α -Glucosidase and *N*-acetylglucosamine-6-sulphatase are the major mannose-6-phosphate glycoproteins in human urine

David E. SLEAT*, Stephen R. KRAUS†, Istvan SOHAR*, Henry LACKLAND* and Peter LOBEL*‡§

*Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08854, U.S.A., †Division of Urology, Department of Surgery, Medical Education Building, New Brunswick, NJ, U.S.A., and ‡Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854, U.S.A.

Most newly synthesized lysosomal enzymes contain a transient carbohydrate modification, mannose 6-phosphate (Man-6-P), which signals their vesicular transport from the Golgi to the lysosome via Man-6-P receptors (MPRs). We have examined Man-6-P glycoproteins in human urine by using a purified soluble fragment of the soluble cation-independent MPR (sCI-MPR) as a preparative and analytical affinity reagent. In a survey of urine samples from seven healthy subjects, the pattern of Man-6-P glycoproteins detected with iodinated sCI-MPR as a probe in a blotting assay was essentially identical in each, regardless of sex or age. Two bands of approx. 100 and 110 kDa were particularly prominent. Man-6-P glycoproteins in human urine were purified by affinity chromatography on immobilized sCI-MPR. Seven distinct bands revealed by SDS/PAGE and Coomassie Blue staining were subjected to N-terminal sequence

analysis. The prominent 100 and 110 kDa Man-6-P glycoproteins were identified as *N*-acetylglucosamine-6-sulphatase and α -glucosidase respectively. This identification was confirmed by molecular mass determinations on the two major bands after deglycosylation. Sequence analysis revealed arylsulphatase A and several previously unidentified proteins as minor species. Man-6-P glycoproteins were also purified on an analytical scale to determine the proportion of a number of lysosomal enzyme activities represented by the mannose-6-phosphorylated forms. The lysosomal enzymes in urine containing the highest proportion of mannose-6-phosphorylated form were β -mannosidase (82 %), hexosaminidase (27 %) and α -glucosidase (24 %). The profiles of Man-6-P glycoproteins detected by blotting in urine and plasma were not similar, suggesting that the urinary species are not derived from the bloodstream.

INTRODUCTION

Newly synthesized lysosomal enzymes contain mannose 6-phosphate (Man-6-P), which is a recognition marker for two high-affinity Man-6-P receptors (MPRs): the cation-dependent MPR and the cation-independent (CI-) MPR (reviewed in [1–3]). MPRs direct the vesicular transport of Man-6-P glycoproteins from the trans-Golgi network to an acidified prelysosomal compartment where the receptor–ligand complexes disassociate, releasing the newly synthesized lysosomal enzymes. This allows the MPRs to recycle back to the Golgi or the cell surface, where the CI-MPR, at least, is capable of mediating the endocytosis of extracellular ligands containing Man-6-P.

Mannose-6-phosphorylation is a transient modification of lysosomal enzymes and the phosphate is normally removed in the lysosome [4]. Thus most mammalian cells contain very low levels of glycoproteins bearing this modification and they are generally considered to be in transit to the lysosome. However, in a recent study, rat brain was shown to contain a considerably higher content of Man-6-P glycoproteins than other tissues. As a first step towards understanding the basis for and biological relevance of elevated Man-6-P glycoproteins in brain, these proteins were purified by immobilized MPR affinity chromatography and analysed by N-terminal sequence analysis [5]. Various lysosomal enzymes were identified, including palmitoyl protein thioesterase, which had not previously been known to be lysosomal. In addition, the proportion of each lysosomal enzyme containing Man-6-P was shown to be elevated in brain compared with other tissues, resulting in higher levels of phosphorylated lysosomal enzymes in brain [5].

Human urine contains numerous lysosomal enzymes [6]. A proportion of some of these are present in urine in what was originally designated ‘high uptake’ forms because, when purified from urine, they were efficiently internalized by cultured fibroblasts. These enzymes include arylsulphatase A [7], α -*N*-acetylglucosaminidase [8,9], α -glucosidase [10] and α -iduronidase [11,12]. Subsequent analysis of these proteins revealed that it was the presence of Man-6-P [13] that conferred the ‘high uptake’ property by allowing interaction with the CI-MPR and subsequent endocytosis.

