Demonstration of Lysosomal Localization for the Mammalian Ependymin-related Protein Using Classical Approaches Combined with a Novel Density Shift Method*

Maria Cecilia Della Valle‡§, David E. Sleat‡§, Istvan Sohar†, Ting Wen*, John E. Pintar¶, Michel Jadot¶, and Peter Lobel‡§¶

From the ‡Center for Advanced Biotechnology and Medicine, Departments of §Pharmacology and ¶Neuroscience and Cell Biology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854 and ¶Laboratoire de Chimie Physiologique, Unite de Recherche en Physiologie Moleculaire, Facultes Universitaires Notre-Dame de la Paix, 61 Rue de Bruxelles, 5000 Namur, Belgium

Most newly synthesized soluble lysosomal proteins are delivered to the lysosome via the mannose 6-phosphate (Man-6-P)-targeting pathway. The presence of the Man-6-P post-translational modification allows these proteins to be affinity-purified on immobilized Man-6-P receptors. This approach has formed the basis for a number of proteomic studies that identified multiple as yet uncharacterized Man-6-P glycoproteins that may represent new lysosomal proteins. Although the presence of Man-6-P is suggestive of lysosomal function, the subcellular localization of such candidates requires experimental verification. Here, we have investigated one such candidate, ependymin-related protein (EPDR). EPDR is a protein of unknown function with some sequence similarity to ependymin, a fish protein thought to play a role in memory consolidation and learning. Using classical subcellular fractionation on rat brain, EPDR co-distributes with lysosomal proteins, but there is significant overlap between lysosomal and mitochondrial markers. For more definitive localization, we have developed a novel approach based upon a selective buoyant density shift of the brain lysosomes in a mutant mouse lacking NPC2, a lysosomal protein involved in lipid transport. EPDR, in parallel with lysosomal markers, shows this density shift in gradient centrifugation experiments comparing mutant and wild type mice. This approach, combined with morphological analyses, demonstrates that EPDR resides in the lysosome. In addition, the lipidosis-induced density shift approach represents a valuable tool for identification and validation of both luminal and membrane lysosomal proteins that should be applicable to high throughput proteomic studies.

Recent genomic and proteomic studies have revealed the presence of numerous previously uncharacterized proteins encoded by the mammalian genome. Determining the biological role of such proteins poses a fundamental challenge. Although sequence and structural similarities to proteins of known function may provide useful information, knowledge of the site of function is essential in understanding uncharacterized proteins. In addition to providing insight into protein function, mapping of proteins to cellular organelles or structures involved in specific biological processes may provide valuable clues to how the organelles carry out these processes.

One cellular compartment that contains protein constituents of characteristic function is the lysosome, an acidic, membrane-delimited organelle present in all eukaryotic cells (1). The lysosome contains over 60 soluble proteins, most of which are hydrolases that function in concert to degrade macromolecules introduced via the endocytic and autophagic pathways into simple constituents that can be reutilized by the cell. A number of recent proteomic studies investigating soluble luminal lysosomal proteins have relied upon the fact that these proteins contain an unusual carbohydrate modification that allows for their specific affinity purification. Most luminal lysosomal proteins are targeted to this organelle by the mannose 6-phosphate (Man-6-P)2 pathway. Here, N-linked glycans on newly synthesized lysosomal proteins are phosphorylated on select mannose residues, allowing binding to intracellular Man-6-P receptors (MPRs) and targeting to the lysosome. These proteins can be affinity-purified on immobilized MPRs, and this has led to the subsequent identification of many known lysosomal proteins and Man-6-P-containing glycoproteins that may represent new lysosomal candidates (2–10).

The identification of new lysosomal proteins is clearly of interest in terms of cell biology. Also, their discovery has considerable biomedical importance, since deficiencies in lysosomal proteins are responsible for over 40 inherited human diseases (11). However, purification by MPR affinity chromatography, although highly suggestive, does not definitively demonstrate lysosomal localization. For example, there are a number of nonlysosomal proteins that may also receive the Man-6-P modification (4). In addition, some proteins (e.g. pro-

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†To whom correspondence should be addressed: CABM, 679 Hoes Ln., Piscataway, NJ 08854. Tel.: 732-235-5032; Fax: 732-235-4466; E-mail: lobel@cabm.rutgers.edu.

2 The abbreviations used are: Man-6-P, mannose 6-phosphate; MPR, Man-6-P receptor; PNGase F, peptide N-glycosidase F; EPDR, ependymin-related protein; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; PBS, phosphate-buffered saline.
tease inhibitors and lectins) that do not contain Man-6-P may specifically associate and co-purify with authentic Man-6-P glycoproteins (7). Finally, as in any proteomic study, as the limits of detection decrease, so does the likelihood of detecting nonspecific contaminants. Thus, there is a clear requirement for the direct experimental verification of the intracellular location of lysosomal candidate proteins identified in MPR affinity purification studies.

