Proteomics Analysis of Serum from Mutant Mice Reveals Lysosomal Proteins Selectively Transported by Each of the Two Mannose 6-Phosphate Receptors* [S]

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Most mammalian cells contain two types of mannose 6-phosphate (Man-6-P) receptors (MPRs): the 300 kDa cation-independent (CI) MPR and 46 kDa cation-dependent (CD) MPR. The two MPRs have overlapping function in intracellular targeting of newly synthesized lysosomal proteins, but both are required for efficient targeting. Despite extensive investigation, the relative roles and specialized functions of each MPR in targeting of specific proteins remain questions of fundamental interest. One possibility is that most Man-6-P glycoproteins are transported by both MPRs, but there may be subsets that are preferentially transported by each. To investigate this, we have conducted a proteomics analysis of serum from mice lacking either MPR with the reasoning that lysosomal proteins that are selectively transported by a given MPR should be preferentially secreted into the bloodstream in its absence. We purified and identified Man-6-P glycoproteins and glycopeptides from wild-type, CDMPR-deficient, and CIMPR-deficient mouse serum and found both lysosomal proteins and proteins not currently thought to have lysosomal function. Different mass spectrometric approaches (spectral count analysis of nano-spray LC-MS/MS experiments on unlabeled samples and LC-MALDI/TOF/TOF experiments on iTRAQ-labeled samples) revealed a number of samples that appear specifically elevated in serum from each MPR-deficient mouse. Man-6-P glycoforms of cellular repressor of E1A-stimulated genes 1, tripeptidyl peptidase I, and heparanase were elevated in absence of the CDMPR and Man-6-P glycoforms of alpha-mannosidase B1, cathepsin D, and prosaposin were elevated in absence of the CIMPR. Results were confirmed by Western blots analyses for select proteins. This study provides a comparison of different quantitative mass spectrometric approaches and provides the first report of proteins whose cellular targeting appears to be MPR-selective under physiological conditions.

The lysosome is a cellular organelle that plays a role in the degradation of macromolecules and contains ~60 known soluble hydrolytic enzymes and associated proteins to achieve this purpose (1). Most of these proteins are targeted to the lysosome by the mannose 6-phosphate (Man-6-P) pathway. Here, shortly after biosynthesis, the N-linked oligosaccharides on lysosomal proteins are selectively modified to generate the Man-6-P recognition marker. The phosphorylated proteins bind to Man-6-P receptors (MPRs) in the trans Golgi network (TGN). The receptors and bound ligands enter transport vesicles and travel to a prelysosomal compartment where the receptor and ligands dissociate. The lysosomal proteins subsequently travel to the lysosome while the MPRs recycle back to the TGN to repeat the process, or to the cell surface.

Most mammalian cells contain two distinct MPRs, a ~46 kDa cation-dependent (CD) MPR and a ~300 kDa cation-independent (CI) MPR (2, 3). Both receptors are type I transmembrane glycoproteins that cycle between the TGN, endosomes, and the plasma membrane. In addition to its role in the transport of newly synthesized lysosomal proteins, the CI-MPR is also involved in the endocytosis of extracellular Man-6-P glycoproteins, whereas the CDMPR, despite being present on the cell surface, does not function in endocytosis under physiological conditions. The CIMPR also binds insulin-like growth factor II (IGF2) (4, 5), a ligand that does not contain Man-6-P, and mediates its endocytosis and clearance from the bloodstream.

The physiological rationale for the existence of two distinct MPRs has long been a question of fundamental interest (6, 7), and one approach to investigate their individual functions has been to generate mutant mice lacking either type of MPR. CIMPR mutants have developmental abnormalities and usually die perinatally (8, 9), but this is independent of disruptions in lysosomal enzyme targeting and results from decreased degradation of specific proteins.
clearance of IGF2 and subsequent embryonic overgrowth. Mice lacking both IGF2 and the CIMPR are dwarfs due to the lack of IGF2 but otherwise viable and fertile (9–11). CDMPR mutants are fertile and have otherwise no obvious defects (12, 13). Mice lacking both the CDMPR and CIMPR in an IGF2-deficient background have a short lifespan and exhibit a lysosomal storage phenotype (10, 14).

Serum from mice lacking either MPR (in an IGF2-deficient background) contain elevated levels of Man-6-P glycoproteins (12, 15), suggesting that both MPRs are required for the complete targeting of all lysosomal proteins under physiological conditions. This conclusion is supported by studies of primary fibroblasts derived from these mice that demonstrate that the lack of either MPR results in the missorting of multiple lysosomal proteins while the lack of both MPRs results in the secretion of even greater amounts (12, 13, 16, 17). In general, the levels of Man-6-P glycoproteins secreted in the absence of the CIMPR appeared higher than those secreted in the absence of the CDMPR. This is consistent with binding studies employing purified receptors that indicate that all of the proteins bound by the CDMPR were also bound by the CIMPR (but not vice-versa) and with higher affinity (18). However, if there are Man-6-P-containing ligands that are preferentially (but not vice-versa) transported by either MPR, they have not been identified to date.

Thus, the precise molecular basis for the requirement for two MPRs still remains to be elucidated. One explanation is that they are required for the targeting of overlapping yet distinct subsets of lysosomal proteins. Previous investigations using a blotting assay revealed that Man-6-P glycoproteins of ~26 and ~114 kDa were selectively elevated in serum of CDMPR- and CIMPR-deficient mice, respectively (15). In the current study, we have used mass spectrometric approaches to confirm and extend these observations and to identify Man-6-P glycoproteins that are selectively elevated in the absence of each type of MPR. Identification of these proteins represents an important step toward understanding the respective function of each receptor in lysosomal protein targeting.

