A Proteomic Analysis of Lysosomal Integral Membrane Proteins Reveals the Diverse Composition of the Organelle*[S]

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Lysosomes are endocytic subcellular compartments that contribute to the degradation and recycling of cellular material. Using highly purified rat liver tritosomes (Triton WR1339-filled lysosomes) and an ion exchange chromatography/LC-tandem MS-based protein/peptide separation and identification procedure, we characterized the major integral membrane protein complement of this organelle. While many of the 215 proteins we identified have been previously associated with lysosomes and endosomes, others have been associated with the endoplasmic reticulum, Golgi, cytosol, plasma membrane, and lipid rafts. At least 20 proteins were identified as unknown cDNAs that have no orthologues of known function, and 35 proteins were identified that function in protein and vesicle trafficking. This latter group includes multiple Rab and SNARE proteins as well as ubiquitin. Defining the roles of these proteins in the lysosomal membrane will assist in elucidating novel lysosomal functions involved in cellular homeostasis and pathways that are affected in various disease processes. ***Molecular & Cellular Proteomics 4:133–143, 2005.***

Lysosomes are single membrane-bound subcellular organelles that are thought to be the chief degradatory compartments in the endosomal pathway. They house a diverse complement of hydrolases that function optimally at acidic pH values. Most resident hydrolytic enzymes are localized to the luminal compartment and are largely soluble glycoproteins capable of hydrolyzing all the major macromolecules of the cell. The simpler molecules arising as products of these digestive events are translocated from the intralysosomal compartment across the membrane and released into the cytoplasm for reutilization. Substances for digestion are acquired by the lysosomes via a series of processes including endocytosis, phagocytosis, and autophagy. The pivotal role lysosomes play in cell metabolism is manifested by the occurrence of at least 40 enzymopathies (1) affecting the catabolism of neutral, phospho-, and glycolipids; complex carbohydrates; and proteins where indigestible macromolecular intermediates become stored inside the organelle, altering the homeostasis of the cell and leading to a disease state. More recently, lysosomes have been found to be highly dynamic organelles involved in regulated secretion (2), repair of damaged plasma membrane (3), and formation of osteoclast ruffled border (4). Classical lysosomes are considered to be part of a family of lysosome-related organelles (for a review, see Ref. 5) that have a range of cell-specific functions, *e.g.* melanosomes, platelet dense granules, and azurophilic granules.

In addition to the storage diseases, lysosomes have been implicated in a wide range of other human pathologies such as Alzheimer's disease (6, 7), autoimmune diseases, and resistance to infectious disease, cancer, and drugs. The roles played by lysosomes in these areas remain poorly defined largely due to a lack of in-depth knowledge concerning the composition and topologies of their integral membrane proteins and the identity of other cytosolic proteins that may form permanent and/or transient macromolecular complexes to facilitate membrane-membrane interactions and fusion events.

While molecular characterization of lysosomal diseases involving the luminal, *i.e.* soluble, enzymes is well advanced, only a few defects associated with lysosomal membrane proteins, such as Danon disease (LAMP-2)[1] (8), Niemann-Pick C (NPC1) (9), cystinosis (cystinosin) (10), and Salla disease (sialin) (11), have been identified. The lysosomal membrane is critical for maintenance of cell homeostasis as it provides the communication link between the degradative milieu of the lysosome and the cytosol. Aside from well known lysosomal membrane proteins, *i.e.* the LAMPs and LIMPs, it can be predicted from physiological uptake and flux studies that

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[1] The abbreviations used are: LAMP, lysosome-associated membrane protein; LIMP, lysosomal integral membrane protein; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; MS/MS, tandem MS; ER, endoplasmic reticulum; NPC1, Niemann-Pick type C1; MGC, Mammalian Gene Collection; V-ATPase, vacuolar-type H^+-translocating ATPase; VAMP, vesicle-associated membrane protein; EMP70, endomembrane protein of 70 kDa; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; CREG, cellular repressor of E1A-stimulated genes; Gl, grey-lethal.

multiple classes of transporter proteins exist.

The goal of our present study was to define the protein composition of the integral membrane fraction of the lysosome. This was achieved by combining subcellular fractionation, differential extraction, and protein separation techniques with proteomic-type protein identification. One of the prerequisites for a comprehensive investigation of lysosomes is the ability to purify the organelle to near homogeneity and in sufficient yield. While many procedures have been used for lysosomal purification, we chose one of the most reliable and best studied, namely, Triton WR1339-filled rat liver lysosomes (tritosomes) as described by Leighton *et al.* (12). Although tritosomes are considered modified lysosomes as they contain a quantity of non-physiological material (Triton WR1339), tritosomes retain lysosomal enzymatic activities (6, 12–14) and perform identically to lysosomes in endosomal and phagosomal fusion assays (15). In this study we identified 215 separate proteins in the integral membrane protein fraction of the lysosome, many not previously associated with the lysosome and several that are still only identified as uncharacterized cDNAs. These data reveal a membrane that has a complex protein composition and support the view that the lysosome is a highly dynamic organelle.

MATERIALS AND METHODS

Lysosome (Tritosome) Isolation—The isolation of Triton WR1339-filled lysosomes has been described previously (6, 12, 13). Briefly rats (200–300-g male Sprague-Dawley from Charles River) were given an intraperitoneal injection of the non-lytic detergent Tyloxapol (85 mg/100 g of animal weight) 5 days prior to sacrifice in a CO_2 chamber. Livers were removed, homogenized, and centrifuged at 1000 × *g* for 10 min to produce a postnuclear supernatant. A crude organellar pellet was generated by centrifuging the postnuclear supernatant at 34,000 × *g* for 10 min. The pellet was resuspended in 45% sucrose, layered underneath a discontinuous gradient of 14.3% sucrose and 34.5% sucrose, and centrifuged at 77,000 × *g* for 2 h. Triton-filled lysosomes (tritosomes) were removed from the 14.3-34.5% interface, diluted with 0.25 M sucrose, and pelleted at 28,000 × *g* for 30 min. Phenylmethylsulfonyl fluoride (0.1 mM) was present in all solutions.

