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Intracellular localization of p40, a protein identified in a preparation of lysosomal membranes

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Unlike lysosomal soluble proteins, few lysosomal membrane proteins have been identified. Rat liver lysosomes were purified by centrifugation on a Nycodenz density gradient. The most hydrophobic proteins were extracted from the lysosome membrane preparation and were identified by MS. We focused our attention on a protein of approx. 40 kDa, p40, which contains seven to ten putative transmembrane domains and four lysosomal consensus sorting motifs in its sequence. Knowing that preparations of lysosomes obtained by centrifugation always contain contaminant membranes, we combined biochemical and morphological methods to analyse the subcellular localization of p40. The results of subcellular fractionation of mouse liver homogenates validate the lysosomal residence of p40. In particular, a density shift of lysosomes induced by Triton WR-1339 similarly affected the distributions of p40 and β-galactosidase, a lysosomal marker protein. We confirmed by fluorescence microscopy on eukaryotic cells transfected with p40 or p40–GFP (green fluorescent protein) constructs that p40 is localized in lysosomes. A first molecular characterization of p40 in transfected Cos-7 cells revealed that it is an unglycosylated protein tightly associated with membranes. Taken together, our results strongly support the hypothesis that p40 is an authentic lysosomal membrane protein.

Key words: lysosome, lysosome membrane protein, proteomic analysis, subcellular localization, transmembrane protein.

INTRODUCTION

Lysosomes are membrane-bound organelles that are responsible for the degradation of intracellular material sequestered by autophagy and molecules internalized by endocytosis. More than 50 hydrolytic enzymes have been described in the lysosomal lumen [1]. These enzymes are transported from the TGN (*trans*-Golgi network) to early and/or late endosomes after M6P (mannose 6-phosphate) tagging followed by their recognition by MPRs (M6P receptors) [2]. These receptors interact with adaptor proteins to mediate the exit of the acid hydrolase–MPR complexes from the TGN in clathrin-coated vesicles [3]. Within endosomes, hydrolases dissociate from the MPRs in response to the acidic pH and are subsequently delivered to lysosomes, while MPRs are recycled back to the TGN [2]. The specific recognition of the M6P proteins by the MPRs provides a convenient means to identify lysosomal proteins [4]. This method was used to characterize the soluble lysosomal proteome [5–8]. It also led to the identification of proteins that are deficient in lysosomal diseases [9,10].

Relative to soluble enzymes, lysosomal membrane proteins remain poorly characterized. Among the best known lysosomal membrane proteins are LAMPs (lysosome-associated membrane proteins) and LIMPs (lysosomal integral membrane proteins), the H^+-ATPase proton pump, and some membrane transporters such as sialin and cystinosin [11]. The newly synthesized lysosomal membrane proteins can be delivered to late endosomes/lysosomes by two major pathways. The first route involves direct intracellular transport from the TGN to the endosomes. This particularly applies to LAMP-1 [12]. In contrast, the membrane precursor of LAP (lysosomal acid phosphatase) traffics primarily via the cell surface and recycles between endosomes and plasma membrane several times before being delivered to lysosomes [13]. The lysosomal targeting of transmembrane proteins depends on two types of sorting signals that are generally localized in their short cytoplasmic tails [14]. The tyrosine-based signals conform to a YXXΦ consensus motif, where tyrosine is the critical residue for the sorting process. The X residues are variable, but tend to be hydrophilic, whereas the Φ residue contains a bulky hydrophobic side chain. The di-leucine-based signals are of [DE]XXXL[LI] type where two consecutive leucine residues (or a leucine residue followed by an isoleucine residue) are preceded by an acidic residue at position -4 from the first leucine residue [14–16].

Low-molecular-mass molecules produced by intralysosomal hydrolysis are transferred from the lumen of the organelle to the cytosol by lysosomal membrane transporters [11]. Transport activities specific for amino acids [17–19], dipeptides [20], monosaccharides [21–23], and nucleosides [24] have been described in isolated lysosomes. The accumulation of digestion products in some lysosomal storage diseases led to the discovery of several lysosomal membrane transporters. For example, the autosomal recessive disorder cystinosis is characterized by an intralysosomal accumulation of cystine. The gene underlying cystinosis encodes a 367-amino-acid transporter called cystinosin [25]. Another transporter, sialin, has been identified and linked to two lysosomal sialic acid storage diseases, namely Salla disease and infantile sialic acid storage disorder [26]. Sialin is a 495-amino-acid protein with a consensus sequence that belongs to a family of ACSs (anion–cation symporters).

Considering the diversity of the products of intralysosomal hydrolysis, it is likely that many lysosomal membrane transporters are yet to be discovered. We decided to use a proteomic strategy applied previously to other intracellular compartments [27–29]

Abbreviations used: BHK-21, baby hamster kidney; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; LAMP, lysosome-associated membrane protein; LIMP, lysosomal integral membrane protein; M6P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; nano-LC–MS/MS, nano-liquid chromatography–tandem MS; ORF, open reading frame; PNGase F, peptide N-glycosidase F; TGN, *trans*-Golgi network.

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with the aim of finding new lysosomal membrane proteins. The membranes of rat liver lysosomes obtained by centrifugation were treated in order to be enriched in highly hydrophobic proteins and were analysed further by MS. This gave rise to the identification of a previously uncharacterized protein, which we have named p40. This candidate protein of the lysosomal membrane is made of 372 amino acids and contains four putative lysosomal-targeting motifs (three of the YXXΦ type and one of the di-leucine type). Our study provides the first biochemical characterization of p40 and demonstrates its residence in lysosomes.

MATERIALS AND METHODS

Preparation of rat liver lysosomal membranes

Lysosomes were purified from the livers of male Wistar rats as described previously [30]. The enriched lysosomal fraction, obtained by isopycnic centrifugation of a light mitochondrial fraction on a discontinuous Nycodenz density gradient [30], was washed in 25 ml of ice-cold isotonic sucrose and centrifuged at 20000 rev./min for 15 min in a Beckman Type 30 rotor. The pellet was resuspended in 500 μl of 0.25 M ice-cold sucrose and progressively mixed with 10 ml of ice-cold deionized water to induce the osmotic lysis of lysosomes. Lysosomal membranes were recovered by centrifugation at 130000 ***g*** for 15 min. This step was repeated twice. The final pellet was resuspended in ice-cold water.