**EXPERIMENTAL PROCEDURES**

**Materials**—Affi-Gel 15, immobilized pH gradient (IPG) strips (11 cm nonlinear pH 3–10) and Triton X-100 were from Bio-Rad (Hercules, CA); leupeptin, pepstatin A, urea, α-dithiothreitol (DTT), CHAPS, iodacetamide and horseradish peroxidase-conjugated secondary antibodies were from Sigma (St. Louis, MO); Pefabloc was from Pentafarm (Basel, Switzerland); SuperSignal West Pico chemiluminescent reagent, Surfact-Amps Triton X-100 and TCEP hydrochloride were from Pierce (Rockford, IL); IPG buffer pH 3–10 was from GE Healthcare (Piscataway, NJ); thiourea was from Fluka (Buchs, Switzerland); ASB-14 and Zwittergent 3–10 were from Calbiochem (San Diego, CA), and iTRAQ reagents were from Applied Biosystems (Framingham, MA).

**Mice**—All experiments and procedures involving live animals were conducted in compliance with approved Institutional Animal Care and Use Committee protocols. Adult mice (2–12 months) were killed as described previously (19). Blood was collected by heart puncture, allowed to clot for 1 h at room temperature, and serum was obtained by centrifugation at 300 × g for 15 min and stored at −80 °C until use. Blood was collected from wild-type (wt) mice (mixed genetic background; strains BALB/c, 129/Sv, and C57BL/6J), or mice deficient in both the CIMPR and IGF2 (designated here as CI(-), official designation Igfl2r(-/-) IGF2(-/-), mixed genetic background; strains C57BL/6J and 129/Sv (15)) or mice deficient in the CDMPR (designated here as CD(-), official designation M6pr(-/-), congenic C57BL/6J (12)). While the genetic backgrounds of the groups of mice were not matched, comparison of current blotting experiments (see “Results”) with previous blotting experiments on serum from 16-day-old littermates of similar genetic backgrounds lacking IGF2 (15) indicated that that strain differences were unlikely to affect the results. (Note that the receptor deficiencies in the juvenile animals analyzed previously appeared to result in a greater magnitude of Man-6-P glycoprotein secretion compared with the current measurements, possibly due to age differences, but the patterns were quite similar.) However, we cannot exclude some minor variations due to strain dependence.

**Purification and Immobilization of Each MPR**—Soluble bovine CIMPR (sCIMPR) was purified from fetal bovine serum by affinity chromatography on immobilized phosphomannan (20) and was coupled to Affi-Gel 15 as described (21) at a density of 2.3 to 4 mg/ml (9 to 16 μg). Soluble bovine CDMPR (sCDMPR) was produced from a Pichia pastoris strain expressing a construct encoding the extracytoplasmic domain engineered to eliminate four of the five N-linked glycosylation sites (generously provided by Dr. Nancy Dahms) (22). sCDMPR was first isolated from culture medium by absorption on immobilized phosphomannan. The protein was eluted with 20 mM sodium acetate pH 4.5 and applied to a Mono S 10/100 GL column (GE Healthcare) which was then eluted with a 0 to 2 mM sodium chloride gradient in 20 mM sodium acetate, pH 4.5. Fractions containing sCDMPR were further purified by gel filtration on a 26 × 60 cm Superdex 75 column (GE Healthcare) equilibrated with 50 mM MES buffer, pH 6.5 containing 100 mM NaCl, 80 °C. Sypro Ruby (Molecular Probes, Eugene, OR) gel stain of purified sCDMPR indicated no visible contaminants. Purified sCDMPR was immobilized on Affi-Gel 15 at a density of 1.4–2.4 mg/ml (80 to 140 μg).

**Affinity Purification of Mouse Serum Man-6-P Glycoproteins**—Serum was thawed at 37 °C and centrifuged at 13,000 × g for 1.5 h at 4 °C. Supernatants were filtered using 6 layers of cheesecloth and diluted into an equal volume of buffer A (100 mM imidazole, pH 6.5, 150 mM NaCl, 20 mM MnCl2, 10 mM β-glycerophosphate, 2% Triton X-100, 2 μM Pefabloc, 2 μg/ml leupeptin, 2 μg/ml pepstatin A). Affinity chromatography was conducted using columns (1 × 10 cm) containing between 2 and 4 ml bed volume of immobilized MPR equilibrated with buffer B (50 mM imidazole, pH 6.5, 150 mM NaCl, 10 mM MnCl2, 5 mM β-glycerophosphate, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) containing 1% Triton X-100 and 1 mM Pefabloc. Diluted serum was loaded onto the columns, which were then washed with eight column volumes of buffer B containing 1% Triton X-100 followed by washing with buffer B until A280 returned to baseline. After washing, the column was mock eluted using four column volumes of buffer B containing 10 mM glucose 6-phosphate (Glu-6-P) followed by specific elution with four column volumes of buffer B lacking MnCl2 and containing 10 mM Man-6-P. Finally, each column was eluted with four column volumes of 100 mM glycine pH 2.5 to remove tightly bound and nonspecifically bound material. Fractions containing Man-6-P glycoproteins were identified by monitoring the activities of β-glucuronidase and β-mannosidase (15). In some cases, Man-6-P-containing glycoproteins were repurified by MPR affinity chromatography after removal of Man-6-P by buffer exchange to buffer B by ultrafiltration (Ultra-15, Millipore, Billerica, MA). The final Man-6-P elutes
were concentrated and buffer exchanged to 100 mM ammonium bicarbonate by ultrafiltration. Protein concentrations were determined using the Bradford method (23) with bovine serum albumin as a standard. Aliquots of purified Man-6-P glycoproteins were stored at –80 °C prior to use.

