online data

Biochemical characterization and lysosomal localization of the mannose-6-phosphate protein p76 (hypothetical protein LOC196463)

Anaïs G. JENSEN*, Magali CHEMALI*, Agnès CHAPEL*, Sylvie KIEFFER-JAQUINOD*, Michel JADOT†, Jérôme GARIN* and Agnès JOURNET*[1]

*Commissariat à l'Energie Atomique, Direction des Sciences du Vivant, Département Dynamique et Réponse Cellulaire, Laboratoire de Chimie des Protéines, Grenoble F-38054, France; INSERM, ERM 0201, Grenoble F-38054, France; Université Joseph Fourier, Grenoble F-38054, France, and †Unité de Recherche en Physiologie Moléculaire, Laboratoire de Chimie Physiologique, Facultés Universitaires Notre-Dame de la Paix, B-5000 Namur, Belgium

Most soluble lysosomal proteins carry Man6P (mannose 6-phosphate), a specific carbohydrate marker that enables their binding to cellular MPRs (Man6P receptors) and their subsequent targeting towards the lysosome. This characteristic was exploited to identify novel soluble lysosomal proteins by proteomic analysis of Man6P proteins purified from a human cell line. Among the proteins identified during the course of the latter study [Journet, Chapel, Kieffer, Roux and Garin (2002) Proteomics, **2**, 1026–1040], some had not been previously described as lysosomal proteins. We focused on a protein detected at 76 kDa by SDS/PAGE. We named this protein 'p76' and it appeared later in the NCBI protein database as the 'hypothetical protein LOC196463'. In the present paper, we describe the identification of p76 by MS and we analyse several of its biochemical characteristics. The presence of Man6P sugars was confirmed by an MPR overlay experiment, which showed the direct and Man6P-dependent interaction between p76 and the MPR. The presence of six *N*-glycosylation sites was validated by progressive peptide-*N*-glycosidase F deglycosylation. Experiments using N- and C-termini directed anti-p76 antibodies provided insights into p76 maturation. Most importantly, we were able to demonstrate the lysosomal localization of this protein, which was initially suggested by its Man6P tags, by both immunofluorescence and sub-cellular fractionation of mouse liver homogenates.

Key words: lysosomal localization, mannose 6-phosphate, post-translational modification, proteomics, sub-cellular fractionation.

INTRODUCTION

Lysosomes are membrane delimited intracellular organelles with an intraluminal acidic pH, which, for a long time, were considered simply as the main digestive compartment of the cell. The degradation of macromolecules inside lysosomes is performed through the action of more than 50 hydrolases and associated accessory proteins. About 40 human disorders are related to mutations in genes encoding lysosomal proteins, and named lysosomal storage diseases, according to their cellular effects [1]. Although the precise mechanisms have not yet been elucidated completely, lysosomes have been shown to be involved in many other cellular processes, such as antigen processing and presentation [2]. Moreover, lysosomes could be involved in programmed cell death [3].

During their biosynthesis, soluble lysosomal proteins acquire, on *N*-linked oligosaccharides, a Man6P (mannose 6-phosphate) marker, which is recognised by two MPRs (Man6P receptors), the small cation-dependent MPR and the large cation-independent MPR [4]. The MPRs sort soluble lysosomal proteins towards the endosomes, where they release their cargo due to the low pH of the organelles. Finally, the soluble lysosomal proteins reach lysosomes where their Man6P tag is dephosphorylated [5].

Over the last decade, proteomic analyses aimed at identifying soluble lysosomal proteins have been performed on Man6P proteins that have been purified by affinity for immobilized MPRs. Several of these studies [6–9] were carried out on proteins purified from cell secretions. Body fluids such as urine [10] or plasma [11] were also used as a source of extracellular Man6P proteins. Studies dealing with intracellular Man6P proteins were performed on a few transformed cell lines [12], but mainly on brain homogenates [13–15]. Indeed, as lysosomal Man6P protein dephosphorylation is less efficient in the brain compared with other organs [13,16], the brain is the most appropriate organ for purification of mature lysosomal proteins through their Man6P sugar.

Our own proteomic studies of Man6P proteins purified from human cell secretions [6,7] led us to the discovery of a novel protein that we named 'p76' because of its apparent molecular mass. In the present paper, we describe its identification as the hypothetical protein LOC196463, its biochemical characterization and, finally, we demonstrate effectively its lysosomal localization.