Characterizing the mannose-6-phosphorylation of individual lysosomal enzymes in urine does not provide an overall picture of the range of Man-6-P glycoproteins present, or what proportion of the total urinary population is composed of each individual protein species. In this study we have addressed these questions by using purified CI-MPR as a tool for the analysis and purification of the total Man-6-P glycoprotein population in urine. Despite the wide range of lysosomal activities [6], we find that relatively few phosphorylated lysosomal enzymes are present in significant mass quantities in urine, with two species predominating.

EXPERIMENTAL

SDS/PAGE and detection of Man-6-P glycoproteins

A portion (0.2 vol.) of 5 × reducing SDS/PAGE sample buffer [300 mM Tris/HCl (pH 6.8)/50 % (v/v) glycerol/10 % (w/v) SDS/5 % (v/v) 2-mercaptoethanol/0.01 % Bromophenol Blue]

Abbreviations used: AAP, alanine aminopeptidase; Man-6-P, mannose 6-phosphate; MPR, Man-6-P receptor; CI-MPR, cation-independent MPR; sCI-MPR, soluble CI-MPR; PNGase F, peptide:N-glycosidase F.

§ To whom correspondence should be addressed.

was added to samples, which were then incubated at 100 °C for 5 minutes. Unless stated otherwise, SDS/PAGE was routinely conducted on 10% (w/v) polyacrylamide gels with a 30:0.8 acrylamide-to-bisacrylamide ratio. Immobilized Man-6-P glycoproteins were detected after quantitative blotting to nitrocellulose [14] by using an iodinated soluble fragment of the cation-independent (sCI-) MPR as described [15]. Blots were analysed with a PhosphorImager and ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and corrected for background binding to nitrocellulose during quantification. Creatinine concentrations were determined with the alkaline picrate method [16]. Man-6-P glycoprotein standards were the secretions of CI-MPR-deficient mouse fibroblasts, which secrete most of their phosphorylated lysosomal enzymes into the culture media [15]. Molecular masses were estimated with high-range prestained molecular mass markers from Gibco-BRL.

Preparative isolation of Man-6-P glycoproteins from urine

Purification of Man-6-P glycoproteins by MPR affinity chromatography was essentially as described [5], with the following modifications. Urine from a single healthy male subject was collected on a daily basis and concentrated 10–20-fold with an Amicon (Beverly, MA, U.S.A.) concentrator with a PM10 or PM30 membrane. Concentrated urine was stored at –70 °C. Concentration, storage at –70 °C and thawing had no effect on the levels and types of Man-6-P glycoproteins detected by MPR affinity blotting (Figure 1, and results not shown). Concentrated urine samples equivalent to 20 litres original volume were thawed, pooled and centrifuged at 5500 *g* for 20 min at 4 °C. The supernatant was removed and further concentrated to a final volume of 100 ml, then dialysed twice against 4 litres of PBS for 15 and 2 h. The centrifugation step was repeated and the supernatant applied to an affinity column (5 ml bed volume) of sCI-MPR immobilized on Affigel 10 (Bio-Rad) at 2 mg/cm³. The column was washed with 5 vol. of PBS; Man-6-P glycoproteins were eluted with 1 × PBS containing 10 mM Man-6-P and 0.02% sodium azide. Fractions containing the highest concentrations of eluted Man-6-P glycoproteins were identified with the protein assay method of Bradford [17], pooled and concentrated to a final volume of 70 µl by centrifugation in a Centricon 10 (Amicon) spin concentrator. The total yield of Man-6-P glycoproteins from 20 litres of urine was 90 µg.

N-terminal sequence analysis

Purified Man-6-P glycoproteins (30 µg) were fractionated by SDS/PAGE (4–12% gel gradient) with premade polyacrylamide gels (Novex). Transfer to Immobilon P^{sq} (Millipore) and N-terminal sequence analysis were as described previously [5]. Sequence data were analysed with the Wisconsin Computer Group Sequence Analysis Software Package, Version 7.2 [18] or the National Center for Biotechnology Information BLAST programs at the National Library of Medicine.