**Two-dimensional Gel Electrophoresis—** IPG strips were rehydrated for 14 h with sample in 200 μl rehydration buffer (7 M urea, 1.25% CHAPS, 2 mM thiourea, 32.5 mM DTT, 0.65% v/v IPG buffer pH 3–10 NL, 0.5% w/v Zwittergent 3–10, 0.5% w/v ASB-14, 0.5% v/v Surfact-Amps Triton X-100, 2.5 mM TCEP hydrochloride, and 0.0002% bromophenol blue). Isoelectric focusing was conducted using a PROTEAN IEF Cell (Bio-Rad) with a current limit of 50 μA per strip for a total of 54,000 voltage hours. IPG strips were stored at –80 °C prior to two-dimensional SDS-PAGE. Proteins in the IPG strips were reduced with DTT, alkylated with iodoacetamide, and fractionated on 11.5% polyacrylamide gels run at constant current (60 mA per gel) for 6 h. For visualization, preparative gels were stained first with Sypro Ruby and then stained with colloidal Coomassie blue (24) and were scanned using either a Typhoon 9400 (GE Healthcare) or an Epson scanner.

**Detection of Man-6-P Containing Glycoproteins—** After fractionation by one- or two-dimensional gel electrophoresis, proteins were transferred to nitrocellulose and glycoproteins containing the Man-6-P modification were detected using 125I-labeled sCIMPR as described previously (25). Signal was visualized and quantified by storage phosphor autoradiography using a Typhoon 9400 scanner (GE Healthcare) and ImageQuant 5.2 software, respectively.

**In-gel Tryptic Digestion—** Gel slices or spots excised from one-dimensional and two-dimensional gels were digested with modified trypsin (Promega, Madison, WI) as described. In addition, for Preparation B, digested gel slices were extracted using 60% acetonitrile (ACN)/5% formic acid (FA) and pooled with the supernatants prior to drying samples and subsequent LC-MS/MS.

**iTRAQ Labeling—** iTRAQ labeling (26) was performed according to the manufacturer’s protocol (Applied Biosystems). We conducted two iTRAQ experiments. The first iTRAQ experiment was conducted on material from B1 and B2 (iTRAQ 2, Fig. 1). In brief, four samples representing equal amounts of protein (20 μg) purified from CD(-) and CI(-) mouse serum were spiked with chicken ovalbumin and processed as described above without generating the pooled standard.

**Two-dimensional Liquid Chromatography of iTRAQ Samples—** Forty micrograms of an iTRAQ-labeled mixture was dissolved in 100 μl 0.1% FA, and loaded onto a pipette C18 tip (SPEC, Varian, Lake Forest, CA) equilibrated with 0.1% FA. The absorbed sample was washed with 300 μl 0.1% FA and peptides eluted with 100 μl of 50% ACN containing 0.1% FA and then 100 μl of 80% ACN/0.1% FA. Eluates were pooled and vacuum-dried. To reduce sample complexity prior to tandem mass spectrometry, iTRAQ labeled peptides were fractionated by two-dimensional liquid chromatography consisting of strong cation exchange (SCX) followed by reverse phase liquid chromatography. For SCX, the dried mixture was dissolved in 25 μl buffer A (5 mM KH2PO4 and 25% ACN, pH 3.0) and applied to a polysulfoethyl A column (PolyLC Inc, Columbia, MD) equilibrated with 0.1% FA. The absorbed sample was washed with 300 μl 0.1% FA, and peptides eluted with 100 μl of 50% ACN, 300 mM KCl, pH 3.0. The column was washed with Buffer A for 5 min; then peptides eluted with a gradient of 0–100% Buffer B in 30 min followed by 100% Buffer B for 5 min. Fractions were collected at 4 min intervals and vacuum-dried. For second dimension reverse phase liquid chromatography, SCX fractions of interest were dissolved in 10 μl 0.1% TFA, and 5 μl was loaded onto a 300 μm × 5 mm C18 trap column (Dionex). The trap column was washed with 0.1% TFA. The flow was reversed and connected to a 75 μm × 12 cm column (in-house packed with Magic C18AQ, 3 μm, 200 Å, Michrom BioResources Inc., Auburn, CA). Peptides were eluted using a linear gradient of 0–50% ACN in 0.1% TFA at a flow rate of 200 nl/min for 30 min with an infusion of 5 mg/ml alpha-cyano-4-hydroxy cinnamic acid in 50% ACN containing 0.1% TFA at a flow rate of 1.5 μl/min. Fractions were collected every 10 s and spotted onto a 384-well MALDI plate using a Probot microfraction collector (Dionex).

**Tandem Mass Spectrometry—** Nanospray LC-MS/MS of tryptic digests was conducted using an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) as described previously (28). Tandem MALDI mass spectrometry was conducted using an Applied Biosystems 4800 MALDI-TOF/TOF. MS spectra were acquired in a window of m/z 800–4000 in positive ion reflectron mode. The 15 most abundant precursor ions with a signal-to-noise ratio >10 were selected for top-down MS/MS scans, excluding identical precursor ions contained in adjacent spots from a given LC-MALDI run. MS/MS was conducted using medium collision energy (1 kV) in positive ion mode.

**Identification of Sites of Man-6-P Modification—** Affinity purified protein samples (10 μg) were denatured, reduced, and alkylated (27). Samples were buffer exchanged by ultrafiltration to 50 mM ammonium bicarbonate and then digested with trypsin alone or trypsin together with Glu-C (Roche). The Man-6-P-containing peptides were purified on an immobilized sCIMPR microcolumn and deglycosylated with Endo H as described previously (29).