EXPERIMENTAL

MS analysis and N-terminal protein sequence determination

Proteins were identified by MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) peptide fingerprinting or LC-MS/MS analysis as described previously [7,17]. For N-terminal sequence analysis, Edman degradation of electroblotted proteins [6] was performed by the Laboratoire d'Enzymologie Moléculaire (Institut de Biologie Structurale, Grenoble, France) using an Applied Biosystems gas-phase sequencer (model 492) and an Applied Biosystems Model 140C

Abbreviations used: 293-EBNA, human embryonic kidney 293 cells stably transformed with Epstein–Barr virus nuclear antigen; 2DE, 2-dimensional electrophoresis; CEA, Commissariat à l'Energie Atomique; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hp76, human p76; HRP, horseradish peroxidase; LAMP1, lysosomal-associated membrane protein 1; LC-MS/MS, LC-tandem MS; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; Man6P, mannose 6-phosphate; mp76, mouse p76; MPR, Man6P receptor; PNGase F, peptide-*N*-glycosidase F; sCI-MPR, soluble cation-independent mannose-6-phosphate receptor; TBST, Tris buffered saline containing 0.1 % Tween-20; UTR, untranslated region.

[1] To whom correspondence should be addressed (email agnes.journet@cea.fr).

HPLC system with the model 610A data analysis software package (version 2.1).

Bioinformatics

Tools found on the Expasy server (www.expasy.org) were used for primary sequence analyses (Blast, Compute pI/Mw, SignalP 3.0 and NetNGlyc 1.0). Genomic, transcriptomic and protein data were obtained from the Ensembl (www.ensembl.org/index.html), Symatlas (www.symatlas.gnf.org) and NCBI (www.ncbi.nlm.nih.gov) web sites.

Molecular cloning and Northern blot analysis

All inserts were generated by PCR using the Expand High Fidelity System (Roche). Sequences of the constructs were verified by DNA sequencing. hp76 (human p76) cDNA was initially obtained by reverse transcriptase-PCR on U937 mRNA with the F1/R1 primers (see Supplementary Table1 at http://www.BiochemJ.org/bj/402/bj4020449add.htm). The vector pFLAG-hp76 was generated by cloning the sequence encoding hp76, from which both the signal sequence and the stop codon had been removed (F2/R2 primers; Supplementary Table 1), into the pFLAG-ATS bacterial expression vector (Sigma–Aldrich). For eukaryotic expression, hp76 cDNA was cloned into the pcDNA3.1-Myc-His(+)A vector (Invitrogen), generating pcDNA3.1-hp76-Myc-His. The DNA sequence encoding the hp76–Myc–His_6 fusion protein (hp76–Myc) was then PCR-amplified from pcDNA3.1-hp76-Myc-His (F3/R3 primer pair; Supplementary Table 1) and inserted into the pCEP4 expression vector (Invitrogen), generating pCEP4-hp76-Myc-His. An expression vector encoding hp76 without a tag was generated, by ligation of the 5′ KpnI–BamHI hp76 fragment, purified from pCEP4-hp76-Myc-His, and of the 3′ BamHI–XhoI hp76 fragment, obtained by digestion of a longer PCR-amplified hp76 fragment (F4/R4 primer pair; Supplementary Table 1), into the pcDNA3.1 vector (Invitrogen).

The plasmid pCMV-SPORT6-mp76 (mouse p76; clone IRAVp968E0290D6), encoding mp76, was obtained from RZPD (Deutsches Ressourcenzentrum für Genomforschung).

Northern blot analysis was performed on a human Multiple Tissue Northern blot (Clontech) by hybridization with a specific hp76 cDNA fragment and with the control human β-actin cDNA, as described previously [18].

Cell lines and transfections

HeLa, U937 and Rat2 cells were from the American Type Culture Collection (ATCC), and 293-EBNA (human embryonic kidney 293 cells stably transformed with Epstein–Barr virus nuclear antigen) cells were from Invitrogen. Murine JR11 fibroblasts [19] were a kind gift from Dr B. Hoflack (Biotechnological Center, Technical University of Dresden, Germany). These cells constitutively secrete Man6P proteins because they have a deficiency in both MPRs.

HeLa, 293-EBNA, Rat2 and JR11 cells were grown in DMEM (Dulbecco's modified Eagle's medium)-GlutamaxI supplemented with 10 % (v/v) FBS (fetal bovine serum), while U937 cells were maintained in RPMI-GlutamaxI medium supplemented with 10 % (v/v) FBS. Media and serum were from Invitrogen.