A number of approaches can be used to investigate the subcellular location of a protein. Double label immunomicroscopy can provide relatively high resolution information but is highly dependent on experimental conditions. Importantly, after fixation, retention of a protein as well as epitope access may vary in different organelles. Thus, it is impossible to be certain if the entire population of the protein of interest is visualized. Another approach that is frequently used is the direct visualization of expressed, tagged proteins. However, a significant drawback of this approach is that overexpression and/or tagging may perturb the normal distribution of the protein. In addition, the tag may be lost in a given cellular compartment, such as the lysosome, which contains a host of proteases.

Many of the respective drawbacks of these methods can be overcome by the quantitative subcellular fractionation methods pioneered by de Duve and colleagues (12). Even so, the resolution of such approaches is limited by the overlapping physical properties of some organelles (e.g. sedimentation coefficient and/or buoyant density). One method for increasing the resolution of subcellular fractionation is to elicit a selective and diagnostic shift in the density of a given organelle. Traditionally, this has been achieved for lysosomes by injecting Triton WR-1339 into the bloodstream of rodents several days prior to tissue harvest (13). The density shift observed when comparing treated and untreated animals is specific to the lysosome and thus, for any given protein, is a hallmark of lysosomal localization. This approach is generally used in characterization of liver organelles, but intravenous injection of Triton WR-1339 does not alter the equilibrium density of brain lysosomes.

One study has demonstrated a shift after intrathecal injection of Triton WR-1339, but only a limited proportion of the brain lysosome population was shifted (14). Therefore, this approach is unlikely to prove useful in terms of validating lysosomal localization for proteins preferentially expressed in brain.

One glycoprotein that has been identified in a number of Man-6-P affinity purification proteomic studies (4, 7, 8, 15) is ependymin-related protein (EPDR). (EPDR has also been referred to as MERP (mammalian ependymin-related protein) and UCC1 (up-regulated in colorectal cancer gene 1). The human and mouse orthologs are encoded by genes EPDR1 and Epdr2, respectively. We have chosen EPDR as a general reference to the mammalian protein and indicate appropriately where more specific reference is meant.) EPDR is of unknown function, is conserved among vertebrates, and has some sequence similarity to fish ependymin, a glycoprotein proposed to be a cell adhesion molecule involved in memory consolidation and learning (reviewed in Ref. 16). The localization of EPDR has not been established, although three lines of evidence suggest that it may be lysosomal. First, the presence of Man-6-P on EPDR has been directly demonstrated, precluding the possibility that this protein represents a contaminant in MPR affinity-purified samples (5). Second, a purified, Man-6-P-containing epitope-tagged derivative of EPDR is endocytosed and delivered to the lysosome by MPRs (8). Third, EPDR was identified as a component of a neuromelanin granule preparation, an organelle related to the lysosome (17).

The aim of this study was to use orthogonal approaches to investigate the subcellular localization of endogenous EPDR in a definitive manner. Although this protein is highly expressed in brain, it is undetectable in liver; thus, the classical triton density shift approach (13) to investigate lysosomal localization is not applicable. We have therefore developed a new strategy for the subcellular localization of brain lysosomal proteins that relies on a density shift of lysosomes due to an accumulation of lipids in mice lacking NPC2, a soluble glycoprotein involved in cholesterol transport (3, 18). Using this novel method, we demonstrate unambiguously that EPDR is a lysosomal protein. In addition, we propose the lipidosis-induced density shift approach as a general method that should be widely applicable for the identification and validation of lysosomal candidates.

**EXPERIMENTAL PROCEDURES**

*Cloning and Expression in Pichia pastoris—*The coding sequence for the mature murine EPDR was PCR-amplified from an expressed sequence tag clone (IMAGE:4223134) and subcloned into the XhoI/XbaI sites of pGAPZa A (Invitrogen). Two constructs were generated. In the first, the α factor signal sequence was present in the vector immediately followed by the amino terminus of mature murine EPDR (forward primer 1, CCGGCTCGAGAAAAAGAACCACAGCCATGCGAGGCA; reverse primer 1, CCGGTCTAGAAGGGAGCAGCCGCGTCACTCATCTT). In the second, an additional Glu-Ala-Glu-Ala sequence was inserted between the α factor signal sequence and the sequence of mature murine EPDR (forward primer 2, CCGGCTCGAGAAAAAGAAGGCTAGCTACCCCACA-GCCATGCCAGGCA; reverse primer as above). Constructs were linearized using AvrII and introduced into competent *P. pastoris* X-33 by electroporation. Transformation, selection, and growth of *P. pastoris* cells was essentially as described in the proprietary pGAPZa A manual (Invitrogen). In brief, cells were selected on plates containing 100 μg/ml Zeocin (Invivogen, San Diego, CA) in YPDS medium. Colonies (~6/construct) were grown in small scale liquid cultures, and supernatants were analyzed by Western blotting with an anti-Myc antibody (Invitrogen) to determine relative levels of recombinant EPDR expression. Overnight cultures (1 ml) of the clones exhibiting the highest level of expression were used to inoculate 2-liter baffled flasks (Nalgene, Rochester, NY) containing 0.4 liters of YPD growth medium and cultured for 4–5 days on a shaking incubator (250 rpm, 30 °C).