Electron Microscopy—Sections of fixed tritosome pellets were processed for electron microscopy according to Leighton *et al.* (12). Acid phosphatase was stained using the Gomori lead phosphate technique (16).

Lysosomal Subfractionation—The isolated tritosomes were subfractionated based on solubility as described previously (6). Briefly washed tritosomes were resuspended in 50 mM Tris-HCl, pH 8.0, 0.2 M NaCl and subjected to five freeze/thaw cycles (frozen with dry ice/ethanol and thawed at 37 °C). A membrane fraction was collected by centrifugation for 15 min at 355,000 × *g* at 4 °C, and the supernatant was removed (soluble/luminal fraction). The crude membrane pellet was treated with 0.1 M Na_2CO_3, pH 11.0 (30 min on ice) and centrifuged as above to give a soluble membrane-associated fraction. The remaining membrane pellet was designated the integral membrane fraction.

Fractionation of Lysosomal Integral Membrane Proteins on CM-Sepharose and Identification by Mass Spectrometry—The integral membrane fraction was dissolved in 6 M guanidine HCl and 40 mM dithiothreitol. The solution was incubated at 45 °C for 30 min to ensure complete reduction of disulfide bonds. Iodoacetamide was added to give a final concentration of 150 mM and was incubated for 1 h in the dark at room temperature. The protein solution was then twice dialyzed overnight against column buffer (50 mM formic acid, pH 3.6, 8 M urea, 1% Elugent, Calbiochem). Conductivity measurements of the dialysis sac contents and the dialysate were made to ensure complete removal of guanidine. The protein solution was centrifuged at 10,000 × *g* for 15 min at 18 °C prior to being loaded on a CM-Sepharose cation exchange column pre-equilibrated in column buffer. Bound fractions were collected with a stepwise gradient of 20, 40, 80, and 250 mM NaCl dissolved in column buffer. The protein fractions were acetone-precipitated, and the pellets were dissolved in Laemmli buffer (17). The proteins were separated by standard one-dimensional SDS-polyacrylamide electrophoresis in 12% tube gels (5 mm). The gels (~12 cm) were recovered; fixed for 2 h in 50% methanol, 10% acetic acid; placed in a gel slicer; and cut into 2–4-mm slices. Slices were then processed for in-gel digestion with trypsin as described previously (18). Recovered peptides were subjected to C_{18} nano-LC-MS/MS-based separation using a Waters CapLC with a PicoFrit C_{18} column (New Objective) in line with a Q-TOF mass spectrometer (Micromass).

Protein Identification—Raw MS/MS data were processed into peak lists (.pkl) using MassLynx Version 3.5 (Micromass). Criteria for this processing were a peptide filter threshold of 2 and a minimum peak width at half-height of 2. The peak lists were analyzed using MS/MS ion search (Mascot, www.matrixscience.com) using the National Center for Biotechnology non-redundant protein (NCBI nr) data base. Some spectra were additionally interpreted by *de novo* sequencing (Pepseq, Micromass) and sequence tags (PeptideSearch, European Molecular Biology Laboratory Bioanalytical Research Group). All proteins that scored as significant data base "hits" by Mascot were verified by manual inspection of MS/MS data. Proteins that were identified with one unique peptide have MS/MS spectra that contain an interpretable y-ion series suitable for a peptide sequence tag search and have "identity" Mascot peptide scores (Supplemental Table 1). Data base search criteria included mass accuracy of 0.3 Da and one possible missed trypsin cleavage. In some instances, methionine oxidation and guanidinylation peptide modifications were found as well as *N*-acetylation of the N-terminal peptide from proteins. A non-redundant list of identified proteins was produced by removing duplications through using BLASTP to search each protein *versus* a data base of all the identified proteins.

Immunoblots—Equal amounts of protein (30 μg) from purified tritosomes and the originating rat liver homogenate were separated by SDS-PAGE and transferred to nitrocellulose. Primary antibodies used were anti-rat LAMP-1 (mouse monoclonal, Calbiochem), anti-sialyltransferase 1 (ST6Gal1, kindly provided by J. Paulson), anti-GPP130 (Covance), and anti-calnexin (H-70) and anti-ribophorin 1 (C-15, both from Santa Cruz Biotechnology). Detection was done using species appropriate horseradish peroxidase-conjugated secondary antibodies with ECL and Hyperfilm (Amersham Biosciences).

RESULTS

Triton WR1339-filled Lysosomes (Tritosomes)—Tritosomes have been extensively characterized as functional secondary (late stage) lysosomes (12, 14, 15, 19). We have previously demonstrated the enrichment of lysosomal markers (β-hexosaminidase activity, Rab7, and LAMP-1) in our tritosome preparations (70-fold enrichment of β-hexosaminidase) and the absence of markers from mitochondria (citrate synthase activity) and ER (calnexin protein) (Refs. 6 and 13 and see below). Electron microscopy of isolated tritosomes (Fig. 1*A*) indicates that almost all of the structures are Triton WR1339-filled lysosomes containing electron dense cores and little or

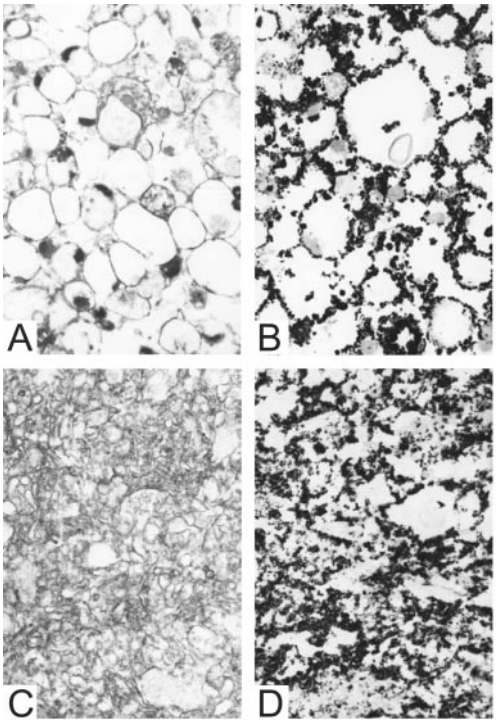

FIG. 1. **Electron microscopy of purified tritosomes.** *A*, intact tritosomes; *B*, intact tritosomes with an acid phosphatase-specific stain; *C*, tritosomal membranes derived from the freeze/thaw fracture procedure; *D*, tritosomal membranes with acid phosphatase stain (images are ×17,800).