**Data Analysis—** For analysis of LTQ data, peak lists were generated using the Sequest module of Bioworks 3.3 (Thermo Electron) using previously described parameters (29). For analysis of 4800 data, peak lists were generated using the “Launch Peaks to Mascot” function of the 4000 Series Explorer software (Applied Biosystems) using the following parameters: minimum signal to noise for both MS and MS/MS peaks was 20; minimum peak area of 1000 for both MS and MS/MS; MS spectra range, m/z from 500 to 4000; MS/MS spectra range, m/z from 60 to 20 units below the m/z of the selected precursor ion. Database searches were conducted using a local version of the Global Proteome Machine (GPM) (30, 31) (GPM-USB, Beavis Informatics Ltd., Winnipeg, Canada) using a combined ENSEMBL mouse
NCBI/36.42 database (29784 unique sequences) and a database of non-mouse protein contaminants (cRAP, GPM common Repository of Adventious Proteins). Data files generated with a given instrument were searched both individually and together to produce a merged output using the “Mupit” option. LTQ LC-MS/MS data were analyzed as described (29), and 4800 MALDI-TOF/TOF data were searched using a precursor ion mass error of 100 ppm and a fragment mass error of 0.4 Da. Thresholds for the minimum number of MS/MS fragments were 5 and 15 for the MALDI-TOF/TOF and LTQ data, respectively. For all MS/MS searches, cysteine carbamidomethylation was a constant modification, oxidation of methionine was a variable modification, and one missed cleavage was allowed during the preliminary model development. The threshold used for model refinement was a peptide expectation score of 0.0001. During refinement, deamidation at asparagine and glutamine residues was allowed. For analysis of iTRAQ experiments, modifications were as described above except a fixed iTRAQ modification at lysines and peptide amino termini were specified during preliminary model development and refinement.

The spectral count method (32) was used to estimate the relative abundance of a given protein in two different sample types analyzed using LC-MS/MS. The total number of spectra assigned to a given gene product was tabulated for each type of sample (note that in this analysis, spectral counts assigned to different accession numbers of the same gene, polymorphisms and splice variants, were combined). The proportion of counts in a given sample was calculated as

$$\hat{\rho} = \frac{a}{a+b}$$  \hspace{1cm} (Eq. 1)

where a and b represent the spectral counts of a given protein in samples a and b, respectively. The statistical tests used are described in (33) unless noted otherwise and computed using the open source language R (R Foundation for Statistical Computing). Upper and lower 95% confidence intervals were calculated using Wilson’s method (34) to estimate counting errors. One-sided p values were calculated using the exact binomial test to determine if values were significantly different from 1, 1.5, and 2 (see “Results”). p values were transformed to q values to control for the false discovery rate among multiple comparisons (35). To estimate fold differences, data are presented as log$_2$(\hat{\rho}), which is formally,

$$\log_2(\hat{\rho}) = \log_2\left(\frac{\hat{\rho}}{1-\hat{\rho}}\right)$$  \hspace{1cm} (Eq. 2)

For clarity of presentation, for a = 0 or a/b ≤ 0.1, the log$_2$(\hat{\rho}) value was replaced by −3.32 (log$_2$ 0.1), while for b = 0 or a/b ≥ 10, it was replaced by 3.32 (log$_2$10). Fisher’s exact test was also used to calculate confidence intervals and significance values for odds ratios, estimated as

$$\text{OR}_i = \frac{a_i/b_i}{a_0/b_0}$$  \hspace{1cm} (Eq. 3)

where a$_i$ and b$_i$ represent the spectral counts of a given protein i in samples a and b, respectively, and a$_0$ and b$_0$ represent the total spectral counts in samples a and b, respectively. Statistics are included in supplemental Table VII.

For calculation of iTRAQ reporter ion ratios, in-house PERL scripts were used to generate a list of identified spectra and associated information from the GPM xml files and to extract relevant peak areas from the Oracle database used by the TOF-TOF for data acquisition. These data were subsequently manipulated using Microsoft Excel. Matrix functions were used to adjust iTRAQ reporter ion peak areas for crossover using correction factors supplied by the vendor. The following inclusion criteria were applied to increase the quality of the data set: 1) only proteins with GPM expectation scores of 10$^{-5}$ or better were chosen for further analysis; 2) for all comparisons, data were excluded if the average iTRAQ reporter ion intensity was below 10,000 or if any individual intensity was below 1000 (see “Results”); and 3) proteins were required to be represented by at least two independent peptides for inclusion in the final analysis. The log$_2$ values of the remaining peak area ratios were used to calculate a mean and standard deviation for each protein. Student’s two-sample $t$ test (assuming equal variance) was used to calculate p values, and q values were subsequently calculated as described above.

**RESULTS**

**Purification and Comparison of Man-6-P Containing Glycoproteins**—The overall goal of this study was to identify proteins that might interact preferentially with one of the two MPRs and thus provide an avenue for the investigation of individual MPR-specific functions. Our initial approach was to fractionate wild-type mouse brain, a rich source of Man-6-P glycoproteins, using columns of either immobilized sCIMP3 or sCDMPR. Equivalent amounts of brain extract were applied to both columns, and after washing, each column was eluted first with Man-6-P and then with 100 mM glycine pH 2.5. The Man-6-P and glycine eluates were analyzed using $^{125}$I-labeled sCIMP3 (supplemental Fig. 1) and LC-MS/MS (supplemental Table I). These analyses helped characterize the brain Man-6-P glycoproteome as well as identifying additional potential ligands for the CIMP3 (see “Discussion”). However, no profound differences were found by $^{125}$I-labeled sCIMP3 blotting, and a serious concern with this purification approach is that the concentration of immobilized MPRs used (micromolar range) greatly exceeded the K$_d$ of most phosphorylated lysosomal proteins for each receptor (typically nanomolar range (18)); thus, biologically relevant differences in affinity would most likely be masked. In addition, conditions used for *in vitro* binding assays may not reflect the physiological conditions that the MPRs experience in the TGN.

Given these concerns, we chose to identify the spectrum of Man-6-P-containing glycoproteins present in serum of mice lacking either type of MPR and to estimate their relative abundances. The rationale was that, if intracellular lysosomal targeting for a given newly synthesized protein is selectively mediated by a single MPR, it would be selectively secreted in the absence of that MPR and thus may be elevated in blood (15). To this end, we conducted two large scale purification experiments on wt, CD(-), and CI(-) serum, with the overall...
Experimental design shown in Fig. 1. The two purifications served as both biological and technical replicates, using pooled sera obtained from different animals (~100 animals of each genotype were used per purification). In addition, the two purifications were designed to evaluate different experimental factors.