*Purification of Recombinant EPDR—*Typically, cleared supernatants were prepared from 1 liter of *P. pastoris* 4–5-day culture by centrifugation at 4 °C at 4000 × g for 15 min followed by a second centrifugation at 13,000 × g for 30 min. Supernatants were filtered through a 0.22-μm membrane and concentrated

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3 M. Jadot, unpublished data.
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10–20-fold by ultrafiltration using an Amicon stirred cell apparatus with a YM10 membrane (Millipore, Billerica, MA). The concentrated supernatant was buffered with the addition of one-tenth volume of 10× HisTrap binding buffer (0.2 M sodium phosphate, 5 M sodium chloride, 0.2 M imidazole, pH 7.4) and filtered through a 0.22-μm membrane. The supernatant was applied at a 1 ml/min flow rate to a 1-ml HisTrap HP column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) equilibrated with 1× HisTrap binding buffer. The column was eluted at a flow rate of 1 ml/min with a 20-ml linear gradient of 0.02–0.5 M imidazole in 500 mM NaCl, 20 mM sodium phosphate, pH 7.4, monitoring protein absorbance at 280 nm, and collecting 0.5-ml fractions. Fractions with the highest absorbance were pooled, concentrated, and buffer-exchanged to column equilibration buffer (125 mM ammonium acetate, pH 5.5, using an Amicon Ultra (Millipore Corp.) with a 10-kDa molecular weight cut-off. Purified protein was digested for 2 h at 37 °C with Endo Hf (10 units/μg of protein) (New England Biolabs, Ipswich, MA). The deglycosylated sample was buffer-exchanged as above to column equilibration buffer (125 mM ammonium acetate, pH 4.5) and filtered through a 0.22-μm membrane. The sample was loaded onto a 1-ml MonoS 5/5 (GE Healthcare Bio-Sciences) and eluted with a mixture of column equilibration buffer and buffer B (5 M ammonium acetate, pH 4.5) at a flow rate of 1 ml/min as follows: 150-ml linear gradient of 0–30% buffer B; 30-ml linear gradient of 30–60% buffer B; 10 ml of 60% buffer B. Fractions (1 ml) were collected and analyzed by SDS-PAGE, and those containing recombinant EPDR protein and lacking Endo Hf were pooled, concentrated, and freeze-dried using a vacuum centrifuge.

**Primary Antibodies**—Rabbits were immunized using purified deglycosylated recombinant EPDR (lacking the Glu-Ala-Glu-Ala sequence), and anti-EPDR antibodies were affinity-purified using recombinant EPDR immobilized on Affi-Gel-15 (Bio-Rad). Hybridoma supernatant containing mouse monoclonal anti-SV2 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and a rabbit polyclonal anti-EPDR antibodies (Abcam Inc., Cambridge, MA) were used at a 1:1000 dilution.

**Western Blotting**—SDS-polyacrylamide gel electrophoresis was conducted using precast 10% reducing Novex bis-Tris membranes or midigels (Invitrogen). After semidry transfer of proteins to nitrocellulose, membranes were baked at 80 °C for 1 h and blocked overnight at 4 °C with PBS containing 3% BSA and 0.2% Tween 20. Blocked membranes were incubated with primary antibodies for either 3 h at 20 °C or overnight at 4 °C and washed four times for 5 min each with PBS containing 0.2% Tween 20. For chemiluminescent detection, blots were incubated for 1 h at 20 °C with a 1:10000 dilution of a goat anti-rabbit IgG or goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Sigma) and visualized using the SuperSignal West Pico chemiluminescent system (Pierce). For radioisotopic detection, blots were incubated for 1 h at 20 °C with a 1:1000 dilution of a goat anti-rabbit antibody (Sigma) iodinated using IODO-GEN (Pierce) and 125I (PerkinElmer Life Sciences), exposed to phosphor storage screens, and scanned using a Typhoon 9400 scanner (GE Healthcare Bio-Sciences Corp.). Quantitation was conducted using ImageQuant 5.2 (GE Healthcare Bio-Sciences Corp.).