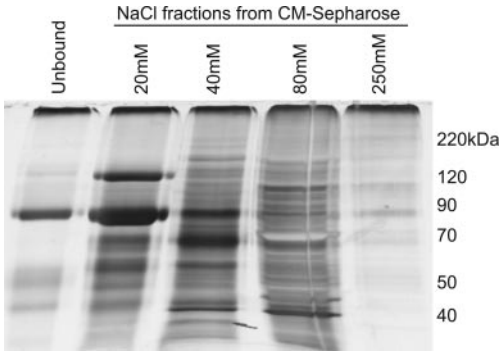

FIG. 2. **Integral membrane protein fraction of rat liver tritosomes separated by CM-Sepharose chromatography analyzed by SDS-PAGE and silver-stained.** The *Unbound* lane is about 5-fold underloaded as compared with the other lanes. Similar preparative SDS-PAGE tube gels were sliced into 2–4-mm slices, and each slice was processed for in-gel digestion and LC-MS/MS.

no other organelles. The membranes of these lysosomes have intense positive staining for acid phosphatase activity (Fig. 1*B*) further characterizing them as *bona fide* lysosomes. Electron microscopy of tritosome membranes obtained by freeze/thaw lysis and centrifugation reveals an absence of the electron dense cores and the presence of amorphous membranes (Fig. 1*C*) that are acid phosphatase-positive (Fig. 1*D*). There is little or no membrane material evident that is negative for acid phosphatase activity. The absence of contaminating organelles and the reproducibility and reliability of the tritosome preparation along with retention of functional and physiological characteristics makes Triton WR1339-filled lysosomes an ideal choice as a proteomic model.

Separation and Identification of Lysosomal Integral Membrane Proteins—Prefractionation of a sample into subsets prior to a proteomic-type analysis generally results in a larger array of proteins being identified. Accordingly, after freeze/thaw lysis of tritosomes to remove the soluble proteins, peripherally bound proteins were removed by treatment with sodium carbonate (20) resulting in an insoluble pellet, *i.e.* the integral membrane fraction. Because of incomplete dissolution of the material in non-ionic detergents (data not shown), likely due to hydrophobic and detergent-resistant domains in the membrane proteins, *e.g.* NPC1 (21), the integral membrane fraction was dissolved, reduced, *S*-alkylated in guanidine HCl, and then dialyzed *versus* 8 M urea and 1% Elugent at pH 3.6. The integral membrane proteins were initially fractionated by cation ion exchange chromatography on CM-Sepharose using a NaCl step gradient. The goal of the fractionation, confirmed by slab gel SDS-PAGE (Fig. 2), was to enrich low abundance proteins and to achieve their segregation from highly abundant acidic proteins, *e.g.* the LAMPs (for reviews, see Refs. 22 and 23). Therefore we chose the pH of the system so that these and other proteins with very low pI values did not bind (Fig. 2, see "*Unbound*") to the adsorbent (under these conditions about 40% of the total protein did not bind).

The proteins from each column fraction were separated by SDS-PAGE in tube gels that were cut into 2–4-mm slices and processed for in-gel digestion, and the peptides were separated and identified by on-line LC-MS/MS. From the thousands of MS/MS spectra acquired, about 13,300 were selected for data base searching resulting in the identification of 215 proteins (non-redundant by BLASTP) in the lysosomal integral membrane protein fraction (Table I). These were organized into ontologies based on their generally accepted biological functions. Hypothetical proteins predicted in the NCBI nr data base from uncharacterized cDNAs were categorized as unknown/uncharacterized unless a conserved domain or orthologue could be found that provided information regarding the function of the protein. For example, "hypothetical protein 5031407H10" was sorted into the vesicular and protein trafficking category as it contained a SAND family (vacuolar targeting) domain (pfam03164).

Proteins were identified in gel slices that roughly corresponded to their calculated molecular weights with some exceptions such as known glycoproteins, *e.g.* macrosialin (predicted to be M_r ~35,000 but found with an apparent M_r of ~80,000) and ubiquitin (~8-kDa monomer but found throughout the molecular mass range). To ensure that our protein separation and identification processes were representative of the true composition of the membrane, we compared the distribution of theoretical protein molecular masses listed in

TABLE I
Proteins identified from the lysosomal integral membrane protein fraction

The table is sorted by functional ontology. Protein name, NCBI accession number, and number of unique peptides identified are given.