Determination of enzyme phosphorylation state

The proportions of different enzymes that contained Man-6-P in urine and plasma were determined by activity measurements on the load, flow-through and 10 mM Man-6-P eluate fractions from analytical sCI-MPR affinity columns. Urine (5 ml) from two healthy male subjects was dialysed three times for 1 h each against 1 litre of 50 mM phosphate buffer, pH 7.2, containing 0.15M NaCl and 0.1% Triton X-100 before fractionation on duplicate MPR columns. Plasma from four healthy male subjects was loaded directly on individual affinity columns. Elution of

Man-6-P glycoproteins and enzyme activity measurements were as described [5].

RESULTS

Mannose-6-phosphorylated glycoproteins in human urine

Proteins in urine samples from four healthy female and three healthy male subjects were fractionated by SDS/PAGE and transferred to nitrocellulose; Man-6-P glycoproteins were detected by using an iodinated soluble fragment of CI-MPR as an affinity probe [15]. Results are presented in Figure 1. The pattern of Man-6-P glycoproteins is clearly conserved in all samples, including those from both male and female subjects, and was independent of age within the range tested (22–45 years old). Freeze-thawing of urine samples had no effect on the levels or pattern of Man-6-P glycoproteins detected (compare lanes 5 and 5' in Figure 1). Two bands of approx. 100 and 110 kDa are particularly prominent although fainter but discernible bands with apparent molecular masses of between 40 and 75 kDa were also present. Detection of Man-6-P glycoproteins was completely abrogated by the presence of free Man-6-P (5 mM), demonstrating the specificity of the detection method (results not shown).

Samples analysed in Figure 1 were volumetric equivalents. Urine samples were also standardized for creatinine content, and total levels of Man-6-P glycoproteins were determined after SDS/PAGE and detection with iodinated sCI-MPR (results not shown). There is quantitative variation in terms of total Man-6-P glycoproteins in urine samples from subject to subject (approx. 4-fold difference between highest and lowest levels for both males and females) but it also seems that female urine contains slightly higher (1.5–2-fold) overall levels than male urine. The latter observation, however, reflects the fact that female urine has a lower concentration of creatinine than male urine. (In this study, the average creatinine concentration was 106 mg/ml for females and 150 mg/ml for males, whereas previous studies found average creatinine production rates of 1.17 and 1.80 g/day for females and males respectively [19]).

Figure 1 Survey of Man-6-P glycoproteins in human urine

Man-6-P glycoproteins were detected by affinity blotting with iodinated sCI-MPR as an affinity probe in morning urine samples from four female (lanes 1–4) and three male (lanes 5–7) subjects, varying in age from 21 to 45 years. Lanes 5 and 5' are duplicates of the same urine sample, except that the sample in lane 5 was frozen on dry ice, stored at –70 °C for 6 h and thawed before electrophoresis. All other urine samples were immediately prepared for electrophoresis. A 40 µl portion of each sample was loaded; standards (Stds.) were 10 and 30 µl secretions from CI-MPR-deficient mouse fibroblasts.

Figure 2 Purification of Man-6-P glycoproteins from human urine monitored by affinity blotting

Samples representing 20 μ l of column load (which was 50 ml of urine concentrated to 1/40 volume in this particular purification), 40 μ l of each of twelve 4 ml flow-through fractions, 40 μ l of each of seven 10 ml glucose 6-phosphate (5 mM) wash fractions and 20 μ l of every other of 45 Man-6-P (5 mM) eluate fractions (2 ml) were detected as described in the Experimental section.

Purification of Man-6-P glycoproteins from human urine

Man-6-P glycoproteins were purified from 20 litres of urine from a healthy 32-year-old male subject by affinity chromatography on immobilized sCI-MPR [5]. To monitor recovery, Man-6-P glycoproteins in each column fraction were determined by SDS/PAGE and affinity blotting [15] with iodinated sCI-MPR (Figure 2). Essentially no Man-6-P glycoproteins were observed in the flow-through fraction, indicating that complete binding to the immobilized MPR was occurring. Free Man-6-P (5 mM) completely eluted glycoproteins bound by the column, whereas glucose 6-phosphate eluted nothing, confirming the specificity of our purification protocol.