Protein extraction, SDS/PAGE, in-gel trypsin digestion and mass spectrometric analysis

The most hydrophobic proteins were extracted from the lysosome membrane preparation using a 5:4 (v/v) chloroform/methanol mixture as described previously [27], except that 1 % (v/v) Triton X-100 was added to the extraction buffer. Proteins present in the organic phase were precipitated with cold acetone (− 20 °C). The protein pellet was re-suspended in 50 μl of SDS/PAGE buffer, and proteins were loaded on a SDS/5–12 % polyacrylamide gel for analysis. Protein migration was stopped just between the stacking and the separating gels so that the proteins were concentrated on a very thin band. Proteins were excised from the Coomassie Blue-stained gel as a single band. The gel band was destained and incubated with a reducing solution (25 mM NH_4HCO_3 containing 10 mM dithiothreitol) and then with an alkylating solution (25 mM NH_4HCO_3 containing 55 mM iodoacetamide). In-gel trypsin digestion was performed using trypsin (Promega, sequencing grade) at a 1:20 protease/protein ratio for 7 h at 37 °C. The dried gel-extracted tryptic peptides were resolubilized in a 95:4.9:0.1 (by vol.) water/acetonitrile/methanoic (formic) acid solution for nano-LC–MS/MS (nano-liquid chromatography–tandem MS) analysis as described previously [28]. MS/MS data were acquired and processed automatically using MassLynx 3.5 software (Waters). Database searching was carried out on an updated compilation of SwissProt and Trembl (http://www.expasy.org/sprot) using the MASCOT 1.7 program. Proteins identified with at least two peptides showing a score higher than 40 were validated automatically. Otherwise, the peptide sequence was checked and/or interpreted manually.

Cell culture and transfection

HeLa and Cos-7 cells were cultured in a humidified atmosphere with 5 % CO_2 in glucose-rich DMEM (Dulbecco's modified Eagle's medium) (Cambrex) containing 10 % foetal calf serum (Greiner Bio-one), 100 units/ml penicillin and 100 μg/ml streptomycin. BHK-21 (baby hamster kidney) cells were maintained in Glasgow minimal essential medium supplemented with 10 % tryptose phosphate broth (Sigma–Aldrich), 0.292 g/l L-glutamine and 5 % foetal calf serum. Transient transfections were performed with the FuGene transfection reagent (Roche Diagnostics) according to the manufacturer's instructions.

Antibody production

Rabbit polyclonal antisera were raised against an internal peptide of mouse p40 (SGNPRGVLEDALDAFC) situated in a hydrophilic loop of the predicted polypeptide (amino acids 239–254) and against a peptide corresponding to the 15 C-terminal amino acids of mouse p40 (ERLLGDSRTPINEAS). Peptides were synthesized by Eurogentec.

Metabolic labelling and immunoprecipitation

Cos-7 cells were transiently transfected with the cDNA of mouse p40 inserted in the pCMV-Sport 6 vector (Resource Centre of the German Human Genome Project RZPD, clone ID IMAG-p998I0410256Q3). At 48 h post-transfection, cells were pulse-labelled with 20–25 μCi/ml of Redivue™ Pro-mix L-[^{35}S]methionine (Amersham Biosciences) for 1 h at 37 °C followed by different chase periods ranging from 0 to 48 h. Cells were lysed in RIPA buffer (50 mM Tris/HCl, 120 mM NaCl, 1 % Triton X-100, 0.1 % SDS and 1 % sodium deoxycholate, pH 7.4) containing a cocktail of protease inhibitors. p40 protein was immunoprecipitated from cell lysates with the antibody raised against an internal peptide (see above) and with Protein A–Sepharose. Immunoprecipitated proteins were solubilized in Laemmli's buffer containing 100 mM dithiothreitol, resolved on SDS/12 % polyacrylamide gels and visualized by autoradiography. p40 bands were detected with the Cyclone™ Storage Phosphor system (Packard BioScience Company) and quantified with the Optiquant™ software. To determine the level of N-glycosylation of p40, the immunoprecipitates were treated with PNGase F (peptide N-glycosidase F) (New England Biolabs) according to the manufacturer's instructions.

Carbonate extraction

Post-nuclear supernatants of metabolically labelled transfected Cos-7 cells were centrifuged at 81000 ***g*** for 40 min to sediment all the membrane structures (MLP fraction). Membranes were then disrupted for 30 min on ice in 0.1 % saponin prepared in a 10 mM Hepes/4 mM EDTA buffer, pH 7. After centrifugation at 100000 ***g*** for 30 min, the membrane pellet was submitted to a 0.1 M sodium carbonate extraction at pH 11.5 for 30 min on ice [31].

Construction of GFP (green fluorescent protein)-chimaeric proteins

Two fusion constructs were generated: GFP was cloned N-terminally (GFP–p40) or C-terminally (p40–GFP) to mouse p40. The full-length p40 ORF (open reading frame) was amplified by PCR with primers containing a SalI and a BamHI restriction sites and inserted into the SalI/BamHI-digested pEGFP-N1 plasmid (Clontech) to obtain the p40–GFP construct. The p40–GFP sequence was then transferred into pCMV-Sport 6 vector by using SalI and NotI. For the GFP–p40 construct, the GFP sequence from pEGFP-C1 was amplified and flanked by EcoRI and XhoI sites by PCR. The p40 ORF was amplified with primers containing an XhoI and a PvuII restriction site. Restriction products were introduced into EcoRI/PvuII-digested pCMV-Sport 6.