Metabolism		
Aldehyde dehydrogenase 3-A2	13929028	2
Apyrase	21426787	2
Arachidonic acid epoxygenase	13929204	4
Aryl sulfotransferase	1091600	1
ATP synthase α subunit	6729934	4
ATP synthase f0 β subunit	19705465	2
ATP synthase F1 complex O subunit	20302061	3
ATP synthase γ chain	39930503	3
Betaine-homocysteine methyltransferase	13540663	5
Carbamoyl-phosphate synthetase 1	8393186	2
Catalase	6978607	2
CYP2A1	6978741	3
CYP2C13	25453406	1
CYP2C22	19924039	2
CYP2C29	9506529	1
CYP2D2	6978747	1
CYP4A3	28461155	2
Cytochrome b_5	11560046	5
Dopa/tyrosine sulfotransferase	11968092	1
Epoxide hydrolase 1	6978813	4
Glyceraldehyde-3-phosphate dehydrogenase	8393418	1
L-Gulono-γ-lactone oxidase	11560006	2
Peroxiredoxin 1	16923958	3
Retinol dehydrogenase type III	31377477	5
Similar to NADH dehydrogenase (ubiquinone) 1 α subcomplex, 9	34858473	4
Immunity		
CD1 antigen	5420461	7
Fc receptor	15375322	6
Macrophage expressed gene 1	12018298	2
MHC class I α chain	940825	1
Polymeric immunoglobulin receptor	27151742	3
Similar to Igh-6 protein	34935297	2
Similar to immunoglobulin joining chain	34876693	3
Biosynthesis		
α-Mannosidase II	34877940	15
Core 1 β-1,3-Galactosyltransferase	12621124	5
Elongation factor Tu, similar to RIKEN cDNA 2300002G02	34859187	1
Eukaryotic translation elongation factor 1 α-2	15805031	2
Heat shock 70kD protein 5, GRP78, BiP	25742763	6
Microsomal glutathione S-transferase 1	19705453	2
N-Acetylglucosaminyltransferase I	13540685	6
N-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 1; heparan sulfate-N-deacetylase/N-sulfotransferase	13242253	5
ppGaNTase-T2	46877109	3
Protein disulfide isomerase (ER60)	91897	1
Ribophorin I	6981486	1
Ribosomal protein L13	13592055	3
Ribosomal protein L6	16758864	1
Ribosomal protein L7, cytosolic [validated]–rat	11383729	2
Ribosomal protein S3, cytosolic [validated]–rat	70850	1
Ribosomal protein S3a, cytosolic [validated]–rat	8394221	1
Ribosomal protein S4	227229	5
Ribosomal protein S6	6677809	1
Sialyltransferase 1 (ST6Gal1)	115446	13
Sialyltransferase 4A (ST3Gal1)	6677957	2
Sialyltransferase 8 (GT3 α-2,8-sialyltransferase)	6981540	1
Similar to 60 S ribosomal protein L3 (L4)	38454246	1
Similar to 60 S ribosomal protein L7a (Surfeit locus protein 3) (PLA-X polypeptide)	34853132	3
Similar to 60 S ribosomal protein L8	27685597	3
Similar to Heparan sulfate 2-sulfotransferase	34860929	2
Similar to ribosomal protein S8	38049330	2
Similar to Vesicular integral-membrane protein VIP36 precursor	27682691	6
UDP glycosyltransferase 2 family, polypeptide B	13928718	1
UDP-glucuronosyltransferase 2 family, member 5	34876712	1
Membrane receptors/Signaling and Cell adhesion		
Asialoglycoprotein receptor 1	7705290	3
Asialoglycoprotein receptor 2	8392926	2
Carcinoembryonic antigen-related cell adhesion molecule	13929060	2
CD59	6978635	1
Cell surface antigen RB13–6–rat	1363274	2
E-Selectin ligand, Golgi apparatus protein 1	6677905	21
Guanine nucleotide-binding protein α-11 subunit	13591951	1
Harvey rat sarcoma oncogene, subgroup R	6677819	1
Lipoprotein receptor-related protein	34865759	6
OX47	111503	2
p21/H-Ras-1 (c-H-ras)	131873	1
Phosphatidylinositol 4-kinase type II	16758554	4
Progesterone receptor membrane component 1	11120720	2
Protein-tyrosine phosphatase, receptor type, F	9507013	2
Purinergic receptor P2X4	13928806	4
Rap2B	13386338	1
Rras2, related RAS viral (r-ras) oncogene homolog 2	13399308	1
Similar to Cux/CDP(1B1); Cux/CDP homeoprotein	34873366	1
Similar to GTP-binding regulatory protein α-13 chain	34875240	1
Similar to MEK binding partner 1	34860783	2
Similar to pituitary tumor-transforming gene 1 protein-interacting protein	34852416	4
Similar to ras-related C3 botulinum toxin substrate 1 isoform Rac1b	34870449	4
Similar to RIKEN cDNA 1300006M19	34870394	1
Similar to transferrin receptor protein 2 (TfR2)	34871536	3
Toll-like receptor 3	33438238	10
Molecular transport		
ATP-binding cassette, sub-family B (MDR/TAP), member 6	18034785	3
ATP-binding cassette, sub-family C (CFTR/MRP), member 2; Canalicular multispecific organic anion transporter; multidrug resistance associated protein 2	6978669	5
Chloride ion pump-associated 55 kDa protein	21489987	1
Niemann Pick C1	6679104	4
Peptide/histidine transporter	21426791	1
Similar to 2810423E13Rik protein	34856883	2

TABLE I—*continued*

Similar to calcium-binding mitochondrial carrier protein Aralar2 (Solute carrier family 25, member 13) (Citrin)	7657583	8
Similar to MLN64 N-terminal domain homolog (STARD3 N-terminal like protein)	27686679	1
Similar to Proteolipid protein 2	46485405	1
Similar to putative protein, with at least 9 transmembrane domains, of eukaryotic origin (43.9 kDa) (2G415)	34863028	1
Similar to Solute carrier 31, member 1	19424310	1
Similar to Tm9sf1 protein	34874167	2
Similar to transmembrane 9 superfamily member 2	34876089	2
Similar to transmembrane 9 superfamily protein member 4	34859018	3
Solute carrier family 25 (mitochondrial phosphate carrier; adenine nucleotide translocator), member 3; phosphate carrier, mitochondrial	20806141	1
Solute carrier family 25, member 4; mitochondrial adenine nucleotide translocator	32189355	2
solute carrier family 29 (nucleoside transporters), member 3	31745142	2
vATPase 100kDa a3 subunit	7140942	3
vATPase subunit B	17105370	2
vATPase subunit D(V0)	3955100	9
vATPase subunit D(V1)	40786463	3
vATPase subunit E	1718091	1
vATPase subunit G	27714615	1
vATPase subunit H	14318722	2
Voltage-dependent anion channel 1	6755963	4
Membrane structure and lipid rafts		
CD36 antigen-like 2 (LIMPII)	16758914	10
DAMP-1 protein	37693510	2
Flotillin-1	13124118	20
Flotillin-2	13124119	15
Golgi-associated protein GCP360	20302065	9
LAMP1	6981144	10
LAMP2	40254785	8
Prohibitin	6679299	9
Similar to band 7 protein (35.3 kDa) (4N53)	34863101	1
Similar to Macrosialin (CD68)	34870966	3
Similar to MAL2A	32693285	1
B-cell associated protein 37; similar to repressor of estrogen receptor activity	34858436	8
Stomatin (Erythrocyte band 7 integral membrane protein 7.2b)	7710018	8
Hydrolases/Co-factors		
5′ nucleotidase, ecto	11024643	2
β-Galactosidase	192185	2
β-Glucuronidase	8393510	2
Cathepsin F	34861419	3
Cathepsin L	67650	2
Cathepsin Y	34328540	1
Dipeptidylpeptidase IV	6978773	12
GM2 ganglioside activator protein	48976085	2
Kidney aminopeptidase M; Leucine arylaminopeptidase 1	13591914	2
Lysosomal acid phosphatase	8392842	9
Nicastrin	27819651	13
Plasma glutamate carboxypeptidase	13928880	1
Prosaposin	6981424	2
Protective protein for β-galactosidase	6679437	3
X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	16924020	6

