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The human urine mannose 6-phosphate glycoproteome

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Abstract

Glycoproteins containing the mannose 6-phosphate (Man-6-P) modification represent a class of proteins of considerable biomedical importance. They include over sixty different soluble lysosomal hydrolases and accessory proteins, deficiencies of which result in over forty different known human genetic diseases. In addition, there are patients with lysosomal storage diseases of unknown etiology and lysosomal proteins have been implicated in pathophysiological processes associated with Alzheimer disease, arthritis, and cancer. The aim of this study was to explore urine as a source for the proteomic investigation of lysosomal storage disorders as well as for biomarker studies on the role of Man-6-P containing proteins in other human diseases. To this end, urinary proteins were affinity purified on immobilized Man-6-P receptors, digested with trypsin, and analyzed using nanospray LC/MS/MS. This resulted in identification of 67 proteins, including 48 known lysosomal proteins and 9 proteins that may be lysosomal. The identification of a large proportion of the known set of soluble lysosomal proteins with relatively few contaminants suggests that urine represents a promising substrate for the development of comparative proteomic methods for the investigation of lysosomal disorders and other diseases involving Man-6-P glycoproteins.

1. Introduction

Biological samples considered for mass spectrometry-based proteomic analysis frequently represent highly complex mixtures of proteins with a wide range in concentration between the most abundant and the least abundant constituents. For example, ~10,000 individual proteins have been identified in human plasma [1] and their concentrations span a range of ~ 10 orders of magnitude from the highest to the lowest abundance [2]. While probably less complex than plasma, urine cannot be regarded as a simple fluid as analyses to date have identified more than 1500 proteins that span at least 3 orders of magnitude in concentration [3]. The complexity and range of protein abundance in such mixtures presents a significant problem in mass-spectrometric analysis. Rare components of mixtures are frequently masked by more abundant components, and, even if proteins or peptides are present at similar levels, sampling considerations often prevent reproducible analysis of a complex mixture. To overcome these intrinsic difficulties, various sample preparation procedures have been developed to simplify analysis of complex mixtures. These include methods where abundant components are removed or those where the relative levels of a specific class of proteins are enriched.

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One useful approach is to enrich glycoproteins or glycopeptides in a sample using lectins or chemical methods [4,5]. While such methods may provide a significant reduction in sample complexity, the glycoproteome itself is still complex and contains many diverse components present at widely different abundances. However, there are also more focused approaches that can selectively enrich specific classes of proteins. For example, it is possible to use affinity purification to enrich for proteins containing the mannose 6-phosphate (Man-6-P) modification on N-linked glycans [6–14]. This modification is characteristic of soluble lysosomal proteins which are typically found at low abundance in biological samples and which play a critical role in the degradation of macromolecules [15].

There are over 60 different known lysosomal proteins that receive the Man-6-P modification. Deficiencies in these proteins cause over forty different human hereditary diseases, the lysosomal storage disorders [16], whose clinical hallmark is an accumulation of undigested material within the lysosomes of affected individuals. These diseases are progressive, often neurodegenerative in nature and usually result in early death. Clinical presentation typically provides sufficient information for biochemical and/or genetic testing to verify the basis for disease in most patients. However, diagnosis can be complicated by an overlap in phenotype between diseases of different genetic bases which is partly due to the presence of late-onset variants of known lysosomal storage diseases caused by hypomorphic alleles. An additional problem is that not all of the genes underlying these diseases have been identified to date and thus new lysosomal disease genes continue to be reported at a regular rate. This is exemplified by the recent discovery that mutations in cathepsin D, one of the longest known and best characterized lysosomal proteases, result in at least two types of human disease [17,18]. Definitive diagnosis of lysosomal storage diseases can therefore be problematic as illustrated by the many patients that clearly have lysosomal disorders based upon cellular pathology but in which the underlying defect cannot be determined.