**Enzyme Assays**—Differential centrifugation and gradient fractions were diluted in cold 0.15 M sodium chloride with 0.1% Triton X-100, except for the catalase assay. Glycosidase and phosphatase measurements using 4-methylumbelliferyl substrates and protease assays using amino-4-methylcoumarin substrates were conducted essentially as described previously (15). The catalase assay was carried out using modifications of described methods (19, 20). Samples were diluted 10–300-fold in ice-cold 0.01% Triton X-100, 1 mM EDTA, 0.1% ethanol, 0.009% sodium bicarbonate, pH 7.5. Diluted samples (15 μl) were mixed with 15 μl of peroxide-free Triton X-100 (Sigma) and incubated for 1 min, and the reaction was initiated by the addition of 300 μl of 3.1 mM H2O2 in 20 mM imidazole buffer, pH 7.0, containing 0.1% bovine serum albumin. The reactions were incubated for 14 h at 0 °C, terminated by mixing an aliquot with an equal volume of 0.45% titanium oxysulfate in 1 M sulfuric acid (a generous gift from Millennium Inorganic Chemicals, MA), and incubated for 10 min at 25 °C, and the absorbance was measured at 415 nm to detect the decrease in H2O2. Dilutions exhibiting 0.2–0.8 fractional consumption of hydrogen peroxide were used to calculate the rate (−log fractional consumption/time), and the values from different dilutions were averaged after correcting for the dilution factor. Alkaline phosphodiesterase activity was measured using a modification of a published method (21). Briefly, 3 mM p-nitrophenyl thymidine-5’-monophosphate sodium salt was used as substrate in 0.1 M Tris, pH 9.0, containing 2 mM zinc acetate reaction buffer, and reactions were terminated by 0.5 M glycine, pH 10.5, and absorbance was measured at 415 nm. Cytochrome c oxidase was measured with a kinetic method (22, 23) at room temperature using 22 μM cytochrome c reduced by sodium dithionite as a substrate in 30 mM sodium phosphate, pH 7.4, containing 1 mM EDTA. The decrease in the logarithm of absorbance at 550 nm was monitored during the first 2 min and is proportional to the activity of cytochrome c oxidase. For all assays, substrates were prepared freshly in reaction buffer or stored in Me2SO and diluted in reaction buffer before use. Fluorescence was measured with a CytoFluor 4000 multiwell plate reader (PerSeptive Biosystems, Framingham, MA), and absorbance was measured with Advanced Protein Assay reagent (Cytoskeleton Inc., Denver, CO).

**Detection of Man-6-P Glycoproteins**—Proteins in subcellular fractions containing Man-6-P were detected as described previously (15, 24). Signal from the radiolabeled probe was detected using a Typhoon 9400 and quantified using ImageQuant 5.2.

**Preparation of Mouse Tissue Extracts for Immunoblotting**—Tissues were obtained from 90-day-old 129/SvEv and Balb/c mice and were frozen on dry ice immediately after dissection and stored at −80 °C prior to use. Frozen tissues were homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) in 5 volumes of extraction buffer (1× PBS, 0.2% Tween 20, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM Pefabloc (Pentapharm Ltd., Basel, Switzerland), 1 mM EDTA). Homogenates were centrifuged at 13,000 rpm for 10 min at 4 °C.
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Immunofluorescence—Brains were fixed as above and used to prepare 25-μm coronal cryostat sections. Histological sections were probed to detect EPDR and, as a neuronal lysosomal marker, Man-6-P glycoproteins (26). Sections were washed twice with PBS and blocked with PBS containing 2% dried milk and 0.2% Tween 20 for 2 h at 20 °C. For double labeling, anti-EPDR antibody (1:50 dilution) and biotinylated-MPR (26) (10 μg/ml) were added to the sections in blocking buffer and incubated overnight at 4 °C. Sections were washed twice with 3× PBS and once with 1× PBS and incubated in PBS containing 2% goat serum and 0.1% Tween 20 for 2 h at 20 °C with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:200) and Cy3-conjugated streptavidin (1:200). Sections were washed three times with PBS and mounted with Vectashield fluorescent mounting medium (Vector Laboratories). Sections were stored in a humid chamber at 4 °C for less than 3 days with light shielding before conducting confocal microscopy at the Keck Center imaging facility at Rutgers University using a Zeiss LSM 510 confocal microscope.

RESULTS

Expression and Purification of Recombinant EPDR—As a first step toward the biochemical and functional characterization of EPDR, we purified recombinant glycosylated protein expressed in P. pastoris. Pilot experiments were conducted with two different constructs consisting of the mature murine EPDR coding sequence cloned into pGAPZα A. (The signal sequence cleavage site of mouse EPDR was based on the experimentally determined amino terminus of mature rat brain EPDR (15) as well as analysis of the mouse protein sequence using SignalP 3.0 (28).) Both constructs contained a Myc epitope tag followed by a hexahistidine sequence at the C terminus (Myc-His) but differed in the presence or absence of a Glu-Ala repeat (Myc-His) but differed in the presence or absence of a Glu-Ala repeat. This Glu-Ala repeat may promote more efficient signal sequence cleavage under certain circumstances (29). After pilot experiments, we chose to use the construct lacking the Glu-Ala repeat for subsequent protein production, because expression levels from both constructs were similar, but N-terminal sequence analysis (data not shown) revealed that the Glu-Ala repeat was retained. The other construct yielded recombinant EPDR with the predicted amino-terminal sequence (TPQPCQAPQ . . . ) of the mature murine EPDR.

Recombinant Myc-His-tagged protein was purified from culture supernatants using nickel affinity chromatography (Fig. 1, lanes 1–3). SDS-PAGE revealed a heterogeneous smear (Fig. 1, lane 3) that collapsed to a single band after deglycosylation with Endo H (Fig. 1, lane 6) or PNGase F (data not shown), indicating the presence of N-linked glycans. The apparent size of the deglycosylated protein estimated from gel electrophoresis (~29 kDa) was somewhat larger than predicted from the amino acid composition of the C-terminally tagged recombinant protein (24,147 Da), probably reflecting anomalous electrophoretic migration or a post-translational modification other than N-linked glycosylation. We further purified the deglycosylated protein from Endo H, and other potential contaminants using ion exchange chromatography (Fig. 1, lanes 7–10). Typi-
cal yields following the final purification were ~3 mg of recombinant EPDR/liter of culture supernatant.