TABLE I—*continued*

Secreted		
α-1-Inhibitor III	12831225	2
α-2-HS-glycoprotein (fetuin)	6978477	7
Apolipoprotein A-V	18034777	12
Apolipoprotein b	34863099	4
Apolipoprotein E	1703338	10
Complement component 9	16924006	1
Dermcidin precursor	16751921	5
Epithelin/granulin precursor	8393493	4
Ferritin light chain–rat	2119695	8
Fibrinogen γ-A chain	71829	2
Fibronectin 1	9506703	4
Inter-α-inhibitor H4 heavy chain	9506819	5
Peptidylprolyl isomerase C-associated protein (mama)	20806135	4
Pregnancy zone protein	21955142	4
Serum amyloid P	8392903	2
Similar to cellular repressor of E1A-stimulated genes CREG	34880832	1
Vitronectin	9507241	3
Unknown/Uncharacterized		
2810022L02Rik protein	15928581	1
Expressed sequence AV006840	20846843	2
Hypothetical protein XP_236205	34863318	2
Mg87 protein	19705543	2
RIKEN cDNA C730027E14	22122497	1
Similar to Ab2–095	34865464	5
Similar to apoptosis related protein APR-3; p18 protein	34862996	3
Similar to cDNA 1810037C20	34881760	1
Similar to CG14980-PB	34870407	2
Similar to Chr14 ORF	34869712	3
Similar to E25B protein	27701907	2
Similar to FAM3C-like protein	38454280	1
Similar to gray lethal osteopetrosis; gray-lethal	34853421	2
Similar to HTGN29 protein; keratinocytes associated transmembrane protein 2	34870715	1
Similar to hypothetical protein FLJ38482	34877746	2
Similar to Hypothetical protein MGC18837	27719651	1
Similar to hypothetical protein MGC29390	34869210	5
Similar to hypothetical protein MGC40107	27672946	2
Similar to RIKEN cDNA 1100001H23	34858551	3
Similar to RIKEN cDNA 1110055L24	34854713	2
Similar to RIKEN cDNA 2010320H07 gene	34853121	1
Similar to TRIM14 α	34868472	7
Unknown (protein for MGC:72560)	40018580	6
Unknown (protein for MGC:72638)	40018550	1
Vesicular and protein trafficking		
Arl10C	12838871	4
Cation-dependent M6PR	27713160	4
Ergicp53, mannose-binding lectin 1	16758758	9
Heat shock protein 8; Heat shock cognate protein 70; heat shock 70kD protein 8	13242237	5
RAB11B, member RAS oncogene family	14249144	3
Rab1A	4758988	7
Rab2	13929006	1
Rab5C	27689505	3
Rab6	17512290	7
Rab7	13027392	6
RIKEN cDNA 1500016L11	21312151	3
RIKEN cDNA 3930401E15	38083573	2
Similar to ankyrin repeat hooked to a zinc finger motif long form	34873095	2
Similar to EH-domain containing 1	34861835	1

TABLE I—*continued*

Similar to Golgi phosphoprotein 4; type II Golgi membrane protein; 130 kDa Golgi-localized phosphoprotein; cis Golgi-localized calcium-binding protein	34857091	6
Similar to Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	34863597	2
Similar to gp25L2 protein	34873639	1
Similar to hypothetical protein 5031407H10	34851786	2
Similar to LRG-47	27670133	2
Similar to NIPSNAP1 protein	34879019	2
Similar to procollagen (type III) *N*-endopeptidase	34851987	1
Similar to Rab18	27687387	1
Similar to Rab5B	34862219	2
Similar to RET-II	34867583	2
Similar to RIKEN cDNA 2010012F05	34859075	2
Sorting Nexin 3A	4507143	2
SNAP α	2143586	2
Syntaxin 7	11177920	4
Syntaxin 8; syntaxin-like protein 3l35	13928908	4
TGN38	20301976	6
Ubitquitin	70637	5
Vamp2B	4894188	2
VAMP8	13929182	8
Vesicle transport through interaction with t-SNAREs 1B homolog	34866243	3
Vps24p protein	27229308	1

Table I to those predicted from translation of the mouse Mammalian Gene Collection (MGC) full-length cDNA data base (including 13,007 cDNAs). The histograms shown (Fig. 3) clearly demonstrate that our approach is not biased with respect to protein size. It should be noted that the number of proteins of M_r >200,000 identified in the present study is somewhat larger than predicted from the MGC collection, suggesting that membrane proteins constitute a higher proportion of large proteins.

Proteins of the Lysosomal Integral Membrane—To determine the most abundant lysosomal membrane proteins in our proteomics survey, we used a pseudoquantitative measure of protein abundance consisting of the number of unique peptides assigned divided by the theoretical molecular weight of the protein. The top 15% of proteins in terms of this abundance measure are indicated in boldface type in Table I. These include LAMP-1, LAMP-2, and LIMPII as expected (for a review, see Ref. 22) and V-ATPase V0 subunit D, Rab7, and lysosomal acid phosphatase. Interestingly the lipid raft constituents flotillin-1, flotillin-2, and stomatin (24, 25) were also found in considerable abundance in the lysosomal membrane (21). Other proteins found in relatively high abundance were nicastrin, ferritin light chain, and apolipoproteins (A-V, E, and B). Although the apoB theoretical gene product is very large (>500 kDa), we have likely found the smaller intestine-specific apoB48 species since the peptides identified for apoB are N-terminal to the putative C-terminal splice site of apoB48 (approximately amino acid 2151 of apoB100) (26).