The mixture of purified urinary Man-6-P glycoproteins was fractionated by SDS/PAGE and individual proteins were detected by either Coomassie Blue staining or affinity blotting with iodinated sCI-MPR (Figure 3). The patterns are essentially identical, demonstrating that each of the purified proteins actually contains Man-6-P. This precludes the possibility that some of the purified proteins are not mannose-6-phosphorylated but co-purify with Man-6-P glycoproteins as part of a multi-component complex. In addition, as even relatively low-affinity ligands are retained on the high-density sCI-MPR affinity column, Coomassie staining of the purified mixture confirms that the prominent species of approx. 100 and 110 kDa in urine do not simply represent higher-affinity ligands for the sCI-MPR probe in the blotting assay than other Man-6-P glycoproteins but are actually present at higher levels.

Sequence analysis of purified Man-6-P glycoproteins

Seven purified Man-6-P glycoproteins were distinctly resolved as shown in Figure 3, with molecular masses ranging from 40 to 130 kDa. After fractionation by SDS/PAGE and transfer to PVDF membrane, each of these bands was subjected to N-terminal sequence analysis. Results are presented in Table 1.

Band 7 was essentially a single species and yielded an interpretable sequence, which, although unidentified, is shown in Table 1. Band 4 yielded a complex mixture of at least four sequences, which is not presented.

Band 1, which was of low abundance in urine, yielded a mixed sequence but contained a predominant species. In eight positions out of thirteen this sequence corresponded to human α -glu-

Figure 3 Affinity blotting and Coomassie staining of purified urinary Man-6-P glycoproteins

Proteins were detected by Coomassie Blue (CB) staining or by affinity blotting with iodinated sCI-MPR as described in the Experimental section. The numbering used for the purified bands is indicated at the right. It should be noted that although the loading of 10 μ g of total purified urinary Man-6-P glycoproteins was not sufficient for detection of bands 6 and 7 by Coomassie staining in this figure, these bands were readily detected when 30 μ g of urinary Man-6-P glycoproteins were resolved for N-terminal sequence analysis (results not shown).

cosidase [20] and aligned with the known propeptide cleavage site [21]. It is possible that the approx. 150 kDa band 1 represents a highly glycosylated form of α -glucosidase but this assignment should be regarded as tentative, given the apparent conflicts with the published sequence.

Band 2, which had the greater molecular mass of the two major species, was a mixture of four sequences, which were unambiguously identified as nested N-terminal variants of α -glucosidase by comparison with the human sequence [20]. Their migration on SDS/PAGE is consistent with the 110 kDa processing intermediate of α -glucosidase, which has been previously purified from human urine [10]. The N-terminus (Ala⁷⁰) of the

Table 1 Sequence assignments for the purified Man-6-P glycoproteins

Cleavage sites are indicated by an asterisk.

Band sequence	Identity	Sequence alignment
Band 1 AGPIRATAVPTIDE GD FV	α -Glucosidase (?)	AGPIRATAVPTIDE GD FV 65: PRDAQ*AHFGRPRAVPTQCDV
Band 2 AHFGRPRAVPTQxD HFGRPRAVPTQxDV AQAHFGRPRAVPTQ DAQAHFGRPRAVPT	α -Glucosidase	AHFGRPRAVPTQxD HFGRPRAVPTQxDV AQ AHFGRPRAVPTQ DAQ AHFGRPRAVPT 65: PRDAQ*AHFGRPRAVPTQCDV
Band 3 TRRPNVLLITDDQDEVLG GTRRPNVLLITDDQDEVLG VAAGTRRPNVLLITDDQDE	<i>N</i> -Acetylglucosamine-6-sulphatase	TRRPNVLLITDDQDEVLG GTRRPNVLLITDDQDEVLG VAAGTRRPNVLLITDDQDE 32: GGCLG*VFGVAAGTRRPNVLLITDDQDEVLGGMTP
Band 4 >4 sequences	Unidentified	
Band 5 RPNIVLIFA	Arylsulphatase A	RPNIVLIFA 13: AGLAVA*RPNIVLIFADDLGY
Band 6 RPNIVLIFA	Arylsulphatase A	RPNIVLIFA 13: AGLAVA*RPNIVLIFADDLGY
Band 7 APAFHREGY D L E	Unidentified	

most abundant species was coincident with the known site of propeptide cleavage [21], which generates the 110 kDa α -glucosidase. The second most abundant species was initiated one residue downstream (His⁷¹) and might represent an alternate cleavage site or it could be the result of N-terminal exopeptidase digestion. The two remaining sequence variants, however, had N-termini (Asp⁶⁷ and Ala⁶⁸) upstream of the propeptide cleavage site (Gln⁶⁹¹-Ala⁷⁰), precluding the possibility of N-terminal degradation. These variants are therefore either processed at an alternative cleavage site or represent transient processing intermediates for the isoforms with N-termini comprising Ala⁷⁰ and His⁷¹. N-terminal heterogeneity at proteolytic processing sites has also been described for other lysosomal enzymes including cathepsin D [5,22] and α -iduronidase [5].