HeLa cells were transiently transfected using the Fugene® 6 reagent (Roche) and processed 3 or 4 days later for analysis. 293-EBNA cells were transfected with pCEP4 or pCEP4-hp76-Myc-His by calcium phosphate precipitation [20], and stable transfectants (293/mock or 293/hp76–Myc) were selected using 0.3 mg/ml hygromycin B (Invitrogen).

Expression and purification of recombinant proteins

The FLAG–p76 recombinant protein was expressed and purified in inclusion bodies from transformed *Escherichia coli* BL21 cells as described previously [21]. For analysis of hp76–Myc, supernatants from confluent 293/mock or 293/hp76–Myc cells, cultivated in DMEM-GlutaMax I without FBS nor hygromycin B, were collected three times a week for a duration of 1 month and stored at − 20 °C.

Production of anti-p76 antibodies

Purified FLAG–hp76 inclusion bodies were injected into rabbits to raise hp76Ab polyclonal antibodies (Charles River Laboratories, Chatillon-sur-Chalaronne, France), as described previously [21].

Two rat peptides TRNPRAKIFQRDQS and SQPDLWMFSPVKVPWD (residues 76–89 and 196–211 respectively according to the incomplete NCBI entry AAM23313), and the human N-terminal peptide IPAPGGRWARDGQVPPASR (residues 42–60, according to LOC196463) were synthesized with an additional cysteine residue (NeoMPS, Strasbourg, France) and coupled to activated maleimide ovalbumin (Pierce). The lyophilized antigens were injected into rabbits (Charles River Laboratories or NeoMPS; [21]) to raise, respectively, the Irp76Ab, Crp76Ab and Nhp76Ab antisera.

hp76Ab and Nhp76Ab react against human p76 only. Irp76Ab reacts against mouse and rat p76. Crp76Ab reacts against the three species. As purification did not improve the reactivity or the specificity of the antisera, the antibodies were used without purification. Non-immune sera did not reveal any significant protein species.

Electrophoresis, Western blotting and MPR overlay

SDS/PAGE separation of the reduced proteins and 2DE (2-dimensional electrophoresis) were performed as described previously [7,22]. Proteins were stained by silver nitrate or Colloidal Blue (Bio Safe; Bio-Rad Laboratories). Western blot analyses [21] were performed with the following primary antibodies: (i) rabbit polyclonal antibodies: hp76Ab (diluted 1:1000), Nhp76Ab (diluted 1:500), Crp76Ab (diluted 1:1000), Irp76Ab (diluted 1:400), anti-mitochondrial ATPase (anti-F_0 subunit) (diluted 1:3000; a generous gift of Drs A. Dupuis and J. Lunardi, CEA-Grenoble, Grenoble, France); (ii) mouse monoclonal anti-LAMP1 (lysosomal-associated membrane protein 1; diluted 1:500; 1D4B, Developmental Studies Hybridoma Bank); and (iii) goat polyclonal anti-mouse CD98, (diluted 1:200; M-20, Santa Cruz Biotechnology). Membranes were then incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies: goat anti-rabbit (diluted 1:50 000; Pierce), goat anti-mouse (diluted 1:3000, Bio-Rad Laboratories), or mouse anti-goat (diluted 1:10 000; Sigma–Aldrich). Signal detection was performed using the SuperSignal West Pico chemiluminescent substrate (Pierce) and exposure to X-ray film.

MPR overlay assays were adapted from Valenzano et al. [23]. sCI-MPR (soluble cation-independent-MPR, purified as described in [6]) was biotinylated using the Biotin Protein Labelling Kit (Roche), according to the manufacturer's protocol. 2DE-resolved Man6P proteins were electro-transferred onto nitrocellulose membranes (Bio-Rad Laboratories) that were blocked in TBST (Tris buffered saline containing 0.1 % Tween-20) containing 3 % (w/v) BSA and then incubated with biotinylated sCI-MPR in TBST and 1 % (w/v) BSA with or without Man6P (Sigma–Aldrich), and then with neutravidin-HRP (diluted 1:75 000; Pierce) in TBST and 1 % BSA. Bound biotinylated sCI-MPR was

revealed using the SuperSignal West Pico chemiluminescent substrate (Pierce) and exposure to X-ray film.