Texas Red–dextran endocytosis and lysosome labelling

Transiently transfected HeLa cells were grown on glass coverslips and incubated 96 h post-transfection for 30 min in serum-free DMEM at 37 °C followed by incubation with Texas Red–dextran (10 000 Da molecular mass; Molecular Probes) for different periods of time. To label early endosomes, cells were fed for 10 min with medium containing 5 mg/ml Texas Red–dextran. Late endosomes and some lysosomes were labelled by a 2 h pulse with 2 mg/ml Texas Red–dextran, followed by a 15 min-chase in dextran-free medium. Late endosomes and lysosomes were detected by a 6 h pulse with 1.5 mg/ml dextran followed by a 2 h chase. Afterwards, cells were washed with PBS, fixed with ice-cold 4 % (w/v) paraformaldehyde and mounted with Mowiol (Sigma–Aldrich). Fluorescence was observed with a Leica DMIRBE confocal microscope (Leica Microsystems). Double-label confocal images were acquired sequentially.

Immunofluorescence

BHK-21 cells were transfected with mouse p40 cDNA. After 48 h, they were fixed and permeabilized with methanol/acetone (80:20, v/v) for 20 min at −20 °C and then with 0.05 % saponin for 10 min at room temperature (20–25 °C). After blocking of the non-specific binding sites with 1 % BSA in PBS, cells were incubated for 2 h with the rabbit p40 antiserum raised against a C-terminal peptide of p40 and then with a mouse anti-(hamster LAMP-1) monoclonal antibody (4A1), generously provided by Dr J. Gruenberg (Department of Biochemistry, University of Geneva, Geneva, Switzerland). Detection of primary antibodies was carried out using an Alexa Fluor® 488-conjugated goat anti-rabbit antibody and an Alexa Fluor® 568-conjugated goat anti-mouse antibody (Molecular Probes). Coverslips were mounted in Mowiol, and fluorescence was observed using a Leica confocal microscope. In HeLa cells transfected with p40–GFP or GFP–p40, human LAMP-1 was detected with a monoclonal anti-LAMP-1 antibody available commercially (H4A3) (Developmental Studies Hybridoma Bank, Iowa City, IA, U.S.A.).

Subcellular fractionation of mouse liver and detection of endogenous p40

Livers from female NMRI mice were fractionated by differential centrifugation as described previously by de Duve et al. [32]. The mitochondrial ML fractions of control mice and Triton WR-1339 (Tyloxapol; Sigma–Aldrich)-injected mice (85 mg/100 g of body mass) [33] were layered on linear sucrose density gradients extending from 1.09 g/ml to 1.26 g/ml. Isopycnic centrifugation was performed at 39 000 rev./min for 150 min in a Beckman SW55.Ti rotor. Fractions were collected by slicing the tube, and the densities were measured by refractometry. β-Galactosidase was assayed in the different fractions with 4-methylumbelliferyl β-D-galactoside (Sigma–Aldrich) as a substrate [34]. Alkaline phosphodiesterase was assayed as described previously [35]. p40 was detected by Western blotting using the rabbit antiserum raised against the C-terminal peptide of p40. Briefly, proteins were resolved by SDS/12 % PAGE under reducing conditions and were electrotransferred on to PVDF membranes (Hybond-P; Amersham Biosciences). Then membranes were incubated for 10 h with the C-terminal p40 antiserum diluted in PBS containing 0.1 % (v/v) Tween 20 and 5 % (w/v) dried skimmed milk, washed extensively, and incubated for 45 min with a horseradish-peroxidase-conjugated goat anti-rabbit antibody (DakoCytomation). Signal detection was performed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Science).

RESULTS

Identification of p40 by MS

Rat liver lysosomes were purified by isopycnic centrifugation on a Nycodenz density gradient [30]. This method allows the recovery of approx. 10 % of all liver lysosomes, purified up to 80-fold relative to the homogenate. This highly enriched lysosomal fraction was subjected to osmotic lysis in water. Pelleted membranes were used for extraction of the most hydrophobic proteins with methanol/chloroform and analysed by MS. Several lysosomal membrane proteins were identified, such as LAMP-1 and the vacuolar ATPase 16 kDa proteolipid. Among all the MS/MS spectra, one MASCOT-unassigned MS/MS spectrum was sequenced *de novo*. The following amino acid sequence was identified unambiguously: GVLEDALDAFCQVGR. This sequence is 100 % identical with an internal sequence corresponding to the RIKEN cDNA 4930471M23 gene (4930471M23Rik). This mouse sequence encodes a hypothetical 372-amino-acid-long protein with a theoretical molecular mass of approx. 40 kDa. This protein was therefore named p40. It contains three potential N-glycosylation sites and four potential lysosomal membrane targeting signals: three tyrosine-based motifs (YXXΦ) and one di-leucine motif (EXXXLL) (Figure 1A). p40 is a putative integral membrane protein with several transmembrane domains. Their number varies from seven to ten depending on the prediction software.

Characterization of p40 in transfected cells

Cos-7 cells were transfected with the cDNA coding for mouse p40. After metabolic labelling with [^{35}S]methionine, p40 was immunoprecipitated with a polyclonal antiserum raised against an internal peptide. As shown in Figure 1(B), a single band with a molecular mass of approx. 33 kDa was observed using SDS/PAGE. This band is specific for p40 since (i) a polypeptide with the same molecular mass was observed after immunoprecipitation with another antiserum raised against a peptide situated at the C-terminal end of the protein (results not shown), and (ii) the 33 kDa band did not appear in mock-transfected cells. After treatment with PNGase F, the immunoprecipitated protein kept the same apparent molecular mass, while the apparent molecular mass of a control glycoprotein, cathepsin D, was reduced as a result of the cleavage of its oligosaccharide chains (Figure 1C). These results suggest that p40 is unglycosylated. The turnover of p40 was studied by pulse–chase experiments (Figure 1D). Based on the quantification of the radioactivity associated with p40, its half-life was estimated to be approx. 10 h.

Metabolically labelled cells were fractionated into a soluble fraction (S) and a fraction containing the membrane structures of a post-nuclear supernatant (MLP). After immunoprecipitation, p40 was exclusively recovered in the MLP fraction (Figure 1E). This fraction was subjected to a carbonate extraction at pH 11.5 to separate soluble and peripheral membrane proteins from those tightly associated with membranes [31]. The result of the immunoprecipitation showed that p40 remained completely associated with the membrane pellet (Figure 1F), suggesting a tight link, such as the presence of at least one transmembrane domain.