The other major species, band 3, was identified as *N*-acetylglucosamine-6-sulphatase by comparison of the N-terminal 20 residues with the human sequence [23]. Interestingly, this protein also had a heterogeneous N-terminus. Three isoforms were identified with N-termini (Val⁴⁰, Gly⁴³ and Thr⁴⁴) that were downstream of the known signal cleavage site (Gly³⁶¹-Val³⁷) [23]. As noted for α -glucosidase, these alternative N-termini could represent cleavage at different sites or N-terminal exopeptidase digestion. The apparent molecular mass of band 3, estimated from its migration on SDS/PAGE, is approx. 100 kDa, which is in agreement with the reported molecular mass of human urinary *N*-acetylglucosamine-6-sulphatase [24] but more than the reported molecular mass of human liver *N*-acetylglucosamine-6-sulphatase, which is 78 kDa [25]. This difference is discussed below.

Minor bands 5 and 6 were both identified as arylsulphatase A by comparison with the known human sequence [26]. The N-termini of both proteins (Arg¹⁹) are consistent with the known signal cleavage site at Ala¹⁸¹-Arg¹⁹ [26]. The estimated molecular masses of bands 4 and 5 from Figure 4 are 65 and 75 kDa respectively. This size difference suggests that these two bands probably represent the 58 kDa single-chain form and the 50 kDa heavy chain of the two-chain form of arylsulphatase A. 'High

uptake' (mannose-6-phosphorylated) arylsulphatase A has been identified in human urine in earlier studies [7].

Deglycosylation of purified Man-6-P glycoproteins from urine

The reported molecular mass of *N*-acetylglucosamine-6-sulphatase from human liver is 78 kDa [25], whereas the polypeptide identified here and previously [24] as *N*-acetylglucosamine-6-sulphatase was approx. 100 kDa. The difference in these molecular masses could potentially reflect an increased carbohydrate content of urinary *N*-acetylglucosamine-6-sulphatase compared with that from liver. This is particularly likely as *N*-acetylglucosamine-6-sulphatase has many (13) potential N-linked glycosylation sites. To test this possibility, the mobilities of the urinary Man-6-P glycoproteins on SDS/PAGE were compared before and after deglycosylation with peptide:*N*-glycosidase F (PNGase F) (Figure 4).

The apparent molecular masses of bands 2 and 3 are approx. 100 and 110 kDa respectively. Incubation of the purified urinary Man-6-P glycoproteins at 37 °C for 1 h did not result in a shift in these apparent molecular masses, indicating a lack of glycosidase or protease activities under these incubation conditions. Incubation at 37 °C for 1 h with PNGase F, however, resulted in a shift in the migration of band 2 to approx. 105 kDa and band 3 to approx. 57 kDa. The predicted molecular masses of deglycosylated α -glucosidase and *N*-acetylglucosamine-6-sulphatase from the amino acid sequences (98 022 Da [20] and 57 706 Da [23] respectively) are in good agreement with Figure 4, confirming the sequence assignments made here.

Determination of phosphorylated proportion of lysosomal enzymes

We have demonstrated here that a small number of urinary lysosomal enzymes are present in identifiable quantities in their mannose-6-phosphorylated form. There are two ways in which the levels could be elevated relative to those of other lysosomal enzymes. First, they could simply reflect relatively large amounts