We identified the membrane-bound form of sialyltransferase 1 (ST6Gal1). It is a type II transmembrane protein originally localized to the Golgi but also shown to reside in several post-Golgi structures including the lysosomal membrane (27). When tritosomes were analyzed by immunoblotting for LAMP-1, sialyltransferase 1, and the Golgi protein GPP130, we found that both LAMP-1 and sialyltransferase 1 were substantially enriched in tritosomes, whereas GPP130 was not (Fig. 4, *A* and *B*). Our peptide coverage of sialyltransferase 1 includes the two tryptic peptides (Gly^{29}–Lys^{46}) on either side of a β-amyloid protein-converting enzyme 1 (Alzheimer's disease β-secretase activity) cleavage site. Cleavage at this site, residue Gln^{38}, followed by exoproteolysis generates soluble sialyltransferase 1 with a new N terminus (Glu^{41}) (28). We also found other glycosyltransferases in the lysosomal membrane with somewhat lower abundances (Table I). The enrichment of sialyltransferase 1 but not GPP130 and the presence of other glycosyltransferases in these membranes suggests that a previously unsuspected biological role for such proteins may exist in the lysosomal membrane.

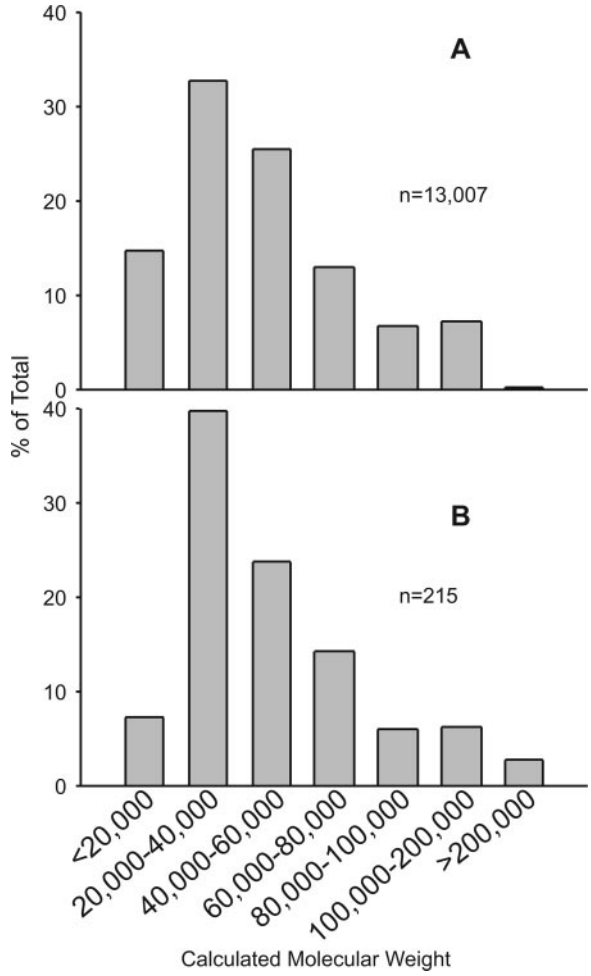

FIG. 3. **Molecular weight distribution of the predicted proteins from the MGC mouse cDNA data base (March 10, 2004) (*A*) and the theoretical molecular weights of the proteins identified in the rat liver lysosomal integral membrane protein fraction (*B*).**

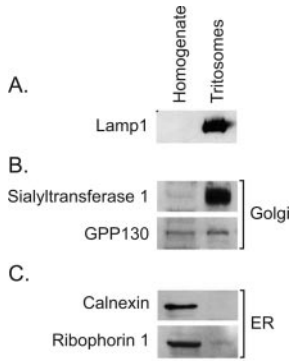

FIG. 4. **Immunoblot of rat liver tritosomes.** *A*, the lysosomal membrane protein LAMP-1; *B*, the Golgi membrane proteins sialyltransferase 1 and GPP130; *C*, the ER membrane proteins calnexin and ribophorin 1. Equal quantities of protein from liver homogenate and tritosomes were used.

We have previously demonstrated the presence of nicastrin and presenilin 1 (components of the γ-secretase complex) as well as acid γ-secretase activity in the lysosomal membrane (6). Nicastrin was confirmed as a major lysosomal constituent in this survey with the assignment of 13 unique peptides (Table I and Supplemental Table 1). However, we did not identify peptides from other putative components of this complex, *e.g.* mAph1 or Pen2. Similarly, nicastrin, but no other components of the γ-secretase complex, was found in a proteomic study of melanosomes (a lysosome-related organelle, Table II) (29).

Seventeen proteins of the lysosomal integral membrane fraction were found to have N-terminal acetylation post-translational modifications (Table I and Supplemental Table 1). This includes several proteins involved in trafficking and three proteins in the "unknown" ontology. N-terminal acetylation is a common post-translational modification of proteins in eukaryotes and is usually indicative of the cytosolic topology of the N terminus of the protein. These findings confirm the N-terminal acetylation of Rab6, originally shown in a proteomic study of Golgi vesicles (30), and we now extend this modification to other Rabs (Rab1A and Rab11B) and SNAREs (syntaxin 7 and VAMP8). Although the functional relevance of *N*-acetylation of these particular proteins is not known, it has recently been reported that *N*-acetylation is required for the correct membrane targeting of the Arf protein family member Arl3p (31, 32).

Ubiquitin (8 kDa) was identified in seven gel slices in the molecular mass range of ~30 to ~150 kDa and therefore exists in the integral membrane fraction as a conjugate to other proteins. Monoubiquitinylation of proteins occurs by conjugation of the C terminus of ubiquitin to a lysine side chain of the target protein, whereas polyubiquitinylation occurs by the successive conjugation of the ubiquitin molecules to lysine residues on the preceding ubiquitin. Lysine 48 of ubiquitin is the most common site for the addition of ubiquitin peptides during polyubiquitin formation (for a review, see Ref. 33). Polyubiquitinylation is generally viewed as a tag for cytosolic proteosome-based degradation, whereas monoubiquitinylation or multimonoubiquitinylation of membrane proteins at the plasma membrane has been reported to be sufficient for their transport to and degradation in the lysosome (34, 35). However, we did not identify the peptide that contains lysine 48 in the present study.