The first large scale preparation was designed to monitor column capacity and specificity of the immobilized MPRs. Mouse serum was applied to two columns connected in tandem, the first containing immobilized sCDMPR (4 ml bed volume, 1.4 mg/ml, ~80 μM), then a portion of the material eluted with Man-6-P was buffer exchanged by ultrafiltration to remove Man-6-P and repurified using a fresh sCDMPR column (2 ml bed volume, 2.4 mg/ml, ~140 μM). MPR blotting and LC-MS/MS analyses were conducted on the Man-6-P eluates of A1, A1′, B1, and B2. Treatment of purified samples prior to MPR blotting and/or LC-MS/MS analyses is indicated as follows: sol, solution trypic digestion; 1D, one dimensional gel electrophoresis; 2D, two-dimensional gel electrophoresis. Man-6-P eluates of B1 and B2 were also subjected to iTRAQ analysis.

Fig. 2. Man-6-P-containing glycoproteins in mouse serum. Wild-type (wt), CD(-), and CI(-) serum (4 μl) or Man-6-P eluates (amount derived from 20 μl serum) from tandem sCDMPR and sCIMPR affinity columns (A1 and A1′, Fig. 1) were fractionated by SDS-PAGE, transferred to nitrocellulose and Man-6-P-containing glycoproteins were detected using 125I-labeled sCIMPR (25). The fraction of total Man-6-P glycoproteins associated with the sCDMPR column was calculated by the specific signal of the phosphorimager counts (background corrected) from the sCDMPR column Man-6-P eluate divided by the sum of the specific signals from the sCDMPR and sCIMPR column Man-6-P eluates. All of the preparations yielded similar values (94–98% of the specific signal associated with the sCDMPR column eluate).

Essentially no signal was detected in material eluting with glycine, indicating that Man-6-P was sufficient to elute all Man-6-P-containing glycoproteins (data not shown). Moreover, there were no unique bands apparent from MPR blotting that were selectively retained by the second sCIMPR column (Fig. 2, compare A1 and A1′). In addition, LC-MS/MS analysis on equivalent amounts of protein from each column eluate did not reveal any lysosomal proteins that did not adhere to the sCDMPR column and that were selectively retained by the sCIMPR column (supplemental Table II).

The yields of MPR-purified protein were ~10–50 μg/ml serum, representing a purification factor >1000, given a total serum protein concentration of ~70 mg/ml. Blotting revealed that, compared with wild-type controls, Man-6-P glycoprotein levels per milliliter of serum were elevated ~3 and ~5 fold in CD(-) and CI(-) mouse serum, respectively (Fig. 2, middle panel, and supplemental Table III). Note that analysis of unfractonated sera by MPR-blotting reveals ~26 kDa and ~114 kDa proteins enriched in the CD(-) and CI(-) sera, respectively, as seen previously in MPR-deficient mice (15). While these bands were also observed in the MPR-affinity purified samples, the MPR purification step also allowed visualization of numerous additional Man-6-P glycoproteins that were previously obscured by albumin and other abundant serum proteins (Fig. 2).
The samples from A1 and B2 were also fractionated by SDS-digests of the total material in preparations A1, B1, and B2. For each of the three mouse genotypes, we analyzed tryptic position of the different purified samples using LC-MS/MS.

Not apparent from this analysis.

ble IV). However, a candidate for the excellent candidate for the 26 kDa protein (supplemental Ta-

While the overall pattern of stained proteins was remarkably similar in all three types of serum samples, MPR affinity purified material was further fractionated by two-dimensional gel electrophoresis and analyzed by MPR blotting and staining (supplemental Fig. 3). While the overall pattern of stained proteins was remarkably similar in all three types of serum samples, sCIMPR blotting revealed a series of spots that appeared to contain the ~26 kDa protein elevated in the CD(-) sample. LC-MS/MS analysis revealed multiple proteins per spot analyzed but clearly indicated that cellular repressor of E1A-stimulated genes 1 (Creg1) was an abundant contaminants from proteins that specifically associate with the immobilized sCDMPR. Here, after application of each type of serum to a single sCDMPR column, a portion of Man-6-P eluate was buffer exchanged by ultrafiltration to remove Man-6-P and repurified using a fresh sCDMPR column. This procedure helped remove some nonspecific contaminants, but comparison of the Coomassie stained gels did not reveal likely candidates that correspond to the selectively elevated Man-6-P glycoproteins identified by blotting (supplemental Fig. 2).

Two-dimensional Gel Electrophoresis Analysis—In an attempt to identify proteins selectively elevated in the different serum sources, MPR affinity purified material was further fractionated by two-dimensional gel electrophoresis and analyzed by MPR blotting and staining (supplemental Fig. 3). While the overall pattern of stained proteins was remarkably similar in all three types of serum samples, sCIMPR blotting revealed a series of spots that appeared to contain the ~26 kDa protein elevated in the CD(-) sample. LC-MS/MS analysis revealed multiple proteins per spot analyzed but clearly indicated that cellular repressor of E1A-stimulated genes 1 (Creg1) was an excellent candidate for the 26 kDa protein (supplemental Table IV). However, a candidate for the ~114 kDa protein was not apparent from this analysis.