Figure 4 Endoglycanase F treatment of purified urinary Man-6-P glycoproteins

Samples (5 μ g) of purified urinary Man-6-P glycoproteins were denatured by incubation at 100 °C for 10 min in 0.5% SDS/1% 2-mercaptoethanol in a total volume of 10 μ l. The incubation volume was then adjusted to a final volume of 20 μ l containing a final concentration of 50 mM sodium phosphate (pH 7.5)/1% Nonidet P40. The addition of 500 New England Biolabs units of PNGase F (New England Biolabs, Beverly, MA, U.S.A.) was as indicated; 5 μ l of 5 $\times$ SDS/PAGE sample loading buffer were added and 5 μ l of each sample was fractionated by gel electrophoresis after incubation at 100 °C for 5 min. Proteins separated by the 10% polyacrylamide gel (Novex, San Diego, CA, U.S.A.) were detected with Coomassie Blue. Samples incubated in the absence of PNGase F for 1 h at 4 °C were identical with those incubated at 37 °C for 1 h (results not shown).

of these proteins in urine with correspondingly large amounts of the mannose-6-phosphorylated form. Secondly, a higher proportion of these enzymes could contain Man-6-P compared with other urinary lysosomal enzymes. To distinguish between these possibilities, we measured the Man-6-P-containing proportion of a number of lysosomal enzymes by measuring enzyme activities in the load, flow-through and Man-6-P eluate of analytical sCI-MPR affinity columns. Total activities are shown in Table 2 and

Table 3 Percentage of lysosomal enzyme activities represented by mannose-6-phosphorylated forms in human urine and plasma

The percentage of each activity represented by the phosphorylated form was calculated as the activity in the Man-6-P eluate divided by the total recovered activity (flow-through plus glucose-6-phosphate eluate plus Man-6-P eluate). Results are means $\pm$ S.D. for duplicate determinations from samples from two subjects (urine) or means $\pm$ S.D. for single determinations from four subjects (plasma). All subjects were healthy males.

	Mannose-6-phosphorylated forms (%)	
	Urine	Plasma
MPR-independent		
Acid phosphatase	0.2 $\pm$ 0.2	1.7 $\pm$ 1.7
AAP	2.5 $\pm$ 0.7	1.4 $\pm$ 0.3
MPR-dependent		
β -glucuronidase	2.5 $\pm$ 0.6	20.7 $\pm$ 1.9
β -galactosidase	8.9 $\pm$ 0.7	15.5 $\pm$ 3.5
Cathepsin C	7.7 $\pm$ 1.0	4.9 $\pm$ 2.0
α -Glucosidase	24.0 $\pm$ 0.1	27.8 $\pm$ 5.0
Hexosaminidase	27.0 $\pm$ 0.1	27.6 $\pm$ 3.0
β -Mannosidase	81.9 $\pm$ 1.6	50.8 $\pm$ 3.5

the proportion of each enzyme activity that can be attributed to the Man-6-P-containing form is shown in Table 3.

Acid phosphatase and alanine aminopeptidase (AAP) were measured as control activities as they do not contain Man-6-P. Neither was significantly retained on the immobilized sCI-MPR affinity column. (Acid phosphatase is targeted to the lysosome by an MPR-independent pathway, and AAP is a cytosolic activity.) Half of the MPR-targeted lysosomal enzymes measured seemed to be present in the non-phosphorylated forms in human urine, with less than 10% of each respective activity being retained on the immobilized MPR column. In an earlier study [27], β -glucuronidase was found to be present in human urine predominantly in the 'low uptake' (i.e. unphosphorylated) form. Our results are in agreement with this finding, with less than 3% activity retained on the affinity column. Significant proportions of three lysosomal enzyme activities, however, contained Man-6-P. These were α -glucosidase, hexosaminidase and β -mannosidase, of which respectively 24%, 27% and 82% of the activity was retained on the affinity column. These results suggest, at least for

Table 2 Total enzyme activities in plasma and urine

Enzyme activities are expressed as nmol of substrate hydrolysed/h per ml of sample. Enzyme activity determinations in urine are the average of duplicate determinations for two subjects and are single determinations on four subjects for plasma.