Production and purification of Man6P proteins from cell lines or mouse brains

Man6P proteins were produced and purified from U937, Rat2, JR11 and 293/hp76–Myc cells as described [6]. Purification was performed either on an Affigel-10–sCI-MPR column or on sCI-MPR immobilized on Epoxy membranes (Sartorius).

Mouse brains were homogenized and Man6P proteins were affinity purified, as described previously [14], on sCI-MPR immobilized on an Epoxy membrane (Sartorius).

Carbohydrate modification analysis

Purified U937 Man6P proteins were deglycosylated by PNGase F (peptide-*N*-glycosidase F; E.C. 3.5.1.52; Roche), according to the manufacturer's instructions. The reaction was stopped by the addition of Laemmli sample buffer [22], or by boiling. In the latter case, proteins were concentrated on Strataclean beads (Stratagene) and eluted by boiling in Laemmli sample buffer prior to Western blot analysis.

Immunofluorescence studies

Transiently transfected HeLa cells were processed for immunofluorescence [21] 4 days after transfection. Cells were fixed at room temperature (25 °C) in methanol/acetone (50:50, v/v) chilled previously at −20 °C. Antibodies were used at the following dilutions: rabbit polyclonal Crp76Ab, 1:40; mouse monoclonal anti-human LAMP1, 1:1000 (CD107a, BD PharMingen); goat polyclonal anti-human cathepsin D, 1:25 (R-20, Santa Cruz Biotechnology); Cy3-conjugated goat anti-rabbit, 1:1500 (Jackson); AlexaFluor® 488-conjugated goat anti-mouse, 1:1000 (Molecular Probes); FITC-conjugated donkey anti-goat, 1:200 (Serotec). Fluorescence was examined using a TSC-SP2 confocal laser-scanning microscope (Leica).

Sub-cellular fractionation and osmotic release experiments

Livers were obtained from female adult NMRI mice (20 g) that received, when specified, an intravenous injection of 17 mg of Triton WR-1339 (tyloxapol; Sigma–Aldrich) 4 days prior to sacrifice. Fractionation of sub-cellular organelles by differential centrifugation was performed according to [24]. Isopycnic centrifugation was carried out on the total mitochondrial ML fraction (heavy and light mitochondrial fractions from density gradient fractionation) as described previously [25].

For osmotic release experiments, an ML fraction from NMRI mouse liver was diluted in sucrose solutions of decreasing concentrations and analysed as described previously [26].

Enzymatic assays

The activities of β-galactosidase, β-hexosaminidase and β-glucuronidase were measured as described previously [27].

RESULTS

Identification of a novel human protein among Man6P proteins: p76 (hypothetical protein LOC196463)

Our previous proteomic analysis of purified Man6P proteins from U937 and MCF7 human cells revealed a few novel proteins such as cystatin F and CREG (cellular repressor of E1A-stimulated genes) [6,7]. A novel protein, which was not mentioned in the previous papers, was initially identified on a 2DE gel from a U937 sample, in a series of spots of neutral to alkaline pI (approx. pH 7–8) and at a molecular mass of 70 kDa by a unique tandem MS peptide sequence (LASDGATWADIFK; residues 355–367 of LOC196463). These spots were barely detected by Colloidal Blue staining (Figure 1A) and were not visible in MCF7 extracts run on 2DE gels. The same protein was also identified in both U937 and MCF7 samples from a series of spots of more acidic pI (approx. pH 5), which migrated at a slightly higher molecular mass of 76 kDa (Figure 1A and see Supplementary Table 2 at http://www.BiochemJ.org/bj/402/bj4020449add.htm). We named it 'hp76', due to its apparent molecular mass. In the U937 sample, two peptides were identified by LC-MS/MS. In the MCF7 sample, 11 peptides of hp76 were identified by MALDI–TOF-MS fingerprinting, leading to a sequence coverage of 20 %. Complementary analyses performed by LC-MS/MS on both U937 and MCF7 samples separated by SDS/PAGE led to the identification of seven tryptic peptides of hp76 in the U937 sample, and nine peptides in the MCF7 sample (respective coverages 13 and 17 %; see Supplementary Table 2).