One approach towards the investigation of these diseases is comparative proteomic analysis of Man-6-P-containing lysosomal proteins in samples from patients and unaffected controls. In concept, this approach is straightforward. Purification of soluble lysosomal proteins by virtue of their Man-6-P modification allows for a subsequent fractionation (by one or two-dimensional gel electrophoresis or liquid chromatography) and identification of proteins that are absent or otherwise aberrant in a patient sample as potential disease gene candidates that can be examined further by molecular genetic methods. In practice, this approach has led to the identification of new lysosomal storage disease genes and identification gene defects in patients that have been incorrectly diagnosed. For example, the defective gene in a fatal human neurodegenerative disease, classical late-infantile neuronal ceroid lipofuscinosis (cLINCL), was identified from a 2D-gel based comparison of Man-6-P glycoproteins in patient and control brain autopsy samples [19]. In addition, patients incorrectly diagnosed with a genetically-distinct disease, juvenile neuronal ceroid lipofuscinosis, were correctly diagnosed with a late-onset form of cLINCL based on initial findings from comparative analysis of Man-6-P glycoproteins [20].

Lysosomal proteins are widespread and potentially any clinical source containing these proteins could be utilized for comparative proteomic approaches. However, there are a number of practical considerations in the choice of patient sample for this sort of study. First, clinical samples should be readily obtainable. Second, lysosomal proteome coverage should be extensive so that a global approach might encompass as many known lysosomal proteins (and associated diseases) as possible. Third, given that almost all enrichment methods are relative rather than absolute, the MPR-affinity purification procedure should yield relatively few contaminants in order to minimize sample complexity.

We previously investigated the plasma Man-6-P glycoproteome using MPR-affinity purification coupled with sensitive nanospray LC-MS/MS methods [13]. However, analysis is complicated by the presence of relatively large numbers of non-lysosomal proteins [13]. This arises from incomplete removal of extremely abundant blood proteins, as well as the fact that some classical plasma proteins contain low levels of mannose 6-phosphorylated glycoforms, which probably reflects some lack of absolute fidelity in the enzyme that confers the Man-6-P modification to lysosomal proteins [13]. Preparations of Man-6-P glycoproteins from human brain autopsy material contain considerably fewer non-lysosomal proteins [11,14] but this is clearly not a readily obtainable sample.

Urine represents a readily available clinical specimen that contains a significant glycoproteome [21], but the representation of Man-6-P glycoproteins in urine has not been adequately addressed. In the past, we identified four lysosomal proteins in an MPR-purified human urine preparation using chemical sequencing [10]. In this study, we reinvestigate the human urine Man-6-P glycoproteome using more sensitive analytical methods and use a combination of MPR-affinity purification and nanospray LC-MS/MS, we have detected a substantial fraction of known soluble lysosomal proteins with few contaminants. This indicates that urine represents a promising source for proteomic investigation of the role of Man-6-P glycoproteins in disease.

2. Materials and Methods

2.1 LC-MS/MS analysis of urinary Man-6-P glycoproteins

A portion (10 µg) of a urinary Man-6-P glycoprotein preparation isolated as described previously [10] was reduced, alkylated, and analyzed by nanospray LC/MS/MS with a Thermo-Electron Finnegan LTQ linear ion trap (Thermo-Electron, San Jose, CA) mass spectrometer following methods described previously [11,13,14]. Duplicate 3 µg samples were analyzed using a 50 min or 135 min gradient. Protein identification was conducted using X! Tandem software [22] (GPM-USB, Beavis Informatics Ltd, Winnipeg, Canada) to search the April 2006 assembly of NCBI 36. This database contains 48,851 total and 43,378 unique accession numbers. Parameters for searching were: no isotope error allowed; fragment mass error of 0.4 Da; parent mass error of +4 and -0.5 Da; cysteine carbamidomethylation and methionine oxidation allowed as fixed and potential modifications during initial model generation and, in addition to deamidation at glutamine and asparagine, during model refinement; one missed cleavage; and the threshold for model refinement was $e < 0.001$. Data were exported into MS Excel spreadsheets and filtered for threshold significance if the respective protein log expectation score was of greater confidence than the highest score obtained at random by searching a reversed human proteome. Several proteins were assigned on the basis of a single peptide – in these cases, identification was accepted if the peptide log(e) score was of greater confidence than a stringent value of -8. In order to avoid redundancy arising from proteins present in the human proteome database under multiple names and/or accession numbers, a list of ENSP numbers was compiled with all numbers corresponding to a single protein referenced to a single primary number. Protein assignments were compared to this database to ensure that multiple accession numbers representing the same protein were not listed.