Purified and deglycosylated recombinant protein was used for the production and affinity purification of rabbit polyclonal antibodies. Western blotting of a purified mouse brain mannosyl-6-phosphorylated glycoprotein preparation known to contain EPDR (7) with this antiserum revealed a band of 28 kDa. Analysis of a series of mouse tissues revealed a band of similar size that is present in brain and muscle but is not detectable in other tissues analyzed (Fig. 2). This tissue distribution is consistent with that observed by Northern blot analysis (30).

Immunohistochemical analysis of the distribution of EPDR in mouse brain (Fig. 3) showed that it is expressed in neurons in different areas of the brain, including cortex, hippocampus, hypothalamus, pons, cranial ganglia, and cerebellum. Staining of tissue sections from liver showed no immunoreactivity (data not shown), which is in agreement with Western (Fig. 2) and Northern (30) blotting data.

Glycosylation of EPDR—Soluble lysosomal proteins typically contain at least one Endo H-sensitive oligosaccharide, since mannosyl-6-phosphorylation inhibits the action of Golgi α-mannosidase I and subsequent oligosaccharide processing to Endo H-resistant structures. We therefore examined the glycosylation of EPDR to determine whether its carbohydrate structure resembled that of known lysosomal proteins. Total extracts or purified Man-6-P glycoproteins from mouse brain and three different types of skeletal muscle (soleus, extensor digitorum longus (EDL), and white gastrocnemius) were deglycosylated with Endo H, or PNGase F. EPDR was detected by Western blotting and chemiluminescence. Different exposure times for different tissue samples were used for clarity of presentation. E, Endo H reaction buffer; P, PNGase F reaction buffer.

FIGURE 1. Purification of recombinant EPDR. EPDR was expressed in P. pastoris X-33 and purified from the culture supernatant. Lane 1, culture supernatant; lane 2, HisTrap HP flow through; lanes 3 and 4, HisTrap HP eluate; lane 5, Endo H; lane 6, pooled HisTrap HP fractions deglycosylated with Endo H; lanes 7–10, MonoS 5/5 fractions containing Endo H (lane 7) and EPDR (lanes 8–10). Arrow, EPDR.

FIGURE 2. Distribution of EPDR in mouse tissues. Extracts of Balb/c mouse tissues (10 μg of total protein/lane) were analyzed for expression of EPDR by Western blotting and chemiluminescence. Epidid., epididymis; Lg int, large intestine; Sm int, small intestine; EDL, extensor digitorum longus; White G., white gastrocnemius; M6PGP, MPR affinity-purified glycoproteins from mouse brain. Similar results were obtained with 129 Sv/Ev mice (data not shown).

FIGURE 3. Distribution of EPDR in mouse brain. Coronal sections of C57BL/6J wild type mouse brain were immunohistochemically stained for EPDR. A, cortex at the level of the striatum; B, hippocampus; C, hypothalamus; D, pons; E, trigeminal ganglia; F, cerebellum. Images were obtained at ×100 magnification.

FIGURE 4. Glycosylation of EPDR. Purified mouse brain Man-6-P glycoproteins (0.1 μg) and tissue extracts (12 μg of total protein/lane) from mouse brain and three different types of skeletal muscle (soleus, extensor digitorum longus (EDL), and white gastrocnemius) were deglycosylated with Endo H, or PNGase F. EPDR was detected by Western blotting and chemiluminescence. Different exposure times for different tissue samples were used for clarity of presentation. E, Endo H reaction buffer; P, PNGase F reaction buffer.
detected by Western blotting, one corresponding to EPDR and one of higher mass, 32 kDa. (The higher mass band was identified using three independent antisera, confirming that it is antigenically related to EPDR rather than a nonspecific artifact of a single antibody (data not shown).) It is possible that this higher mass protein is not actually EPDR but an antigenically related protein that is specific to these muscle types, but, more likely, this protein may actually represent another EPDR isoform. The molecular basis for this heterogeneity in muscle is not clear, although treatment with PNGase F did not collapse the two isoforms to a single species (Fig. 4), indicating that it does not result from differences in N-linked glycosylation. Thus, the higher mass EPDR isoform in muscle could result from the presence of PNGase F-resistant oligosaccharides (e.g. O-linked glycans). Alternatively, there may be two independent but related EPDR polypeptide chains arising from alternate splicing of the EPDR transcript.

In order to determine the proportion of EPDR that contains Man-6-P, a mouse brain homogenate was applied to an immobilized MPR affinity column, and EPDR levels were measured by Western blotting in the total homogenate and in column fractions (Fig. 5). Approximately 60% of the EPDR was retained on the column and specifically eluted with Man-6-P. Analysis of lysosomal marker enzymes indicated that similar proportions contained Man-6-P.