Subcellular localization of wild-type p40 and p40–GFP chimaeric proteins in transfected cells

The discovery of a previously unidentified protein in a fraction of lysosomes purified by subcellular fractionation does not guarantee its residence in lysosomes. Indeed, the best preparations of so-called purified lysosomes still contain various contaminant organelles [30]. It was therefore of the utmost importance to study the

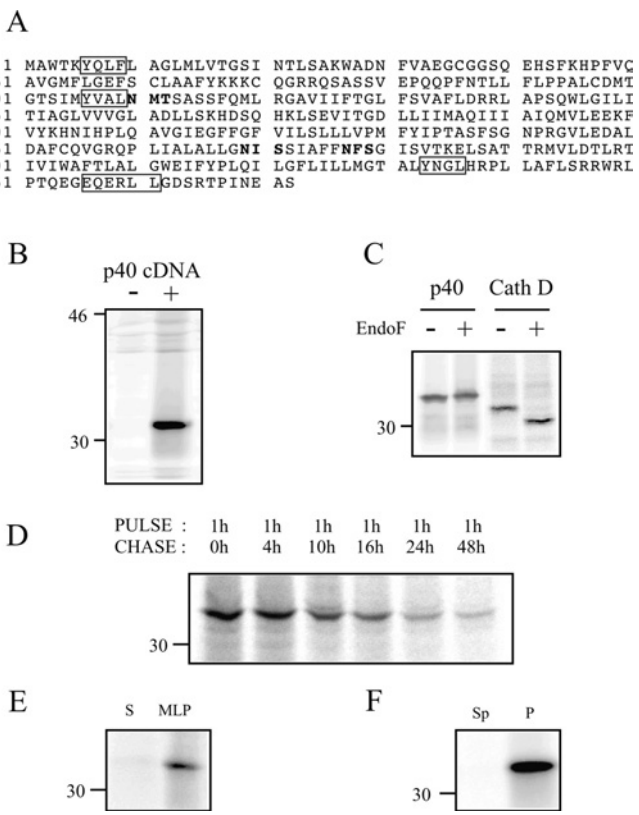

Figure 1 Characterization of p40

(**A**) p40 is a protein of 372 amino acids containing three potential N-glycosylation sites (NXS/T, indicated in bold) and four potential lysosomal membrane targeting signals (three YXXΦ motifs and a di-leucine motif, all boxed). (**B**) Non-transfected and transfected Cos-7 cells were labelled for 4 h with [^{35}S]methionine, lysed and subjected to immunoprecipitation with a rabbit p40 polyclonal antiserum. After separation using SDS/PAGE, p40 was visualized by autoradiography. (**C**) At 48 h post-transfection, p40 proteins were immunoprecipitated and subjected to PNGase F (endo F) treatment. Cathepsin D (Cath D) was used as a control glycoprotein. (**D**) At 48 h post-transfection, cells were pulsed for 1 h with [^{35}S]methionine and chased for the indicated time period. p40 was immunoprecipitated and separated by SDS/12 % PAGE. Signals were detected by autoradiography and quantified with the Cyclone™ Phosphorimager software. (**E**) At 48 h post-transfection, cells were labelled for 4 h, and a post-nuclear supernatant was prepared and subjected to centrifugation at 81 000 ***g*** for 40 min in a Beckman 50Ti rotor to separate the membrane structures (MLP) from the cytosolic fraction (S). p40 was immunoprecipitated in these fractions, and samples were resolved by SDS/12 % PAGE. (**F**) The MLP fraction was submitted to a carbonate extraction at pH 11.5 and centrifuged at 100 000 ***g*** for 30 min to obtain a supernatant (Sp) and a pellet (P). Results of p40 immunoprecipitation are presented. Molecular masses are indicated in kDa to the left of the gels.

subcellular localization of p40. To achieve that goal, we first engineered two GFP-chimaeric proteins where GFP was fused either N-terminally (GFP–p40) or C-terminally (p40–GFP) to mouse p40, and we expressed these constructs into HeLa cells. At 96 h post-transfection, the endocytic compartments of these cells were visualized by incubation with Texas Red-conjugated dextran in a kinetic study. Dextran is a polysaccharide of high molecular mass that enters the cells by fluid-phase endocytosis and accumulates in lysosomes owing to its resistance to intralysosomal hydrolysis. As shown in Figure 2, p40–GFP proteins displayed a punctate labelling throughout the cytoplasm with a predominance in the perinuclear region. No signal was detected at the plasma membrane or in the nucleus. After 10 min of incubation with Texas Red–dextran, no superimposition between this marker and the p40–GFP staining was observed (Figure 2A), indicating that p40 does not reside in the early endosomes [36]. This was corroborated by the absence of co-localization between p40–GFP and Texas Red-conjugated transferrin, a marker of early and recycling endosomes (results not shown). When cells were incubated for 2 h in the presence of Texas Red–dextran to allow its delivery to late endosomes and partially to lysosomes, followed by a 15 min chase in the absence of the fluorescent marker to remove it from early endosomes [36], some co-localization of Texas Red–dextran with the p40–GFP fluorescence could be seen (Figure 2B). The co-localization was maximal when the bulk of Texas Red–dextran had accumulated in lysosomes, i.e. after a 6 h pulse and a 2 h chase [37] (Figure 2C). Similar results were obtained with GFP–p40 (results not shown).

To analyse further the intracellular distribution of p40 in transfected HeLa cells, we carried out immunofluorescence co-localization experiments. Extensive co-localization was observed between both GFP-conjugated p40 proteins and either LAMP-1 (Figures 3A–3B) or LAMP-2 (results not shown), two late endosomal/lysosomal markers [11]. The distribution of wild-type p40 was also investigated in transfected BHK-21 cells using a rabbit p40 antiserum. The same punctate cytoplasmic labelling with a predominance in the perinuclear region (Figure 3D) was observed. This labelling is specific since it did not appear in non-transfected cells (Figure 3C). Again, there was an extensive overlap of p40 and LAMP-1 labelling, suggesting that p40 resides in lysosomes.