In accordance with the major role of the lysosome in transporting small molecules out of its degradative milieu, we identified (*a*) four proteins that contain an endomembrane protein of 70 kDa (EMP70) domain, which may have small molecule membrane transport functions (36), (*b*) proteins of the solute carrier family 25 (including the adenine nucleotide transporter and phosphate transporter), (*c*) the putative copper uptake transporter SLC31 member 1 and the histidine transporter SLC15 member 14, and (*d*) members of the ATP-binding cassette family. As well, the NPC1 cholesterol transporter and components of the vacuolar ATP proton pump were also found. Aside from the V-ATPase, most of the transporters identified were assigned five or fewer peptides suggesting that these are low abundance proteins in the lysosomal membrane.

Membrane Proteins Common to Lysosomes, Phagosomes, and Melanosomes—We compiled a BLAST data base of the proteins identified from published proteomic studies of the phagosome (37) and melanosome (29) and searched it with each of the 215 proteins from our present survey. Forty proteins in our lysosomal integral membrane fraction were found also either in phagosomes and/or melanosomes (Table II). Proteins such as LAMP-1, subunits B and E of the V-ATPase, voltage-dependent anion channel 1, Rab7, flotillin-1, stomatin, and ubiquitin were common to all these organelles. The predominantly ER proteins GRP78 (BiP) and protein-disulfide isomerase were also found in phagosomes and melanosomes. Three other Rab proteins, Rab2, Rab5C, and Rab11B, were also in common as well as flotillin-2, LIMPII, and NPC1. In our survey, we identified a number of other ER, cytosol, and Golgi proteins (mostly classified as biosynthesis or metabolism in Table I) in the lysosomal membrane protein fraction. Although these proteins had somewhat lower abundances in terms of the number of peptides identified, some of these proteins have been reported to be associated with phagosomes and/or melanosomes (Table II) and are therefore likely not from vesicular contaminants of the preparations but arise in lysosomes and these related organelles through common trafficking pathways. It has been demonstrated previously that phagosomal membrane may arise from ER membrane, and the maturation of the phagosome to phagolysosome coincides with a decrease in the quantity of ER proteins (37, 38). In agreement with its level of maturation, immunoblots of tritosomal proteins show that the ER membrane protein calnexin is undetectable, while ribophorin 1 is present at a low level (Fig. 4*C*). This suggests that ER membrane and its associated proteins may be involved in early lysosomal struc-

TABLE II

Common proteins from lysosomes, phagosomes, and melanosomes

Common proteins from lysosomes, phagosomes, and melanosomes were found by comparing lists of proteins from published proteomic studies. MHC, major histocompatibility complex; MDR, multidrug resistance; TAP, transporter associated with antigen processing; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein.

Protein Name	Lysosome GI number	Phagosome GI number	Melanosome GI number
Metabolism			
CYP4A3	28461155	296449	
Glyceraldehyde-3-phosphate dehydrogenase	8393418	P16858	
Peroxiredoxin 1	16923958	P35700	
Immunity			
Macrophage expressed gene 1	12018298	I52603	
MHC class I α chain	940825	CAA06194	
Biosynthesis			
Eukaryotic translation elongation factor 1 α2	15805031	P10126	
Heat shock 70-kDa protein 5, GRP78, BiP	25742763	P20029	p11021
Protein disulfide isomerase (ER60)	91897	P27773	P30101
Ribophorin I	6981486		P04843
Membrane receptors/signaling and cell adhesion			
Phosphatidylinositol 4-kinase type II	16758554		13559514
Similar to ras-related C3 botulinum toxin substrate 1 isoform Rac1b	34870449		P15154
Molecular transport			
ATP-binding cassette, sub-family B (MDR/TAP), member 6	18034785		11245444
Niemann-Pick C1	6679104		O15118
V-ATPase Subunit B	17105370	P50517	P21281
V-ATPase Subunit D	3955100		P12953
V-ATPase Subunit E	13097342	P50518	P36543
Voltage-dependent anion channel 1	6755963	Q60932	P21796
Membrane structure and lipid rafts			
CD36 antigen-like 2 (LIMPII)	16758914	P27615	
Flotillin-1	13124118	O08917	3599573
Flotillin-2	13124119		Q14254
LAMP-1	6981144	P11438	P11279
LAMP-2	8393690	P17046	
Prohibitin	6679299	P24142	
Stomatin (erythrocyte band 7 integral membrane protein 7.2b)	7710018	P54116	P27105
Hydrolases/cofactors			
β-Glucuronidase	893510	P12265	
Cathepsin L	67650	P06797	
Cathepsin Z	34978341	CAB44494	
Nicastrin	27819651		Q92542
Prosaposin	9438805		1360694
Protective protein for β-galactosidase	6679437	P16675	
Secreted			
Ferritin light chain, rat	2119695	P29391	
Peptidylprolyl isomerase C-associated protein (mama)	20806135	397800	
Vesicular and protein trafficking			
Arl10C	12838871	AI006608	
heat shock protein 8; Heat shock cognate protein 70; heat shock 70-kDa protein 8	13242237	P08109	
RAB11B, member RAS oncogene family	14249144	P46638	
Rab2	13929006	P53994	
Rab5C	27689505	P35278	
Rab7	13027392	P51150	P51149
SNAPα	2143586	P54921	
Ubiquitin	70637	1167510	P02248
Number of proteins reported and used for comparson	215	117	68

tures and are lost at variable rates during maturation of the organelle.