Subcellular Localization of EPDR in Rat Brain—We conducted a series of subcellular fractionation studies on rat brain to determine the intracellular location of EPDR. Western blot analysis of differential centrifugation fractions revealed that the bulk of EPDR was present in the M (heavy mitochondria) and L (light mitochondria) fractions, which also contained the bulk of the lysosomal marker enzymes (data not shown; see below for mouse brain fractionation). The distribution of EPDR was then examined after isopycnic centrifugation of the ML fraction in sucrose and Nycodenz density gradients (Fig. 6). In both cases, the distribution of EPDR paralleled that of the lysosomal marker β-galactosidase and was distinct from plasma membrane and ER markers. EPDR and the lysosomal marker had a slightly lower density than the mitochondrial marker in the Nycodenz gradient. In contrast, EPDR and the lysosomal marker had a slightly higher density than the mitochondrial marker in the sucrose gradient. This is consist-

**FIGURE 5.** Mannose 6-phosphorylation state of mouse brain proteins. Protein species lacking and containing Man-6-P were separated by MPR affinity chromatography. Fractions (FT, flow through; W, wash; G6P, glucose 6-phosphate elution; M6P, Man-6-P elution; Gly, glycine elution) were analyzed by Western blotting for EPDR (inset) and for lysosomal enzyme activities (β-gal, β-galactosidase; β-hex, β-hexosaminidase; β-man, β-mannosidase).

**FIGURE 6.** Subcellular distribution of EPDR in rat brain. An ML (M, heavy mitochondrial; L, light mitochondrial) fraction obtained by differential centrifugation of a rat brain homogenate was further fractionated by top loading on sucrose (A) or Nycodenz (B) density gradients. Gradient fractions were analyzed by Western blotting for EPDR (inset) and by enzyme assays for organellar markers. For clarity of presentation, different enzymes measured on an individual gradient are split between the top and bottom graphs and compared with EPDR.
ent with the expected osmotic behavior of lysosomes and mitochondria in these two different media (31), but a higher degree of organelle separation is desirable for unambiguous assignment of localization (32).

Subcellular Localization of EPDR in Wild Type and Mutant Mouse Brain—A traditional method to verify the subcellular localization of putative lysosomal proteins exploits the selective shift in the buoyant density of liver lysosomes elicited by treatment of animals with Triton WR-1339 (13). However, this approach is not applicable to EPDR, given that it is not expressed in liver. We have therefore developed a novel approach based on similar principles that allows a selective shift of brain lysosomes.

This new approach relies upon the fact that in mice lacking NPC2, a protein involved in lysosomal sterol transport (3), cholesterol and other lipids accumulate in the lysosome (25). The distributions of marker enzymes in NPC2 mutant and wild type brain homogenates were similar in differential centrifugation fractions (Fig. 7). However, analysis of the brain L fractions using isopycnic sucrose density gradient centrifugation revealed that the lipid storage accompanying the NPC2-deficiency elicits a dramatic shift in the buoyant density (from equilibrium density ~1.19 g/cm^3 (wild type) to ~1.10 g/cm^3 (NPC2 mutant) of an organelle that contains numerous known lysosomal markers. (For clarity, the distribution of a single lysosomal marker, β-galactosidase, is shown in Fig. 8A, whereas the distributions of others (β-glucosidase, β-hexosaminidase, β-glucuronidase, α-glucosidase, α-mannosidase, β-mannosidase, tripeptidyl-peptidase I (TPP I), and acid phosphatase) are shown together in Fig. 8B). In addition, the same density shift is observed with Man-6-P glycoproteins (Fig. 8A), which were previously shown to co-distribute with lysosomal markers (26). In contrast, the distributions of markers for mitochondria, peroxisomes, ER, plasma membrane, and synaptic vesicles are unaffected by the NPC2-deficiency. Importantly, the distribution of EPDR shows an NPC2 mutation-dependent density shift that parallels that of bona fide lysosomal enzymatic markers and Man-6-P glycoproteins. This shift is more clearly illustrated when the distribution of EPDR and lysosomal markers in control and NPC2-deficient brain are graphed together, with the abscissa representing fraction density rather than fraction number (Fig. 8C).

Immunofluorescent Localization of EPDR—In another approach to investigate its subcellular localization, morphological studies of EPDR in mouse brain were conducted by immunofluorescence confocal microscopy. EPDR exhibits a punctate cytoplasmic distribution that is consistent with a lysosomal localization (Fig. 9). We have previously determined that a biotinylated MPR derivative colocalizes with different lysosomal markers in rodent brain (26). Here there is complete overlap in distribution between Man-6-P glycoproteins and EPDR.

