin D (MPR 300− MEF), to 57%, in secretions from control MEF that contain less than 90% of the newly synthesized procathepsin D due to MPR-dependent sorting. Thus, the majority of procathepsin D, which escapes sorting via either MPR, contains at site II oligosaccharides with two phosphomonoesters. Furthermore, the oligosaccharide pattern at the glycosylation site I is not affected by the MPR-dependent sorting of procathepsin D. It appears unlikely, therefore, that the small fraction of procathepsin D, which ends up in the secretions of control MEF, is missing due to a low affinity to MPR 46 and/or MPR 300. It is more likely that most of this procathepsin D is secreted because it fails to encounter a free receptor in the trans-Golgi network (TGN) or is sorted via an MPR, but it is released from the receptor at a site (e.g. early endosomes) from where it can exit to the medium.

**What Causes the Differential Sorting Efficiency of MPR 46 and MPR 300 for Procathepsin D?**—Previous studies have shown that both MPRs participate in general in the sorting of individual lysosomal proteins, albeit with variable efficiency (2, 9). Procathepsin D is an example of a lysosomal protein that is poorly sorted via the MPR 46 and efficiently sorted via the MPR 300. While until now it was assumed that preferential binding of a lysosomal protein to either MPR may be related to the structure of its Man-6-P recognition marker, this study demonstrates that structural differences of the recognition marker concerning the number of phosphate residues and their presence as a monoester- or diester-linkage cannot account for the MPR-dependent sorting efficiency.

The majority of procathepsin D forms that escape sorting by MPR 46 and/or MPR 300 contain, at site II, oligosaccharides with two phosphomonoesters. Among the phosphorylated oligosaccharides, the latter have the highest affinity for MPRs 46 and 300. This makes it unlikely that the missorting of the procathepsin D, which is recovered in the secretions of the MEF expressing MPR 46 and/or MPR 300, is due to a low affinity of the phosphorylated oligosaccharides unless structural parameters not analyzed in this study are critical for the distinct affinity of diphosphorylated oligosaccharides in procathepsin D to MPRs. Such parameters are the structure of the underlying oligosaccharides and the position of the phosphorylated mannose residues within the oligosaccharides. An alternative possibility is that the polypeptide backbone of procathepsin D, rather than its phosphorylated oligosaccharides,
determines its distinct affinities to the two MPRs. For procathepsin L, a negative modulation of the affinity of its phosphorylated oligosaccharides by protein determinants has been reported. Mouse procathepsin L contains a fairly homogenous population of phosphorylated oligosaccharides with two phosphomonoesters. While native or denatured procathepsin L interacts poorly with MPR 300, its oligosaccharides show high affinity binding to MPR 300 (16). The polypeptide backbone may modify the affinity by interacting with the phosphorylated oligosaccharides themselves or with the MPR. It remains, however, to be demonstrated that the polypeptide backbone of a lysosomal protein such as procathepsin D can regulate the affinity of its phosphorylated oligosaccharides in a receptor-type-dependent manner.

REFERENCES
1. Kornfeld, S., and Mellman, I. (1989) Ann. Rev. Cell Biol. 5, 483–525
2. Kornfeld, S. (1992) Annu. Rev. Biochem. 61, 307–330
3. Chao, H. H. J., Waheed, A., Pohlmann, R., Hille, A., and von Figura, K. (1990) EMBO J. 9, 3507–3513
4. Gabel, C. A., Goldberg, D. E., and Kornfeld, S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 775–779
5. Kaster, A., Saftig, P., Matzner, U., von Figura, K., Peters, C., and Pohlmann, R. (1993) EMBO J. 12, 5219–5223
6. Ludwig, T., Ovitt, C. E., Bauer, U., Hollinshead, M., Remmler, J., Lobel, P., Ruther, U., and Hoflack, B. (1993) EMBO J. 12, 5225–5235
7. Wang, Z. Q., Fung, M. R., Barlow, D. P., and Wagner, E. P. (1994) Nature 372, 464–467
8. Ludwig, T., Munier-Lehmann, H., Bauer, U., Hollinshead, M., Ovitt, C., Lobel, P., and Hoflack, B. (1994) EMBO J. 13, 3430–3437
9. Pohlmann, R., Wendland, M., Boeker, C., and von Figura, K. (1995) J. Biol. Chem. 270, 27311–27318
10. Varki, A., and Kornfeld, S. (1983) J. Biol. Chem. 258, 2808–2818
11. Hoflack, B., Fujimoto, K., and Kornfeld, S. (1987) J. Biol. Chem. 262, 123–129
12. Tong, P. Y., Gregory, W., and Kornfeld, S. (1989) J. Biol. Chem. 264, 7962–7969
13. Tong, P. Y., and Kornfeld, S. (1989) J. Biol. Chem. 264, 7970–7975
14. Kaspar, D., Dittmer, F., von Figura, K., and Pohlmann, R. (1996) J. Cell Biol. 134, 615–623
15. Conner, G. E. (1989) Biochem. J. 263, 601–604
16. Lazzarino, D., Gabel, C. A. (1990) J. Biol. Chem. 265, 11864–11871
17. Schmidt, B., Selmer, T., Ingendoh, A., and von Figura, K. (1995) Cell 82, 271–278
18. Cantor, A. B., Baranski, T. J., and Kornfeld, S. (1992) J. Biol. Chem. 267, 23349–23356
19. Varki, A., and Kornfeld, S. (1980) J. Biol. Chem. 255, 10847–10858
20. Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Geuze, H., and von Figura, K. (1988) EMBO J. 7, 2551–2555
21. Weitz, G., and Priia, R. L. (1992) J. Biol. Chem. 267, 10039–10044
22. Sonderfeld-Fresko, S., and Priia, R. L. (1988) J. Biol. Chem. 264, 7692–7697
23. Goldberg, D. E., and Kornfeld, S. (1981) J. Biol. Chem. 258, 3159–3165
24. Shipley, J. M., Grubb, J. H., and Sly, W. S. (1993) J. Biol. Chem. 268, 12193–12198
25. Gieselmann, V., Schmidt, B., and von Figura, K. (1992) J. Biol. Chem. 267, 13262–13266
26. Munier-Lehmann, H., Mauxion, F., Bauer, U., Lobel, P., and Hoflack, B. (1996) J. Biol. Chem. 271, 15166–15174