Spectral Count Analysis—We also characterized the composition of the different purified samples using LC-MS/MS. For each of the three mouse genotypes, we analyzed tryptic digests of the total material in preparations A1, B1, and B2. The samples from A1 and B2 were also fractionated by SDS-

PAGE, and 32 gel slices from each were analyzed following in-gel digestion with trypsin. After excluding likely contaminants such as highly abundant mouse proteins known not to be lysosomal and non-mouse proteins introduced during sample handling, 271 gene products in the mouse database were identified, including 60 known or likely lysosomal proteins (henceforth referred to as lysosomal, see supplemental Table V). Finally, to help identify true Man-6-P glycoproteins from potential contaminants, we digested the MPR-purified proteins with trypsin and isolated and identified Man-6-P glycopeptides as described previously (29). This resulted in the identification of 57 phosphorylation sites on 37 lysosomal proteins (including palmitoyl protein thioesterase 2 and de-oxyribonuclease 2, which were not identified in the total purified protein mixture) and 81 phosphorylation sites on 52 additional proteins (supplemental Table VI).

We analyzed the LC-MS/MS results using the spectral count method (32) to identify serum proteins that are specifically elevated in the absence of either MPR. To conveniently visualize fold-differences in the relative abundance of a given protein in two different samples, data are presented as the log2 of the spectral count ratio (see “Experimental Procedures”), with a value of 1 representing a relative 2-fold in increase in abundance and a value of ~1 representing a relative 2-fold decrease in abundance. Moreover, we can determine confidence intervals based on the total number of spectral counts and the relative ratios (see “Experimental Procedures”). The distribution of all proteins in the different serum samples is shown in supplemental Fig. 4. The relative levels of lysosomal proteins in CD(-) and CI(-) samples are indicated in Fig. 3. We consider proteins to be highly elevated in the CD(-)
due to inadequate reporter ion statistics arising from insufficient, alkylation, digestion, and labeling); and 2) variation in steps prior to sample pooling (see "Experimental Procedures"). While the vast majority of MS/MS analyses yielded reporter ions with the expected intensity ratio, there were a number of outliers, particularly at low signal intensities (supplemental Fig. 6). Given this, we filtered the data based on signal threshold as described under "Experimental Procedures," which excluded ~5% of the total spectra (supplemental Fig. 6).

The relative abundance of the Man-6-P forms of lysosomal proteins in the three types of serum samples is shown in Fig. 4. As expected, the relative abundance of most lysosomal proteins present in the wild-type serum sample (hatched bars) was lower when compared with samples from the MPR-deficient animals. In contrast, when comparing the CI(-) (open bars) and CD(-) (filled bars) samples, the relative abundance was highly dependent on the individual lysosomal protein analyzed.

A second iTRAQ experiment analyzing CI(-) and CD(-) samples from preparations B1 and B2 was conducted to validate the first data set. Comparison of individual spectra indicated similar CD(-)/CI(-) ratios for the B1 samples analyzed in iTRAQ Experiments 1 and 2 and for the B1 and B2 samples (supplemental Fig. 7 and supplemental Table VIII). Given that the 95% confidence interval decreases with the number of spectra analyzed, we chose to use the combined analysis to directly compare proteins. This revealed that TPP1, Hps, Rnaset2, and Creg1 were ≥2-fold elevated in the CD(-) samples while Man2b1, Ctsd, and Psap were ≥2 fold elevated in the CI(-) samples (Fig. 5).

Results from the iTRAQ and spectral count analyses are generally in excellent agreement (Fig. 6 and supplemental Table IX).
The second iTRAQ experiment also helped distinguish proteins of interest that are currently not known to be lysosomal from likely contaminants. Preparations B2 and B1 differ in that B2 should be depleted of nonspecific contaminants. A plot of the log₂ B2/B1 ratios for the CD(-) and CI(-) specimens indicate that, as expected, lysosomal proteins are enriched in B2 (Fig. 7, filled circles). While some of the proteins not known to be lysosomal are depleted in B2, some are enriched (Fig. 7, open circles). The latter represent proteins that are worth evaluating further with regard to mannose 6-phosphorylation status and potential lysosomal localization (see “Discussion”).

Verification of Quantitative Mass Spectrometry by Western Blotting—We conducted Western blot analysis on purified samples for several of the proteins that appear to be elevated in the absence of either MPR. Both the spectral count and iTRAQ mass spectrometric analyses indicate that Man2B1 and Ctsd are elevated in the absence of the CIMPR and that Tpp1 and Creg1 are elevated in CD(-) serum. In terms of general trend, all these results were corroborated by the Western blotting experiments (Fig. 8). In addition, the Niemann-Pick type C2 protein (Npc2) appeared to be moderately elevated in CD(-) serum from the iTRAQ analysis, and this elevation was also corroborated by Western blotting (Fig. 8).

DISCUSSION

The central focus of this study was to identify proteins that are specifically targeted by each MPR with the added benefit of expanding our knowledge of the Man-6-P glycoproteome. In the course of this research, we have explored different mass spectrometric-based analytical methods for proteome comparison. Application of such methods is not limited to the current study and may be of general interest.

Spectral Count Analysis—Our initial data sets were generated using essentially qualitative LC-MS/MS methods. However, we explored the use of the spectral count method (32) in an attempt to abstract quantitative information from these
data. There are several statistical methods that could be used to analyze spectral count data (39, 40). We have chosen simply to compare the spectral counts in two specimens of interest without normalization and calculate confidence intervals using the Wilson’s method and significance using the exact binomial test with a false discovery rate correction (see “Results” and supplemental Table VII). We also calculated an odds ratio, taking into account the total spectral counts associated with each specimen, and determine confidence intervals and statistical significance using Fisher’s exact test (supplemental Table VII). For the serum samples, both methods yield similar results, which is expected because the total number of spectral counts were similar in the wt, CD(-), and Cl(-) data sets. However, when comparing levels of proteins in Man-6-P and glycine eluates of MPR-purified brain samples, the odds ratio analysis appears to overestimate the abundance of proteins in the glycine sample where there were far fewer total protein assignments (see below).