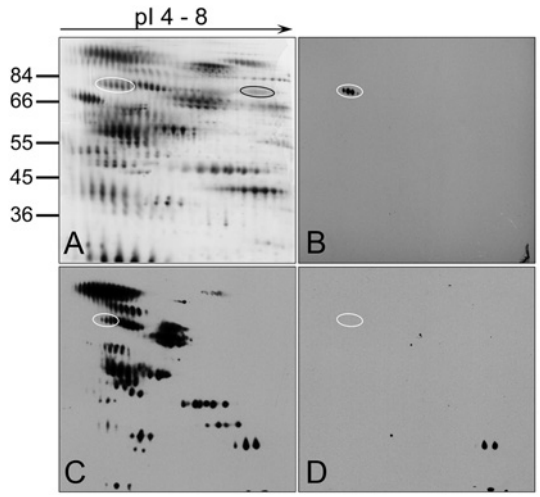

Figure 1 Identification of hp76 as a Man6P protein

(**A**) Man6P proteins purified from 2.5×10^9 U937 cells were resolved by 2DE over a 4–8 linear pH range on an SDS/10 % (v/v) PAGE gel (20 × 20 cm) before Colloidal Blue staining (modified from [7] with permission). (**B**–**D**) Man6P proteins purified from 2×10^8 U937 cells were resolved by 2DE over a 4–8 linear pH range on an SDS/10 % (v/v) PAGE minigel (9 × 9 cm), transferred onto nitrocellulose membranes and probed with hp76Ab (**B**) or with 3.3 nM of biotinylated sCI-MPR without (**C**) or with (**D**) 5 mM Man6P. The spots inside the white ovals show the main series of hp76 spots; the black oval shows the position of the minor series of hp76-containing spots, which could only be detected with high protein amounts. Positions of molecular-mass markers (kDa) are shown to the left-hand side of (**A**).

This novel protein, reported in the NCBI protein database as the 'hypothetical protein LOC196463', is 589 amino acids in length with a predicted signal peptide of 41 amino acids (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/402/bj4020449add.htm). Since six potential *N*-glycosylation sites have been predicted for this protein, carbohydrate modifications are likely to explain the difference between the apparent and predicted molecular masses of the secreted protein, i.e. 76 and 61 kDa respectively.

hp76 appears to be evolutionarily conserved, as proteins exhibiting high amino-acid sequence similarity are found in several mammals and other species, namely *Caenorhabditis elegans*, *Dictyostelium discoideum* and *Trypanosoma brucei*

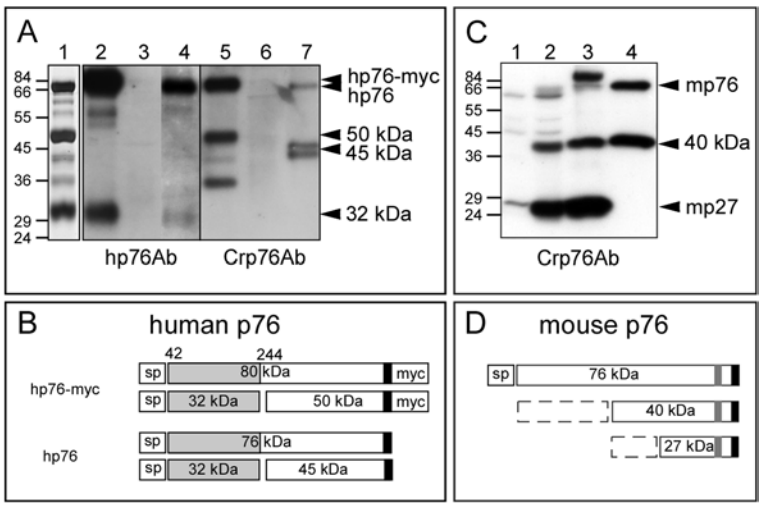

Figure 2 Human and mouse p76 maturation

(**A**) Characterization of hp76-derived polypeptides. Protein samples were loaded onto a SDS/12 % (v/v) PAGE gel and either Silver-stained (lane 1) or transferred for Western blot analysis with hp76Ab (lanes 2–4) or with Crp76Ab (lanes 5–7). Lane 1, Man6P proteins purified from 293/hp76–Myc supernatant; lanes 2 and 5, 293/hp76–Myc supernatant; lanes 3 and 6, 293/mock supernatant; lanes 4 and 7, Man6P proteins purified from U937 cells. Lane 4 is overexposed compared with the other lanes. (**B**) Hypothetical maturation scheme for hp76. hp76Ab targets epitopes in the N-terminal part of hp76 (light grey). Crp76Ab targets the C-terminal peptide of hp76 (black). (**C**) Characterization of mouse p76-derived polypeptides. Protein samples were loaded onto a SDS/12 % (v/v) PAGE gel and transferred for Western blot analysis with Crp76Ab. Lane 1, untransfected HeLa cell lysate; lane 2, lysate of HeLa cells transfected with wild-type mp76 cDNA; lane 3, purified mouse brain Man6P proteins; lane 4, purified JR11 Man6P proteins. (**D**) Hypothetical maturation scheme for mp76. Crp76Ab targets the C-terminal peptide of rat and mouse p76 (black). Irp76Ab targets an internal peptide of rat and mouse p76 (dark grey).