Intracellular distribution of endogenous p40 in mouse liver

Next, we investigated the intracellular distribution of endogenous p40 in mouse liver using biochemical approaches. First, p40 was detected by immunoblotting on fractions obtained by differential centrifugation of a mouse liver homogenate [32]. As shown in Figure 4(A), the vast majority of endogenous p40 was recovered in the heavy (M) and light (L) mitochondrial fractions that contain the bulk of lysosomes, as judged by the β-galactosidase activity. The amount of p40 in the microsomal fraction (P) was low. Histograms representing the relative specific signal intensity of p40 (i.e. the percentage of the signal intensity assigned to p40 to the percentage of proteins in the corresponding fraction) plotted against the percentage of proteins in the different fractions displayed a typically lysosomal profile, characterized by a peak in the L fraction (Figure 4A), as observed for β-galactosidase (Figure 4C) and for the late endosomal/lysosomal marker LAMP-1 (Figure 4B). In contrast, the pattern of distribution of alkaline phosphodiesterase, a plasma membrane marker, was quite different, with the highest specific activities in N and P fractions (Figure 4D). An ML fraction was submitted to isopycnic centrifugation on a linear sucrose density gradient (Figure 5A). Lysosomes, represented by the β-galactosidase activity, were recovered in the high-density region of the gradient, while the plasma membrane marker, alkaline phosphodiesterase, was mainly detected in more buoyant fractions (Figure 5A). The distribution pattern of p40 was similar to that of β-galactosidase (Figure 5A). Semi-quantitative analysis of two independent experiments indicated that the p40 median equilibrium density was 1.20 g/ml and that of β-galactosidase was 1.21 g/ml. Nevertheless, the co-distribution of a protein with a lysosomal marker does not provide definite proof of its lysosomal localization. Intravenously injected Triton WR-1339 induces a striking reduction in the density of liver lysosomes [33]. Therefore, if the distribution of a liver protein follows the shift of lysosomal hydrolytic activities after Triton WR-1339 injection, this provides a strong argument in favour of its lysosomal localization. As shown in Figure 5(B), after Triton WR-1339 injection, the β-galactosidase distribution exhibited the expected shift towards lower densities and p40 distribution shifted similarly towards the upper fractions of the sucrose density gradient.

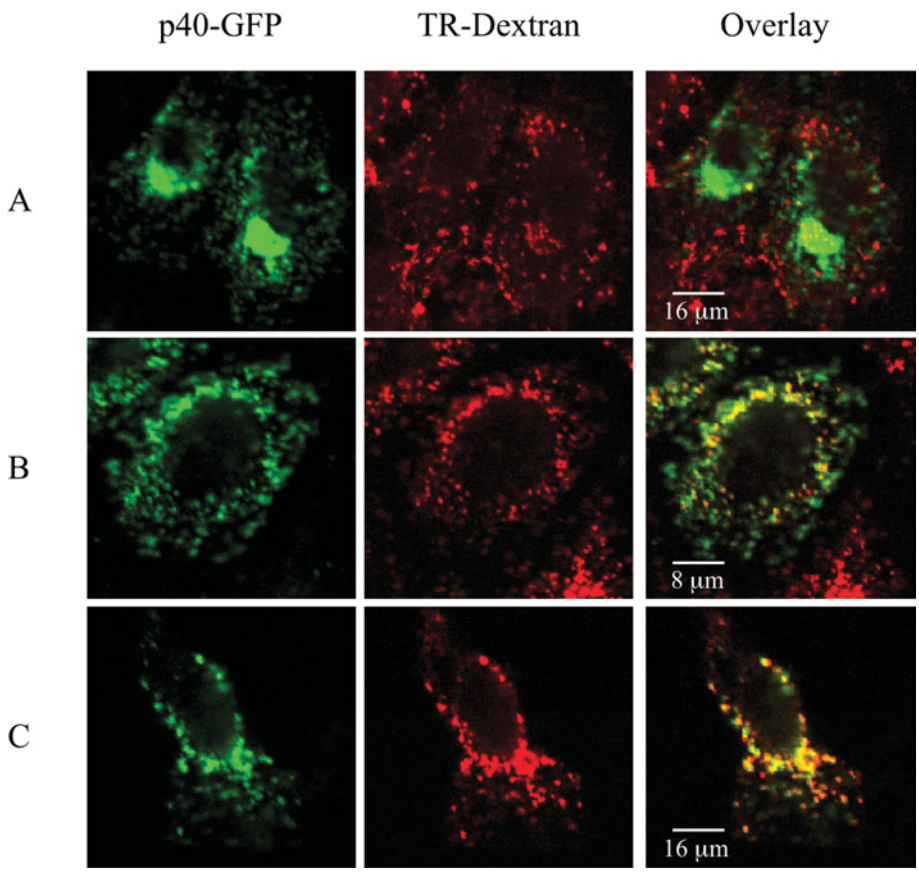

Figure 2 Subcellular localization of p40–GFP in transfected HeLa cells

At 96 h post-transfection with p40–GFP, HeLa cells were incubated with Texas Red–dextran (TR-Dextran), fixed with 4 % paraformaldehyde and processed for confocal microscopy. (**A**) Cells were pulsed for 10 min with Texas Red–dextran to label early endosomes. (**B**) Cells were pulsed for 2 h with Texas Red–dextran and chased for 15 min in dextran-free medium to label mostly late endosomes. (**C**) Cells were pulsed for 6 h with Texas Red–dextran and chased for 2 h in marker-free medium to label mostly lysosomes.