Enzyme	Activity (nmol/h per ml of sample)						
	Plasma					Urine	
	Subject 1	Subject 2	Subject 3	Subject 4	Plasma average	Subject 1	Subject 2
α -Glucosidase	17	29	19	20	21 $\pm$ 5	34	33
β -Galactosidase	16	12	16	12	14 $\pm$ 2	104	68
β -Mannosidase	189	229	160	207	196 $\pm$ 25	23	12
β -Glucuronidase	38	34	25	62	40 $\pm$ 14	29	29
Hexosaminidase	38	39	45	40	41 $\pm$ 3	11	7
Cathepsin C	188	130	140	168	156 $\pm$ 23	408	159
Acid phosphatase	269	248	247	269	258 $\pm$ 11	17296	1974
AAP	230	204	156	248	210 $\pm$ 35	179	61

Figure 5 Man-6-P glycoproteins in human plasma

Purified urinary Man-6-P glycoproteins (0.01, 0.04 or 0.1 μ g) or plasma Man-6-P glycoproteins (adjacent affinity column eluate fractions) were fractionated by SDS/PAGE, transferred to nitrocellulose and detected with iodinated sCI-MPR as an affinity probe. Plasma was prepared from 5 ml of blood taken from a healthy male subject by the addition of EDTA, sodium phosphate (pH 7) and Tween-20 to concentrations of 3 mM, 50 mM and 0.2% respectively, followed by centrifugation at 800 g for 10 min. Plasma Man-6-P glycoproteins could not be detected directly because the high concentration of albumin caused distortion of the gel. Instead, plasma Man-6-P glycoproteins were purified by affinity chromatography on immobilized sCI-MPR. The buffered plasma was loaded on an analytical column (1 ml bed volume) and washed with 30 vol. of PBS/0.2% Tween-20. Removal of albumin from the column was monitored by protein assays on each wash fraction. Man-6-P glycoproteins were eluted with six 1 ml aliquots of PBS/0.2% Tween-20 containing 5 mM Man-6-P. Each eluate fraction was concentrated to 100 μ l with Centricon 10 spin concentrators; 30 μ l of each was loaded. Eluate fractions 1 and 2 are shown.

α -glucosidase, that high levels of the phosphorylated form in urine reflect a higher proportion containing Man-6-P compared with other lysosomal enzymes. Earlier studies of urinary α -glucosidase found that approx. 45% of total activity could be attributed to the Man-6-P-containing form [10]. None of the sequenced proteins was identified as β -mannosidase, even though it is predominantly mannose-6-phosphorylated in urine (Table 1), suggesting that it is present at a very low concentration relative to other Man-6-P glycoproteins.

In a parallel study we determined the Man-6-P-containing proportion of a number of lysosomal enzymes in plasma (Table 3). Again, enzymes whose trafficking is MPR-independent (acid phosphatase and AAP) were not retained on the affinity column. Significant proportions (more than 10%) of most MPR-targeted lysosomal enzymes in plasma were bound to the immobilized sCI-MPR, including β -galactosidase, β -glucuronidase, hexosaminidase, α -glucosidase and β -mannosidase. In both urine and plasma the percentage of lysosomal activities that correspond to the mannose-6-phosphorylated form in humans is considerably higher than in most rat tissues, with the exception of brain [5].

There are two possible ways in which the phosphorylated and total lysosomal enzyme populations could accumulate in the urine: (1) they could reflect passive or active clearance from the bloodstream or (2) they could be actively secreted into the urine by cells within the kidney or urinary epithelia. There is no significant correlation (Table 2; see also [28]) between total activities in urine and plasma, suggesting that the appearance of

urinary Man-6-P glycoproteins does not reflect non-selective clearance from the blood. To investigate this possibility further, we directly compared the array of Man-6-P glycoproteins in urine and plasma by affinity blotting with iodinated sCI-MPR as a probe (Figure 5). Proteins of molecular mass corresponding to the two major species in urine (i.e. α -glucosidase and *N*-acetylglucosamine-6-sulphatase) were present in plasma but were not predominant. Other mannose-6-phosphorylated glycoproteins of molecular masses approx. 40, 60 and 75 kDa were also relatively abundant in plasma but were not observed in urine. Similar analyses of Man-6-P glycoproteins in the plasmas of wild-type and transgenic mice lacking the CD-MPR have revealed a complex pattern of eight or more phosphorylated lysosomal enzymes [29]. Given the differences in the patterns of Man-6-P glycoproteins, it seems unlikely that phosphorylated lysosomal enzymes in urine are derived from the bloodstream. Interestingly, however, there is significant correlation ($P < 0.001$) between the proportion of each enzyme that contains Man-6-P in plasma and in urine (Figure 5). This might indicate that phosphorylated and non-phosphorylated lysosomal enzymes are secreted into the urine and bloodstream by similar mechanisms and perhaps by similar cell types.