(see Supplementary Table 3 at http://www.BiochemJ.org/bj/402/bj4020449add.htm). However, no similar protein is found in yeast. All these proteins are members of a family, based on the presence of a Laminin A domain. The name Laminin A domain probably originates from the organ where the first p76 encoding gene was identified, namely in the *Drosophila* 'lamina' glia [14,28]. The human and rodent p76 proteins are highly similar (see Supplementary Figure 1). The mouse hypothetical protein LOC71772 is 594 amino acids long and shares 82 % sequence identity and 91 % similarity with hp76 over a 558 amino acid alignment, whereas the rat hypothetical protein LOC246120 (Ensembl peptide ID ENSRNOP00000001872) is 585 amino acids long and shares 82 % sequence identity and 92 % similarity with hp76 over a 547 amino acid alignment. The similar protein expressed in the amoeba *D. discoideum* is of particular interest, because it is the only one for which a function (phospholipase B) has been so far demonstrated *in vitro* [28].

Expression of hp76 in human tissues

The *loc196463* gene is located on the long arm of chromosome 12 (12q24.13) and is predicted to contain 12 exons generating a transcript of 2584 bp. The sequence of the corresponding cDNA (BC030618) is composed of a 28 bp 5′-UTR (untranslated region), a 1767 bp open reading frame and a 789 bp 3′-UTR containing a polyadenylation signal and a short poly(A) stretch.

Northern blotting (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/402/bj4020449add.htm) showed that a transcript migrating at 2.4 kb, consistent with the hp76 cDNA length, was found in all tissues studied, with significantly higher expression in heart, brain and liver. The ubiquitous expression correlates well with the transcriptomic data provided by the Symatlas website. Two additional transcripts were observed, a longer one of 4.4 kb, whose expression roughly follows that of the 2.4 kb transcript and a shorter one of 0.24 kb only detected in the liver. The origin of these two additional transcripts is unknown.

Characterization of human and mouse p76 maturation fragments

Human p76

As recombinant hp76–Myc is secreted in large amounts by 293-EBNA cells transfected with pCEP4-hp76-Myc-His (293/hp76–Myc cells), we used this model for initial analysis of hp76 maturation. Comparison by SDS/PAGE of protein profiles of culture supernatants from mock-transfected 293-EBNA cells (results not shown) and 293/hp76-Myc cells showed that three major hp76–Myc-related polypeptides were secreted (Figure 2A, lane 1). N-terminal sequencing of the largest (80 kDa) form indicated that it corresponded to the hp76–Myc precursor, after cleavage of its signal peptide between positions 41 and 42 as predicted (see Supplementary Table 4 at http://www.BiochemJ.org/bj/402/bj4020449add.htm). Edman analysis of the 32 kDa species identified position 42 at its N-terminus, indicating that this fragment is in the N-terminal half of the protein (Figure 2B and Supplementary Table 4). As for the 50 kDa fragment, it starts at position 244 of the pre-protein (Figure 2B and Supplementary Table 4). Western blot analysis of the 293/hp76–Myc supernatant with hp76Ab, an antibody raised against the recombinant FLAG–hp76 protein, revealed the 80 kDa and the 32 kDa bands, but not the 50 kDa one (Figure 2A, lane 2). Thus, hp76Ab targets hp76 N-terminal epitopes only. The same sample was then analysed with Crp76Ab, an antibody that specifically recognises the C-terminus of rodent p76 and cross-reacts with human p76. Crp76Ab revealed the 80 kDa precursor and the 50 kDa fragment, but not the 32 kDa N-terminal one (Figure 2A, lane 5), indicating that the 50 kDa fragment is C-terminal. Other polypeptides at 40 kDa and 35 kDa were also revealed and these might be additional C-terminal fragments generated through limited proteolysis. These fragments could also be seen in the silver-stained gel (Figure 2A, lane 1). Among U937 Man6P proteins, hp76Ab detected a strong band at 76 kDa as well as a faint band at 32 kDa (Figure 2A, lane 4), whereas Crp76Ab detected the same 76 kDa band and a 45 kDa doublet (Figure 2A, lane 7).