DISCUSSION

A proteomic analysis of highly enriched lysosomal membrane fractions reveals the presence of p40, a previously uncharacterized protein

The proteome of the lysosomal membrane is far from elucidated. Our study concerns a previously uncharacterized protein (p40) identified by MS from membranes of normal rat liver lysosomes purified by isopycnic centrifugation on a discontinuous Nycodenz density gradient [30]. p40 was found through the nano-LC–MS/MS identification of a single 15-mer peptide (GVLEDALDAFCQVGR) recovered in a population of hydrophobic proteins extracted from these membranes. Another way to prepare lysosomes is to rely on the density shift induced by an injection of Triton WR-1339 (Tyloxapol) into rats or mice [38]. After endocytosis by the liver cells, this non-haemolytic detergent induces a specific reduction in the equilibrium density of lysosomes recovered after isopycnic centrifugation in a sucrose density gradient. These 'tritosomes' are *bona fide* lysosomes. This method was recently applied by Bagshaw et al. [39] to establish a list of more than 200 putative lysosomal membrane proteins. Based on the identification of the peptide (R)QSDSSVEPR(Q), p40 was suggested to be part of the tritosomal membrane [39]. In the present study, p40 was also detected in tritosomes (see Figure 5). The discontinuous Nycodenz density-gradient approach and the tritosome approach both give rise to lysosomes that are purified 50–100-fold over the homogenate [30,33,38]. With such modest purification factors, a validation of the subcellular localization of any candidate lysosomal protein is crucial.

p40 is a highly hydrophobic and unglycosylated membrane protein

In transfected Cos-7 cells, p40 protein displayed an apparent molecular mass of approx. 33 kDa, while the theoretically expected value is 41 kDa. This difference could be explained by the high hydrophobicity of the protein. Computational analysis revealed that p40 is probably an integral membrane protein, but the exact number of transmembrane domains varies from seven to ten depending on the software used. As expected from this predicted topology, p40 was recovered in the membrane pellet after high-speed centrifugation of a post-nuclear supernatant prepared from transfected Cos cells. Carbonate extraction of this pellet confirmed the tight association of p40 with membranes. Although p40 contains three potential N-glycosylation sites, the protein seems to be unglycosylated, as suggested by its resistance to PNGase F treatment. This observation is not surprising since the three potential sites are predicted to be located either in a transmembrane domain (Asn^{269}-Ile-Ser and Asn^{277}-Phe-Ser) or very close to it (Asn^{110}-Met-Thr). It was quite unlikely that the Asn^{110}-Met-Thr consensus site could be recognized by the oligosaccharyltransferase since a minimum of 12–15 residues is required between an N-glycosylation consensus site and a transmembrane

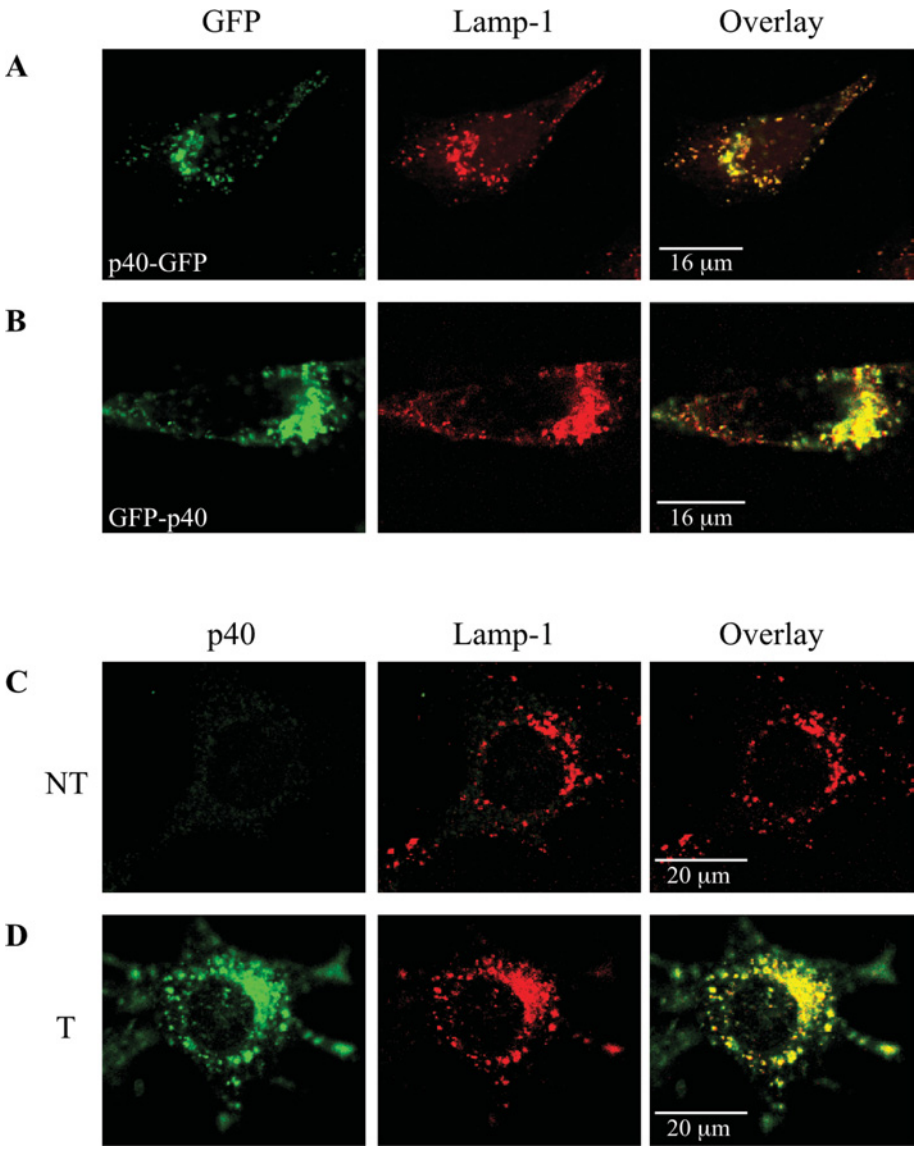

Figure 3 Co-localization of p40, p40–GFP and GFP–p40 with LAMP-1

HeLa cells were transfected with the p40–GFP (**A**) or the GFP–p40 (**B**) construct. At 96 h post-transfection, cells were fixed and processed for GFP (green) and LAMP-1 (red) detections using confocal microscopy. BHK-21 cells were transiently transfected (T) with the wild-type p40 cDNA (**D**). p40 (green) and LAMP-1 (red) were detected as described in the Materials and methods section. Non-transfected (NT) BHK-21 cells are shown in (**C**).