3. Results and Discussion

We were able to unambiguously identify a total of 67 proteins in the mixture of MPR-affinity purified proteins from human urine (Table 1). A summary of identification scores, sequence coverage and number of peptides assigned for each respective protein identified is provided in Online Supplementary Data Table 1. In addition, xml files are also provided for the database searches that can be uploaded for viewing all supporting data and spectra in the GPM (http://human.thegpm.org/tandem/thegpm_upview.html).

As expected, the majority (48/67) of the identified proteins are known lysosomal proteins, all of which were previously identified in human plasma [13]. It is worth noting that these lysosomal proteins are frequently purified from urine and other sources as Man-6-P glycoproteins that have not undergone the proteolytic processing that is characteristic of the mature active enzymes. For example, for tripeptidyl peptidase I (TPP1), a semi-tryptic peptide was clearly assigned as starting at the mature N-terminus (L196), indicating the presence of the processed form. However, a number of other peptides were assigned to the proregion of TPP1, indicating the presence of the proform.

Nineteen proteins were identified that are currently assigned neither lysosomal localization nor function (Table 1). A number of these have been previously identified in MPR-affinity purified mixtures from human brain and/or plasma [11,14] and about half have been previously demonstrated to contain Man-6-P using direct mass spectrometric methods [13,14]. Based upon either predicted or known biological function, some of these proteins probably represent previously uncharacterized lysosomal proteins e.g. acid sphingomyelinase-like 3A and retinoid inducible serine carboxypeptidase. However, their precise cellular location requires further investigation. Others represent abundant plasma proteins known to contain Man-6-P (e.g. AMBP and zinc alpha glycoprotein 2). We also isolated the abundant urinary protein uromodulin, which contains Man-6-P within the glycan portion of its carboxy-terminal glycosylphosphatidylinositol anchor [23]. The only proteins that appear to represent simple contaminants are albumin and the immunoglobulins.

There have been several recent studies of the total human urinary proteome [3,21,24–29] and the greatest number of the Man-6-P containing lysosomal proteins (40 proteins in total; Online Supplementary Data Table 1) was identified by Adachi *et al* [3]. Only two lysosomal proteins that are known to contain Man-6-P (GM2A activator protein and galactocerebrosidase) were identified in one or more of the previous studies but not found in this present study. Taken together, results from this and previous studies indicate the presence of a total of 50 lysosomal proteins detectable in urine. The source of these proteins is not clear (reviewed in [10]) but they may be derived from the bloodstream, or more likely, appear in urine as a result of active secretion by kidney cells or exocytosis of lysosomes [30,31].

In conclusion, we identify a representative selection of known lysosomal proteins in human urine, including those involved in 28 human diseases. The ability to detect of a wide variety of lysosomal proteins with relatively few contaminants indicates that urine is a promising sample for the investigation of lysosomal diseases. These results therefore provide a rationale for the development of protocols for the small-scale purification of urinary Man-6-P glycoproteins with mass-spectrometric based quantitative analysis after appropriate normalization (e.g., to concentration of creatinine, total protein or total lysosomal protein). Such methods will allow a detailed investigation of individual variation in the urinary Man-6-P glycoproteome. In particular, investigation of the role of lysosomal proteins as urinary biomarkers [32] will be possible in more widespread human disease, for example cancer, where alterations in Man-6-P glycoproteins have already been suggested to have predictive value [33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

MPR-affinity purified proteins identified in human urine.