We chose to merge results from separate MS-based analyses (solution digests and in-gel digests) and purifications (A, B1, and B2, Fig. 1). We think that this is justified as analysis of each individual experiment reveals a similar trend in terms of relative abundance of a given protein in the wt, CD(-), and Cl(-) samples (Supplemental Table V). Data pooling tends to average out random variation between experiments and also increases the total number of spectral counts. This is important, as there is considerable inherent uncertainty in the calculated ratios when the spectral counts are low. For instance, for the case where the spectral counts for a given protein in two samples differ by a factor of 2, then one would need at least 42 total spectral counts (a ratio of 28:14 or 14:28) in order to achieve statistical significance (two-sided p < 0.05). (For examples of different simulated ratios, see (40)). Nonetheless, the spectral count method clearly revealed a number of candidates for MPR-selective ligands.

iTRAQ Analysis—The iTRAQ method produced a high-quality data set after filtering to remove spectra that gave low reporter ion intensities (see “Experimental Procedures” and “Results”). Low ion intensities are expected to yield poorer estimates of abundance due to signal to noise considerations, as has been observed previously (41). In Experiment 1, we had an internal control that could be used to filter the data either independently or in addition to the intensity criteria that we applied. However, this required the use of one of the reporter ions and decreases the numbers of comparisons that can be made in a given iTRAQ experiment, and thus increases the expense and effort associated with a project. However, either filtering criteria generated similar results (data not shown) so we chose to apply intensity thresholds established from analysis of iTRAQ Experiment 1 to filter the data from iTRAQ Experiment 2.

Comparison of Analytical Methods—The results generated using the iTRAQ and spectral count methods were in excellent agreement. One advantage in the iTRAQ approach is that a single spectrum yields abundance ratios; and in cases where the abundance ratios of different spectra or peptides assigned to a given protein vary, it is possible to inspect each on a case-by-case basis. One limitation was that the proteome coverage in the LC-MALDI experiments was less than that obtained by LC-MS/MS on unlabeled samples (e.g. only 39 lysosomal proteins were identified in the LC-MALDI/TOF/TOF experiments on iTRAQ labeled samples, two of which did not yield usable iTRAQ ratios, while 60 proteins were found using LC-MS/MS on unlabeled samples.). This in part may arise from oversampling the more abundant proteins at the expense of detecting less abundant proteins. For example, we collected 434 acceptable spectra for murinoglobulin 1 but only 2 for beta-galactosidase. This redundancy might be reduced by implementing improvements in precursor ion selection for MS/MS analysis such as excluding repeated analysis of peptides that elute in multiple ion exchange fractions or limiting the number different peptides analyzed that are derived from a single protein. In addition, we typically only obtain ~3 usable MS/MS spectra (identified peptides with acceptable reporter ion statistics) per MALDI spot, and this is open for further optimization.

Despite the difference in protein coverage using LC-MALDI/TOF/TOF on iTRAQ labeled samples and LC-MS/MS on unlabeled samples, both yielded similar quantitative results in terms of discovering true differences in protein abundances. (supplemental Table IX). For instance, a total of 10 lysosomal proteins were identified as having a 1.5-fold difference when comparing the CD(-) and Cl(-) samples by the spectral count and/or iTRAQ approaches (one-sided q < 0.025). Eight proteins were identified using both approaches, one was only identified using iTRAQ, and one was only identified using the spectral count analysis. In the latter two cases, both approaches yielded similar trends, but data from only one of the approaches reached the specified significance level (supplemental Table IX).

In addition to the agreement of the two MS-based approaches, these results also were supported by the Western blot analysis. It is important to note that the Western blot experiments were not quantitative, as revealed by the nonlinearity of the response from the different amounts of a given sample loaded on the gel, despite a wide range of exposure times (Fig. 8 and data not shown). This is not simply due to nonlinearity of chemiluminescence and film detection, there are cases where the same amount of a given protein loaded on two different lanes of a gel yields dramatically different signals in Western blotting (42, 43). There are a variety of factors that can account for this, including differences in transfer efficiency of the protein from gel to membrane (42). Our impression is that the MS-based results more accurately reflect the relative abundance of the proteins of interest in the different samples, but further work is required to test this rigorously.

Potential New Man-6-P Glycoproteins and Other Ligands for the MPRs—In the course of this study, we analyzed MPR-
purified preparations from mouse brain and serum. As mentioned under “Results,” the brain Man-6-P glycoproteins were affinity purified on sCDMPR or sCIMPR columns, but data from these experiments were not used to classify ligands as CDMPR or CIMPR specific due to the high concentration of immobilized MPRs. In addition, the brain Man-6-P glycoproteins are largely in their processed forms (21, 44, 45), and as processing typically occurs after targeting, these do not reflect the forms that the receptors would encounter in the TGN. This also could affect interaction with the immobilized receptors in different ways. For instance, by 125I-labeled sCIMPR blotting, we found that in the analysis of brain extracts, a ~46 kDa Man-6-P protein was predominantly located in the glycine eluates from both of the MPR affinity columns. This contrasts markedly with the serum samples, where the levels of Man-6-P glycoproteins detected in the glycine eluate by MPR blotting were extremely low (data not shown). MS analysis indicated that the ~46 kDa brain protein represents the mature form of Tpp1 (supplemental Fig. 1 and supplemental Table I). The inactive precursor form of this protein is soluble and can be eluted from MPR affinity columns using Man-6-P (46) but, after low pH-triggered autocatalytic processing, converts to a form that tends to aggregate and absorb to surfaces (46).