domain to achieve efficient N-glycosylation [40]. The lack of glycosylation is unexpected for a lysosomal membrane protein. Indeed, it has been demonstrated that the oligosaccharide chains can protect lysosomal membrane proteins against proteolysis [41,42]. However, the estimated half-life of p40, approx. 10 h, is consistent with the turnover of lysosomal membrane glycoproteins such as LIMP-1, -2 and -3, and LAMP-1, with half-lives of 8, 20, 10 and up to 15 h respectively [41,43]. It should be stressed that other known lysosomal proteins, such as the lysosomal sialic acid transporter, sialin, and the lysosomal apyrase-like protein-70, seem to be unglycosylated [44,45]. Both sialin and p40 possess a high number of predicted transmembrane domains and relatively short loops exposed to the lysosomal lumen. Therefore the putative membrane topology of these unglycosylated proteins could afford them an efficient protection against proteolysis.

The lysosomal localization of p40 is supported by biochemical and morphological evidence

Our subcellular fractionation studies provide strong evidence that, in mouse liver, endogenous p40 resides within lysosomes. After differential centrifugation of the homogenate, the distribution pattern of p40 is similar to that of the lysosomal marker enzyme β-galactosidase. Both proteins were mainly recovered in the M and L fractions and gave rise to similar enrichment in the lysosomal fraction L. A co-distribution of p40 and β-galactosidase was observed after isopycnic centrifugation of an ML fraction in a sucrose density gradient. Even more significantly, injection of Triton WR-1339 into mice induced the same density shift for p40 and for β-galactosidase in sucrose density gradients.

We also analysed the intracellular localization of p40 in cultured cells by confocal microscopy. Two GFP-constructs with GFP

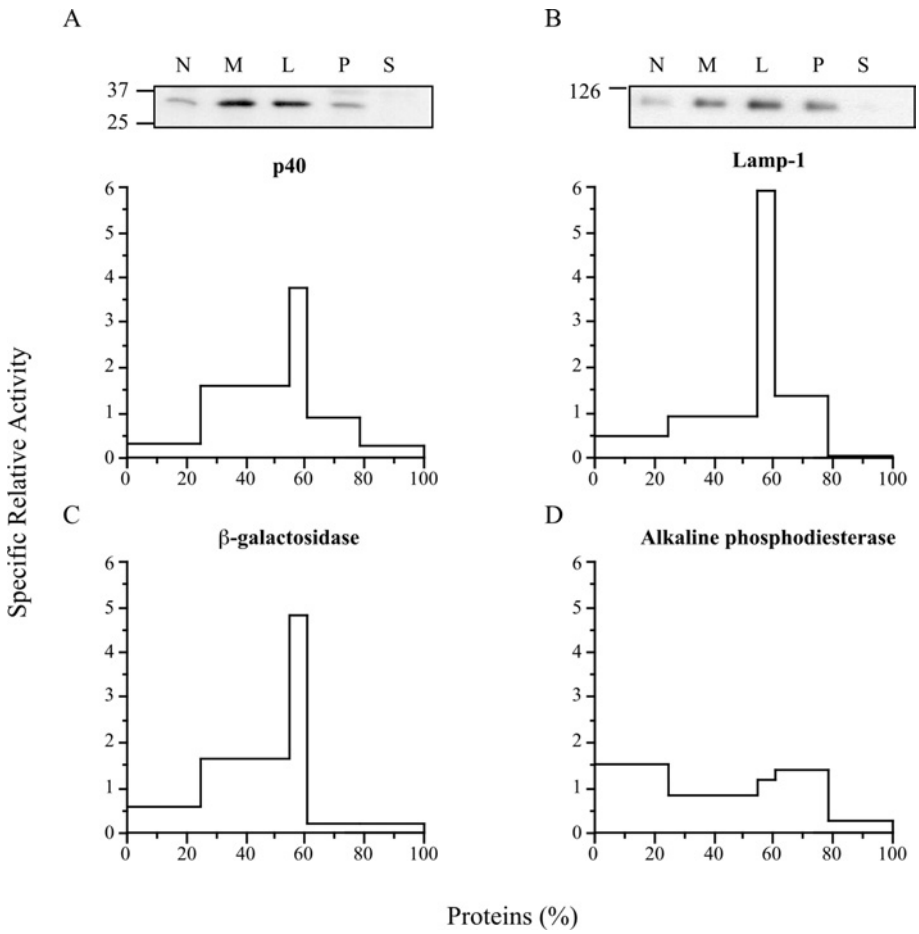

Figure 4 Distribution of endogenous p40, LAMP-1, β-galactosidase and alkaline phosphodiesterase after differential centrifugation of mouse liver homogenate

p40 (**A**) and LAMP-1 (**B**) were detected by Western blotting using the same amount of tissue homogenate for each fraction obtained by differential centrifugation, according to the method of de Duve et al. [32]. Molecular-mass sizes are given in kDa to the left of the gels. Quantitative analysis of their distribution was performed with the NIH (National Institutes of Health) Image software. Enzymatic activities of β-galactosidase (**C**), a marker of lysosomes, and of alkaline phosphodiesterase (**D**), a marker of plasma membranes, were measured as described in the Materials and methods section. For all graphs, the ordinate is the relative specific activity and the abscissa is the relative protein content plotted cumulatively from left to right.

either at the N-terminal or the C-terminal end of p40 were engineered. These GFP-conjugated p40 proteins expressed in HeLa cells exhibited a punctate cytoplasmic labelling profile with a predominance in the perinuclear region and co-localization with the late endosomal/lysosomal marker LAMP-1. Similar results were obtained with an immunostaining of p40 expressed in BHK-21 cells. Furthermore, GFP-chimaeric proteins co-localized with endocytosed Texas Red–dextran, provided this probe was chased into the latest compartments of the endocytic pathway.