DISCUSSION

Although human urine contains a wide range of lysosomal enzyme activities, we demonstrate here that only a few lysosomal enzymes are present in significant quantities as their mannose-6-phosphorylated form. α -Glucosidase and *N*-acetylglucosamine-6-sulphatase were the most abundant Man-6-P glycoproteins, whereas arylsulphatase A and three unidentified species represented minor mannose-6-phosphorylated species. The source of these Man-6-P glycoproteins and their non-phosphorylated counterparts remains unknown. Clearance from the bloodstream seems unlikely in view of the size of the proteins identified here (40–130 kDa), which seems to argue against passive filtration. The lack of correlation between total or mannose-6-phosphorylated activities in urine and plasma (results not shown) [28] also seems to rule out this possibility. In addition we demonstrate here that the profiles of Man-6-P glycoproteins in urine and plasma are dissimilar. Another potential source of urinary Man-6-P glycoproteins is the male genital system, which contains relatively high levels of these proteins [5,30,31]. However, in this report we show identical patterns of Man-6-P glycoproteins in urine from male and female subjects, ruling out this potential source.

The most likely source for Man-6-P glycoproteins in human urine is the kidney or the epithelia lining the urinary tract; there is experimental evidence to support this. Urinary α -glucosidase is identical with kidney α -glucosidase [32], which is located primarily in the proximal convoluted tubule brush border [33]. Immunohistochemical studies in human kidney reveal that, whereas total α -glucosidase is located in both the lysosomes and brush border of proximal tubule epithelial cells, the 70 and 76 kDa proteolytically processed forms, which are unphosphorylated in urine [34], are restricted to the former compartment. This suggests that some or all incompletely proteolytically processed α -glucosidase isoforms are present in significant amounts at the surface of the brush border of the proximal tubule epithelial cells [35]. In COS cells transfected with α -glucosidase cDNA, a fraction of α -glucosidase is membrane-bound via its uncleaved signal peptide and presented at the cell surface [21]. It is possible that in kidney, unprocessed α -glucosidase is also anchored via its signal peptide and that the mannose-6-phosphorylated α -glucosidase in human urine represents a proteolytic product of this.

Whether the other Man-6-P glycoproteins identified here, such as *N*-acetylglucosamine-6-sulphatase and arylsulphatase A, enter the urine in a similar manner remains to be determined.

An interesting possibility is that the phosphorylated and non-phosphorylated forms of lysosomal enzymes appear in the urine via two different pathways, one involving direct secretion and the other involving defecation of lysosomal contents [36,37]. Proteins arising from the lysosome would be Man-6-dephosphorylated and extensively proteolytically processed. The fact that there are mannose-6-phosphorylated forms of lysosomal enzymes in urine that are partly proteolytically processed is consistent with this model. In addition there are mouse pigment mutants that are defective in secretion of lysosomal enzymes from the kidney into the urine [38]. Normal urinary production of mannose-6-phosphorylated lysosomal enzymes in these mice would represent direct evidence for two pathways of lysosomal enzyme secretion.

In addition to furthering our understanding of the biology of urinary lysosomal enzymes, our observations here might also prove to have clinical value in two respects. First, we have sequenced previously unidentified proteins that, because they contain Man-6-P, are presumably lysosomal enzymes (band 7 and possibly band 1). Deficiencies in lysosomal enzyme activities underlie a wide range of inherited diseases (lysosomal storage disorders); thus functional characterization of the unidentified species described here could potentially reveal biochemical bases for disorders for which the cause is currently unknown. Secondly, in recent studies, we have demonstrated that elevated levels of Man-6-P glycoproteins are associated with an aggressive subset of breast and prostate tumours ([38]; J. K. Raska, D. E. Sleat, R. DiPaola, T.-L. Chen, B. Holland, R. M. Maliniak, N. J. Barnard, R. V. P. Hutter and P. Lobel, unpublished work). Our finding here that Man-6-P glycoproteins can be readily detected in normal urine samples with an affinity blot assay provides a useful first step towards developing a prognostic MPR-affinity urine test for clinical use. A potential application for such a test might be in the diagnosis and/or monitoring of malignancies of the urogenital system such as bladder and prostate cancer.

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