DISCUSSION

The aim of this study was to investigate the subcellular localization of endogenous EPDR. For detection of this protein, we generated a specific polyclonal antibody against recombinant EPDR expressed in P. pastoris. As a first step, we used this antibody to investigate if the glycosylation of EPDR was characteristic of lysosomal proteins before initiating an in depth analysis of subcellular localization. We demonstrated by endoglycosidase analysis that both predicted glycosylation sites are used and that EPDR always contains at least one Endo H-sensitive high mannose oligosaccharide that is typical of soluble lysosomal proteins. In addition, to test the possibility that the Man-6-P glycoform represents a very minor fraction of the total EPDR population in brain, which would suggest a nonlysosomal localization (4), we directly determined the proportion of
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EPDR that contains Man-6-P. Approximately 60% of mouse brain EPDR contains this modification, similar to that of other lysosomal proteins.

Although the glycosylation properties of EPDR were consistent with lysosomal localization, additional experimental evidence is required to demonstrate its true intracellular location, including subcellular fractionation (see Introduction). Classical subcellular fractionation schemes have been optimized for liver (12). Given that the expression of EPDR in liver was below the limits of detection as determined by Western blotting (Fig. 2) and that muscle requires harsh homogenization methods that can disrupt the integrity of organelles, we chose to use brain as a tissue source for our experiments.

Localization of EPDR in rat brain sucrose and Nycodenz density gradients demonstrated a similar distribution to lysosomal markers (Fig. 6). However, the difference in the distribution of lysosomal markers and that of other organelles, in particular mitochondria, was relatively subtle, and a method with increased resolving power was highly desirable. We therefore developed a novel approach to elicit a diagnostic density shift in brain lysosomes (see below) and found that the distribution for EPDR is identical to that of other lysosomal proteins. This is strongly supported by the double label confocal microscopy analysis and provides unambiguous demonstration that EPDR represents a novel soluble lysosomal protein.

The knowledge that EPDR is a lysosomal protein represents an important step toward elucidating its biological role, but its precise biochemical function remains unknown. Previously, based on bioinformatic analysis of the primary sequence, EPDR was predicted to be a type II transmembrane protein (33), but this now seems unlikely given data presented here. Experiments conducted with mouse brain homogenates showed that the bulk of EPDR appears in the

FIGURE 8. Subcellular distribution of EPDR and organellar markers in wild type and NPC2 mutant mouse brain. A, fractions (light mitochondrial) obtained by differential centrifugation of control and mutant mouse brain homogenates were further fractionated by bottom loading on a sucrose density gradient. $A$ gradient fractions were analyzed by either Western blotting for EPDR ($\bullet$), PMP-70 (a peroxisomal marker), and SV2 (a synaptic vesicle marker), radiolabeled MPR blotting for Man-6-P glycoproteins ($\triangledown$) (a global marker for lysosomal proteins), or enzyme assays for organellar markers ($\beta$-galactosidase ($\bigtriangleup$), lysosomes), cytochrome oxidase ($\blacklozenge$, mitochondria), catalase ($\bigtriangledown$, peroxisomes), alkaline phosphodiesterase ($\blacklozenge$, plasma membrane), and $\alpha$-glucosidase II ($\bigtriangleup$, endoplasmic reticulum). $B$, enzymatic assays for additional lysosomal markers ($\bullet$, $\beta$-hexosaminidase; $\blacksquare$, $\beta$-glucuronidase; $\bigblackdiamond$, $\alpha$-mannosidase; $\blacklozenge$, tripeptidyl-peptidase I; $\blacktriangle$, $\beta$-mannosidase; $\blacklozenge$, $\alpha$-glucosidase; $\blacklozenge$, $\alpha$-glucosidase; $\bigtriangleup$, acid phosphatase). $C$, plots represent $\beta$-galactosidase ($\bigtriangleup$, wild type; $\blacklozenge$, NPC2 mutant), Man-6-P glycoproteins ($\blacksquare$, wild type; $\blacklozenge$, NPC2 mutant), and EPDR ($\bullet$, wild type; $\blacklozenge$, NPC2 mutant). Note that the density limits of the two gradients are different to optimize separation of normal and lipid-laden lysosomes from other organelles.
EPDR is a lysosomal protein, coupled with the observation that
unique protein, the definitive demonstration in this study that
and genetic studies are now required to fully understand this
(Fig. 3). However, although further biochemical, biophysical,
sion in brain and the fact that it is widely expressed in neurons
important neurological function, given its high levels of expres-
protocol that is secreted into the cerebrospinal fluid (34) that
have an important function, since it is highly conserved in all vertebrates, with the amino acid identity between human EPDR
function that may provide a useful clue. However, it is likely to
have an important function, since it is highly conserved in all vertebrates, with the amino acid identity between human EPDR
and mouse, chicken, and zebrafish proteins being 80, 75, and 60%, respectively.

Fish, but not mammals, have an ortholog of EPDR, ependy-
min, for which EPDR was initially named. Ependymin is a gly-
coprotein that is secreted into the cerebrospinal fluid (34) that
has been proposed to be involved in memory consolidation and learning in fish (16), but its biochemical function in these pro-
cesses is unknown. It is difficult to know to what extent these observations can be extrapolated for EPDR, especially given the relatively low degree of similarity between this protein and ependymin (e.g., the amino acid sequences of zebrafish EPDR
and zebrafish ependymin are only 23% identical and 43% simi-
lar). Conversely, it is quite possible that both mammalian and fish EPDR have a conserved lysosomal function that is unre-
lated to the function of ependymin.