Taken together, the results of our biochemical and morphological analyses of the intracellular localization of p40 strongly support its residence in lysosomes.

p40 might be a lysosomal membrane transporter

So far, the function of p40 is unknown. Northern blot analysis revealed that p40 transcript is widely expressed in human tissues and is most abundant in the heart, the skeletal muscle and the liver (see the Supplemental Data at http://www.BiochemJ.org/bj/395/bj3950039add.htm). When the mouse p40 sequence (TrEMBL accession number Q8VE96) is introduced into the NCBI Conserved Domain Search program [46] (without filter for low complexity regions), a domain of low and partial homology (E value $= 10^{-5}$) is found between the peptide region extending from Gln^{83} to Gln^{170} and Pfam 04142.6, which is a conserved domain in several members of the nucleotide-sugar transporter family (SLC35). These proteins are antiporters that transport nucleotide sugars from the cytosol into the lumen of the Golgi and/or the endoplasmic reticulum in exchange for the corresponding nucleoside monophosphates [47]. Hydrophobicity analyses of the nucleotide-sugar transporters cloned to date suggest between six and ten transmembrane domains [48,49]. Using an epitope-insertion approach, it has been shown that the CMP-sialic acid transporter contains ten transmembrane domains and that its N- and C-termini are oriented towards the cytosol [49]. Our observations suggest the same orientation for p40 since no difference in fluorescence intensities could be detected when comparing p40 with either its C- or its N-terminus fused to GFP, while residence of the GFP moiety in the lysosomal lumen could have induced a significant quenching of the fluorescence [50].

Based on its subcellular localization, its predicted multispanning topology, and the presence of a domain exhibiting a sequence homology with some solute carriers, it is tempting to suggest

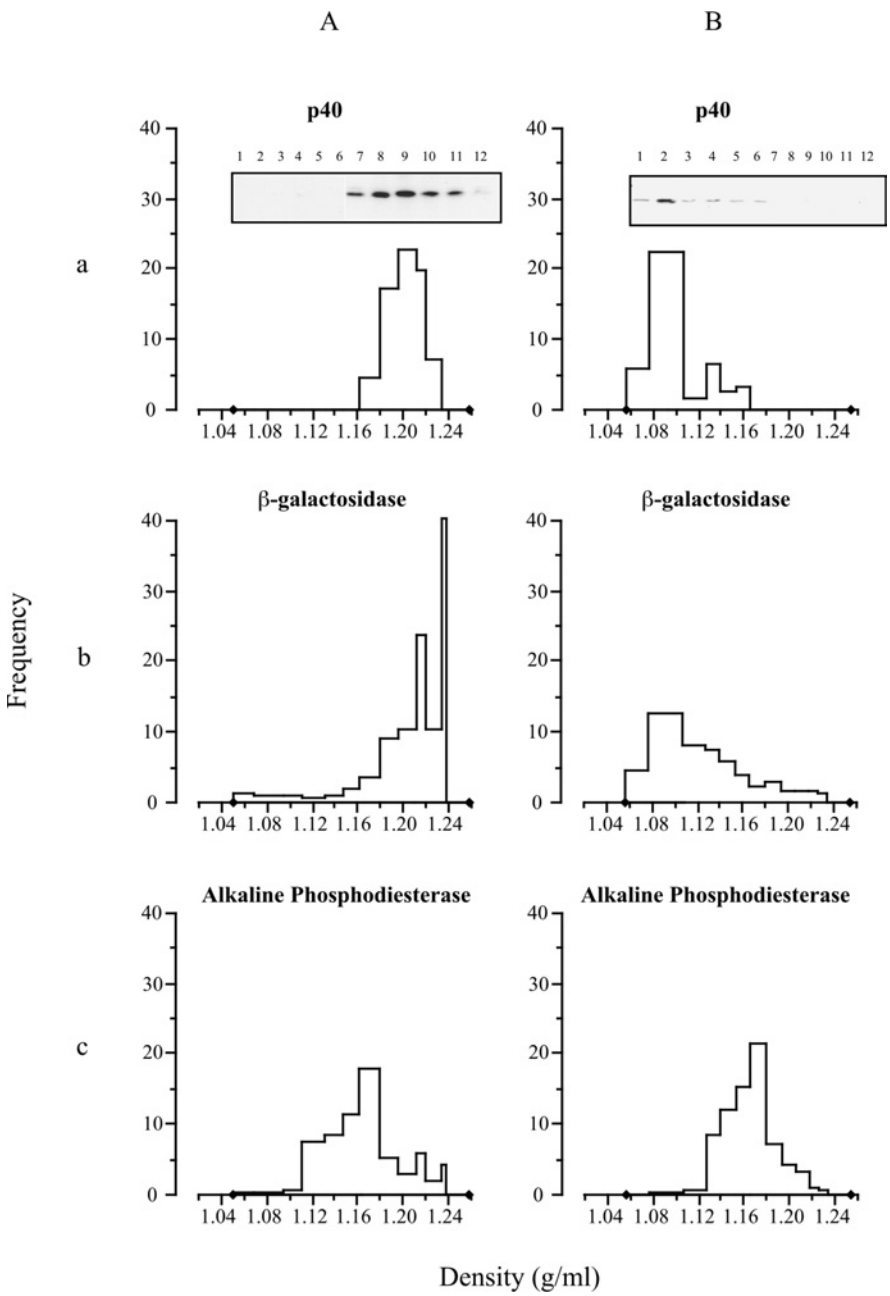

Figure 5 Distribution of endogenous p40, β-galactosidase and alkaline phosphodiesterase after isopycnic centrifugation on a linear sucrose density gradient

ML fractions prepared from liver homogenates of either normal mice (**A**) or Triton WR-1339-injected mice (**B**) were layered on top of a sucrose density gradient and centrifuged at 39 000 rev./min for 150 min in a Beckman SW55.Ti rotor. The distributions of p40 (a), of β-galactosidase (b) and of alkaline phosphodiesterase (c) among the 12 collected fractions were determined by Western blotting or by enzymatic assays. Histograms represent the frequency (percentage of activity divided by the increment of density) as a function of the density.

that p40 corresponds to some previously unidentified lysosomal membrane transporter.

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