Data from the mouse brain and serum analyses can contribute to ongoing studies to identify novel components of the luminal lysosomal proteome (21, 28, 29, 44, 47–53). LC-MS/MS analysis of MPR affinity purified proteins from mouse brain and serum samples have revealed a total of 63 different lysosomal proteins as well as numerous others that are not known to reside in the lysosome (supplemental Tables I and V). The latter may represent Man-6-P modified proteins, proteins that specifically interact with Man-6-P modified proteins (e.g., protease inhibitors and lectins), contaminants, or proteins that interact with a given MPR in a Man-6-P-independent manner (i.e., IGF2). Distinguishing between these possibilities is a formidable challenge. Most known lysosomal proteins were found in both the brain and serum samples (Fig. 9, left). In contrast, of the total 313 different proteins (after excluding likely contaminants) not known to be lysosomal, only 29 were found in both types of samples (Fig. 9, right and supplemental Tables I and V). These proteins are thus worthy of further consideration. Other criteria can also be used for prioritization (discussed in [28]). These include the detection of Man-6-P glycopeptides and enrichment of proteins following two sequential rounds of MPR-affinity purification. When combining these classification strategies, 5 proteins meet all three criteria, 11 meet two of the three criteria, and 68 meet one of the criteria (supplemental Table X).

The data from the protein identification experiments conducted on brain samples contain information that could provide clues toward additional biological functions of the CIMPR. The CIMPR is a multifunctional protein (2, 3). The luminal region of the CIMPR consists of 15 contiguous ~147 amino acids units that are 14–38% identical to each other and that are similar to the 159 residue luminal domain of the CDMPR. The luminal CDMPR monomer contains a single Man-6-P binding site while two of the repeating units on the CIMPR contain the high affinity binding sites (domains 3 and 9) and domain 11 contains the IGF2 binding site. The CIMPR may also bind urokinase-type plasminogen activator receptor at a site distinct from the Man-6-P and IGF2 binding sites (54), but this is controversial (55). Given the multiple domains of the CIMPR, it is possible that there are additional, as yet unidentified, ligands for this receptor. We would expect that these would be enriched in the CIMPR glycine eluate compared with the glycine eluate of the CDMPR column or the Man-6-P eluates of both MPR columns. Interestingly, IGF2 as well as four IGF2 binding proteins were found only in the CIMPR column glycine eluate, and there were a number of other proteins that had similar behavior that may be worthy of further investigation (supplemental Table I).

Relative Functions of the Two MPRs—The two MPRs are present in a wide variety of vertebrates (56), suggesting that each has a specific and conserved biological function. However, despite over 20 years of investigation (6, 7), the specific role of each MPR in lysosomal protein targeting remains unclear. Studies using mutant mice indicate that animals lacking either the CDMPR (12, 13) or the CIMPR (9) exhibit no apparent health defects, whereas the combined MPR deficiency results in a profound lysosomal storage phenotype and early death (10, 14). This indicates that each MPR can partially compensate for the absence of the other, which complicates determination of MPR-specific functions. Previous studies have directly examined the affinities of the two MPRs for different Man-6-P-containing ligands with the aim of identifying differences that might have functional significance. However, apart from the observations that the CDMPR binds Man-6-P-containing ligands with lower affinity than the CIMPR (18, 57), and that the CIMPR but not the CDMPR can bind mannose 6-phosphodiesters (57), no clear differences were found that suggest specialized biological functions. Other studies have examined Man-6-P glycoproteins secreted from cultured cells lacking either MPR with the rationale that ligands specific to either MPR should be secreted only in the absence of that MPR (16, 17). These studies conclude that, for the most part, the two MPRs direct the lysosomal targeting of distinct but overlapping populations of Man-6-P glycoproteins and that neither MPR can fully compensate for...
the absence of the other. The CIMPR appears more efficient in targeting newly synthesized lysosomal proteins to the lysosome (17, 58), but this in part may be due to its specific ability to function in endocytosis and thus recapture secreted lysosomal proteins (59). In particular, cathepsin D was preferentially secreted in the absence of the CIMPR (17). However, to our knowledge, no Man-6-P glycoproteins that appear preferentially secreted in the absence of the CDMPR have been identified to date.

One indication that there may be Man-6-P glycoproteins that are specifically targeted by each MPR arose from analysis of MPR-deficient mice (15). Using $^{125}$I-labeled sCIMPR as an affinity probe to visualize Man-6-P glycoproteins, bands migrating at $\sim 26$ kDa and $\sim 114$ kDa were highly elevated in the CD(-) and CI(-) serum samples, respectively. However, there is some uncertainty in interpretation of these blotting experiments, as an elevated signal could be due to increased protein levels and/or the number of phosphorylated oligosaccharides on the protein. Our quantitative MS-based and Western blotting analyses demonstrate that levels of multiple Man-6-P glycoproteins in serum vary in an MPR-specific manner. Of these, alpha-mannosidase 2B1 is likely to represent the $\sim 114$ kDa Man-6-P glycoprotein detected in unpurified CI(-) serum, whereas Creg1 is likely to represent the $\sim 26$ kDa Man-6-P glycoprotein in CD(-) serum.

The molecular basis for the selective secretion of these proteins in an MPR-specific manner remains to be elucidated. It is possible that there is a significant difference in the respective affinities of the MPRs for these proteins. For example, a protein bound with high affinity and targeted efficiently by one MPR but not the other would be predicted to be secreted only in the absence of its high affinity receptor. Proteins that are recognized in a comparable manner by both MPRs would be secreted into the bloodstream to a similar extent in the absence of either MPR, and this appears to be the case with most of the Man-6-P glycoproteins identified. It is possible that both protein and carbohydrate determinants could modulate receptor specificity (58, 60, 61). Another possibility is that serum Man-6-P glycoprotein levels could reflect differences in the cell or tissue distribution of the MPRs and their ligands. For example, a lysosomal protein that is highly expressed in a cell type that predominantly contains a single type of MPR would appear disproportionately elevated in serum in the absence of that MPR. Support for this possibility comes from the observation that, while the MPRs are expressed in most cell types, their relative expression is tissue-dependent, with heart and spleen containing 3–4-fold higher molar levels of CDMPR than CIMPR (62). While the molecular bases for the selective secretion of these various proteins in the absence of either MPR is not yet apparent, we anticipate that the identification of these proteins in this study will provide a useful basis for the future investigation of the relative functions of the two MPRs.

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