Protein	Gene	Lysosomal#	Man-6-P*	Associated lysosomal disease
I-O-acylceramide synthase	LYPLA3	yes	+	Farber Granulomatosis
Acid ceramidase	ASAH1	yes	+	Hurler Syndrome
Alpha-L-iduronidase	IDUA	yes	+	Schindler Disease, Type I
Alpha-N-acetylgalactosaminidase	NAGA	yes	+	Mucopolysaccharidosis Type IIIB
Alpha-N-acetylglucosaminidase	NAGLU	yes	+	Metachromatic Leukodystrophy
Arylsulfatase A	ARSA	yes	+	Mucopolysaccharidosis Type VI
Arylsulfatase B	ARSB	yes	+	Gangliosidosis, Generalized GM1, Type I
Beta-galactosidase	GLB1	yes	+	Mucopolysaccharidosis Type VII
Beta-glucuronidase	GUSB	yes	+	Tay-Sachs Disease
Beta-hexosaminidase alpha chain	HEXA	yes	+	Sandhoff Disease
Beta-hexosaminidase beta chain	HEXB	yes	+	Mannosidosis, Beta A, Lysosomal
Beta-mannosidase	MANBA	yes	+	Ceroid Lipofuscinosis, Neuronal, 10
Cathepsin D	CTSD	yes	+	
Cathepsin F	CTSF	yes	+	
Cathepsin H	CTSH	yes	+	
Cathepsin L	CTSL	yes	+	
Cathepsin O	CTSO	yes	+	
Cathepsin Z	CTSZ	yes	+	
Ceroid-lipofuscinosis neuronal protein	CLN5	yes	+	Ceroid Lipofuscinosis, Neuronal, 5
Deoxyribonuclease II alpha	DNASE2	yes	+	
Di-N-acetylchitinase	CTB	yes	+	
Dipeptidyl-peptidase I	CTSC	yes	+	Papillon-Lefevre Syndrome
Dipeptidyl-peptidase II	DPP7	yes	+	
Epididymal secretory protein E1	NPC2	yes	+	Niemann-Pick Disease, Type C2
Epididymis-specific alpha-mannosidase	MAN2B2	yes	+	
Gamma-glutamyl hydrolase	GGH	yes	+	
Iduronate 2-sulfatase	IDS	yes	+	Mucopolysaccharidosis Type II
Legumain	LGMN	yes	+	
LOC196463	LOC196463	yes	+	
Lysosomal acid lipase	LIPA	yes	+	Wolman Disease
Lysosomal alpha-glucosidase	GAA	yes	+	Glycogen Storage Disease II
Lysosomal protective protein	PPGB	yes	+	Galactosialidosis
Lysosomal Pro-X carboxypeptidase	PRCP	yes	+	
Mammalian ependymin-related protein	EPDR1	yes	+	
Myeloperoxidase	MPO	yes	+	Myeloperoxidase Deficiency
N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	AGA	yes	+	Aspartylglucosaminuria
N-acetylglucosamine-6-sulfatase	GNS	yes	+	Mucopolysaccharidosis Type IIID
N-sulphoglucosamine sulphotransferase	SGSH	yes	+	Mucopolysaccharidosis Type IIIA
Palmitoyl protein thioesterase 2	PPT2	yes	+	
Plasma glutamate carboxypeptidase	PGCP	yes	+	
Proactivator polypeptide precursor	PSAP	yes	+	Saposin deficiencies
Probable serine carboxypeptidase CPVL	CPVL	yes	+	
Ribonuclease T2	RNASET2	yes	+	
Sialate O-acetyltransferase	SIAE	yes	+	
Sialidase-1	NEU1	yes	+	Neuraminidase Deficiency
Sphingomyelin phosphodiesterase	SMPD1	yes	+	Niemann-Pick Disease, Type A
Tissue alpha fucosidase	FUCA1	yes	+	Fucosidosis
Tripeptidyl-peptidase I	TPPI	yes	+	Ceroid Lipofuscinosis, Neuronal, 2
Acid sphingomyelinase-like phosphodiesterase 3A	SMFDL3A	likely	+	Biotinidase Deficiency
Biotinidase	BTD	likely	+	
CREG1 protein	CREG1	likely	+	
Retinoid-inducible serine carboxypeptidase	SCPEP1	likely	+	
Deoxyribonuclease-1	DNASE1	possibly	+	

Protein	Gene	Lysosomal [#]	Man-6-P [*]	Associated lysosomal disease
Granulins	GRN	possibly		
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	PLOD1	possibly		
Prostaglandin-H2 D-isomerase	PTGDS	possibly	+	Nevo Syndrome
Ribonuclease pancreatic	RNASE1	possibly	+	
AMBp protein	AMBp	possibly	+	
CD59 glycoprotein	CD59			
Galectin-3-binding protein	LGALS3BP			
Ig alpha-1 chain C region	IGHV			
Ig gamma-4 chain C region	IGH4			
Kininogen-1	KNG1			
Oxidized low-density lipoprotein receptor	OLR1			
Uromodulin	UMOD		+	
Zinc-alpha-2-glycoprotein	AZGP1		+	
Serum albumin	ALB	no		

^{*} experimentally demonstrated to contain Man-6-P
[#] categorization based upon review of literature and/or known function of homologs.