One similarity between EPDR and ependymin may be an
important neurological function, given its high levels of expres-
sion in brain and the fact that it is widely expressed in neurons
(Fig. 3). However, although further biochemical, biophysical,
and genetic studies are now required to fully understand this unique protein, the definitive demonstration in this study that
EPDR is a lysosomal protein, coupled with the observation that
>90% of soluble lysosomal proteins are hydrolases, raises the
intriguing possibility that EPDR may represent a novel and as yet
undetermined hydrolytic enzyme.

Key to the demonstration that EPDR is a lysosomal protein has been the development of a method here to verify the localization of brain lysosomal proteins. This new approach relies upon a mouse mutant (25) in which a lysosomal
defect alters the biophysical properties of this organelle. These mice lack NPC2, a small soluble lysoso-
mal glycoprotein (3) that is involved in cholesterol binding
(18) and sterol transport. The NPC2 and NPC1 proteins are
defective in the two complementation groups underlying Niemann-
Pick type C disease, a fatal neuro-
visceral disorder characterized by accumulation of cholesterol and other lipids within the lysosome. It
is worth noting that earlier experi-
ments directed at characterizing NPC1-deficient mice revealed a similar shift in density in liver, lung, and spleen lysosomes (35,
36), and thus the lipidosis-induced density shift method is likely to be applicable to a wide variety of cell types and tissues.

It is important to consider the possibility that the accumu-
lation of lipids within the lysosome would disrupt normal

targeting pathways and result in the transport of some lysos-
omal proteins to alternate destinations. However, this appears unlikely, given that we have measured a wide spec-
trum of lysosomal activities, and all demonstrate the same density shift in the absence of NPC2 (Fig. 8B). In addition, as
a general demonstration that the loss of NPC2 does not appear to perturb lysosomal targeting, the distribution of
Man-6-P glycoproteins, which are a global lysosomal marker
in brain (26), also shifts in accordance with the individual
activities (Fig. 8A). Conversely, the lysosomal lipidosis could
result in mistargeting of nonlysosomal proteins to the lysosome. For example, previous studies have indicated that
annexin II, a cytosolic and membrane-associated protein, redistributes to a cholesterol-laden late endosomal/lysoso-
mal compartment in NPC1-deficient cells (37). However,
annexin II is a cholesterol-binding protein; thus, there is a
clear molecular basis for mis-targeting, and this would be a
highly specialized circumstance that would apply to very
few, if any, other cellular proteins. Also, such mis-targeting
would not necessarily result in incorrect assignment of lysoso-
mal localization, since our approach measures distribu-
tion in both wild type and mutant mice, and bona fide lysoso-
mal proteins will co-distribute with known lysosomal markers in both situations. Therefore, like the experimen-

FIGURE 9. Immunolocalization of EPDR. C57BL/6 wild type Bouin’s fixed mouse brain 25-μm sections were
labeled with antisera against EPDR and fluorescein isothiocyanate-conjugated secondary antibodies (top) and
a biotinylated MPR derivative and Cy5-avidin (middle). In the superimposed images (Merged, bottom), the signal
from the anti-EPDR probe is in green, and the Man-6-P glycoprotein (M6PGP) probe is in red, with yellow
indicating co-localization.

soluble fraction after disruption of the membranes by freeze/
thawing and subsequent centrifugation, indicating that the
protein is soluble (data not shown). Sequence data base analyses do
not reveal similarities between EPDR and any protein of known
function that may provide a useful clue. However, it is likely to
have an important function, since it is highly conserved in all vertebrates, with the amino acid identity between human EPDR
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EPDR is a lysosomal protein, coupled with the observation that
>90% of soluble lysosomal proteins are hydrolases, raises the
tally induced shift elicited in Triton WR-1339 liver, the lysosomal lipidosis-induced density shift promises to be a powerful and specific tool for investigating the subcellular location of lysosomal proteins in the brain and other tissues.

In this study, we have utilized the approach based on the shift in buoyant density of brain lysosomes that is associated with NPC2 deficiency to demonstrate that EPDR is a lysosomal protein and have corroborated this using morphological approaches. This study has relied upon the generation of specific antibody reagents, an endeavor that, in general, can be time- and resource-consuming with no guarantee of success. In the future, we anticipate that the lysosomal lipidosis-induced density shift approach could be combined with appropriate quantitative mass spectrometric methods to measure protein distribution across the gradient, thus avoiding the need for antibodies. Such a combined approach should be widely and globally applicable to investigate novel Man-6-P glycoproteins that are lysosomal candidates. In addition, these methods may allow a thorough characterization of the lysosomal membrane proteome, a class of proteins that lack any specific characteristic (e.g. Man-6-P) that allows their selective purification. Quantitative analysis of the distribution of protein in shifted versus nonshifted organelles will be an important step in distinguishing true resident lysosomal proteins from the contaminants that inevitably are present in any subcellular fraction.

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