DISCUSSION

The approach of organelle-based proteomics allows the concentration and thus the identification of otherwise rare cellular proteins while at the same time offering clues to their *in vivo* function based on their localization. This technique is dependent on the quality and yield of the organelle in question. Since lysosomes are heterogeneous in terms of the native densities, they are difficult to purify by conventional methods. Triton-filled lysosomes overcome this problem by decreasing the overall densities of a large population of lysosomes in rat liver, allowing milligram quantities of highly purified organelles to be isolated in a single step. Additionally since tritosomes are secondary lysosomes they represent a population of relatively stable, end stage organelles. Thus their protein composition may be considered constant and constitutive, *i.e.* they are less a reflection of the transitional phase(s) of the life cycle of lysosomes.

The 215 proteins reported here to our knowledge represent the largest number of proteins identified from a membrane fraction of the lysosome to date. A previous study identified 27 proteins from a soluble lysosomal fraction that bound to a mannose 6-phosphate affinity column (39). However, lysosomal membrane proteins are generally not transported to the organelle via this pathway as reflected by the highly sialylated state of their *N*-linked oligosaccharides. Our combination of ion exchange chromatography and one-dimensional SDS-PAGE followed by in-gel trypsin digestion and mass spectrometry (LC-MS/MS) provides a high degree of protein separation. It is robust, offers reasonable reproducibility, and can be used for comparing protein profiles from multiple complex protein samples.

One interesting finding was the relatively high abundance of lipid raft proteins in the lysosomal membrane. Lipid rafts are small membrane domains enriched in cholesterol and sphingolipids that have the ability to sort, concentrate, and organize proteins for various cell signaling events. They have recently been shown to concentrate SNARE proteins involved in vesicle fusion (40) and have been found associated with late endosomal (41), lysosomal (21), and phagosomal membranes (42). Interestingly a coincident increase in the quantity of lipid rafts in phagosomes with their maturation into phagolysosomes was demonstrated (42); this is in agreement with our present finding of an apparent high abundance of lipid rafts on mature lysosomes. Their presence in the lysosomal membrane suggests major signaling and sorting functions for even this late stage organelle. Consistent with this we have identified the rat orthologue of MEK binding partner 1 protein, a scaffold component of the endosomal ERK pathway of the mitogen-activated protein kinase cascade and which is known to bind to p14 (not identified in this study) on the cytosolic face of late endosomes/lysosomes (43), a process required for the endosomal activation of ERK1/2 (44). Similarly, cellular repressor of E1A-stimulated genes (CREG) was identified confirming a previous study of lysosomal mannose 6-phosphate receptor-binding proteins (45). CREG is a secreted glycoprotein that promotes differentiation of pluripotent stem cells (46) and inhibits cell growth in a mannose 6-phosphate/insulin-like growth factor II receptor-dependent manner (47). We also identified LRG-47, a macrophage interferon-γ-inducible GTPase required for pathogen-containing phagosomes to fuse with lysosomes (48). Also identified were syntaxin 7, syntaxin 8, vti1b, and VAMP8. These occur in a complex that forms on late endosomes, and this complex is required for fusion events with other endosomes (49) and lysosomes (50). Collectively these findings suggest a crucial role for the lysosomal membrane in intracellular communication and signaling events.

A well described function of the lysosomal membrane has been the transport of the products of degradation to the cytosol for reuse by the cell. However, few proteins linked to these activities in the lysosome have been identified. A notable group of proteins that we have identified in this report are those predicted to have transport functions, *e.g.* EMP70 domains, or the those in the solute carrier family. These proteins can now be assessed for molecular transport function with respect to the lysosomal membrane and its luminal contents.

Although *de novo* biogenesis of lysosomes is not well understood, identification of proteins in common among lysosomes and their related organelles may help elucidate how they are formed. As a starting point, many well known proteins of the lysosomal membrane identified in this study were also identified in other proteomic surveys of the phagosome and melanosome (Table II). Another clue to begin to understand the processes of lysosome biogenesis and maintenance arises from our finding of the so-called "grey-lethal" (Gl) protein. Gl has been implicated in a severe form of human malignant autosomal recessive osteopetrosis (51). In mice, mutation of this gene causes a defect in coat color through "clumping" of pigment granules (melanosomes) in melanocytes (52) and a defect in bone resorption caused by defective osteoclast maturation (53), *i.e.* this protein affects the function of specialized lysosomes (for reviews, see Refs. 4 and 54). The localization to the lysosomal membrane of Gl protein and a large number of other proteins with no previously ascribed function will help greatly in elucidating their biological roles.

From this proteomic survey, it is clear that the protein composition of the lysosomal membrane is influenced by contributions from Golgi, ER, and plasma membrane components. Highlighting this was the identification in the integral membrane fraction of Golgi transferases, *e.g.* sialyltransferase 1 (27) and other glycosyltransferases, some of which have been shown to have post-Golgi localizations (for a review, see Ref. 55). As well, some abundant ER proteins were found, *e.g.* protein-disulfide isomerase, ribophorin 1, and cytochrome P450s, but interestingly no calnexin and plasma membrane

receptor proteins, *e.g.* asialoglycoprotein receptors and Toll-like receptor 3, were found. Although delivery of proteins and cargo to the lysosome from endosomes and the trans-Golgi network is well established, autophagy, a process of sequestering cytoplasm and organelles for degradation, also delivers protein to the lysosome presumably using LAMP-2 as a receptor (56). Many abundant ER and cytosolic proteins such as GRP78, protein-disulfide isomerase, cytochrome P450, and betaine-homocysteine *S*-methyltransferase have been found to be associated with autophagic vacuoles (57–59).

In summary, the lysosome is traditionally thought to be the end point of the endocytic pathway and to have a chiefly nonspecific degradative capacity for its luminal contents. Through our lysosomal proteomic strategy it seems clear that the membrane is a very diverse structure that receives membrane and protein contributions from a variety of subcellular sources and pathways. Through vesicular trafficking and intracellular membrane dynamics, the high degree of diversity of the proteins of the lysosomal membrane is indicative of a highly complex organelle. The diverse set of proteins in the lysosomal membrane could be organized into distinct domain structures comparable to those currently envisioned for earlier endosomal compartments (60) that are demarcated by at least two different Rab proteins (61). Since we identified several different Rabs in the lysosomal membrane, it remains to be determined whether each of these Rab proteins is involved in the organization of additional protein sets and different domains in the lysosomal membrane.

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S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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