Therefore, in both our models, that is the U937 Man6P proteins and 293/hp76–Myc secreted proteins, the hp76 precursor seems to be matured by a cleavage event occurring after signal peptide cleavage (Figure 2B). An N-terminal 32 kDa fragment is produced identically from hp76 or hp76–Myc, whereas the C-terminal fragment appears as a doublet of 45 kDa from U937 Man6P proteins or as a unique 50 kDa polypeptide in 293/hp76–Myc secretions. The presence of the Myc tag on hp76–Myc explains the difference in migration observed between the hp76 precursors (76 kDa compared with 80 kDa) and between the C-terminal polypeptides (45 kDa compared with 50 kDa) from U937 or 293/hp76–Myc samples respectively.

Mouse p76

Rat and mouse p76 proteins were analysed by immunoblotting and detected with Crp76Ab and Irp76Ab, two antibodies that recognise both rat and mouse p76. They were raised against different peptides of the rat p76 sequence, targeting the last 16 C-terminal residues and an internal peptide located in the C-terminal part of the protein respectively. Western blot analysis of lysates of HeLa cells overexpressing mp76 allowed us to visualize three major bands migrating at 76 kDa, 40 kDa and 27 kDa (Figure 2C, lanes 1 and 2). These same polypeptides were detected in Man6P proteins purified from rat (results not shown) or mouse brain (Figure 2C, lane 3), although the 76 kDa precursor was present as a minor species. In Man6P proteins purified from secretions from a rat cell line, Rat2 (results not shown), or from a mouse cell line, JR11 [19], only the 76 kDa and 40 kDa polypeptides were detected (Figure 2C, lane 4). The presence of an additional 27 kDa p76 polypeptide fragment in the HeLa cell lysate and the brain samples is probably due to an additional maturation event occurring only inside the cell. As the antibodies target C-terminal peptides, the 40 kDa and 27 kDa polypeptides are C-terminal fragments of the 76 kDa precursor. The 40 kDa polypeptide, which is probably the equivalent of the 45 kDa doublet observed for human p76, must be cleaved to generate a major intracellular 27 kDa fragment. This 27 kDa fragment will now be referred to as mp27 (Figure 2D).

hp76 precursor glycosylation

All potential glycosylation sites are glycosylated

To determine the actual number of occupied *N*-glycosylation sites, endogenous Man6P proteins purified from U937 cells were subjected to a time-course deglycosylation by PNGase F and subsequently analysed by Western blotting using three anti-p76 antibodies, the hp76Ab and Crp76Ab antibodies mentioned previously and Nhp76Ab, an antibody raised against the N-terminal peptide of hp76. Several bands of decreasing molecular masses ranging from 76 kDa to approx. 60 kDa were progressively produced (Figure 3A). Progressive deglycosylation of the 45 kDa C-terminal doublet gave rise to two additional bands with lower molecular masses, with the fully deglycosylated polypeptide migrating at 38 kDa (Figure 3B). Therefore, the initial doublet probably represents di-glycosylated and tri-glycosylated forms of the 38 kDa fragment, which were present in approximately equivalent amounts in U937 Man6P protein populations. Similarly, deglycosylation of the 32 kDa N-terminal fragment produced three additional species with decreasing molecular masses, indicating the presence of three glycosylation sites on the 22 kDa polypeptide backbone (Figure 3C). Taken together, these results indicate that all six predicted hp76 *N*-glycosylation sites are actually glycosylated.

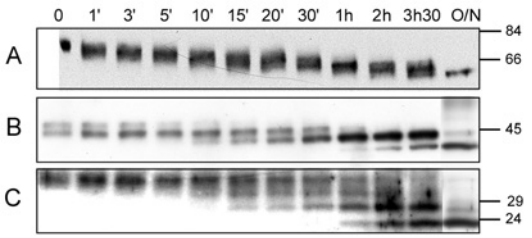

Figure 3 *N*-Glycosylation of hp76

Man6P proteins from U937 cells were subjected to deglycosylation by PNGase F for the indicated times, and resolved by SDS/PAGE using 7.5 % (v/v; **A**; equivalent of 6×10^6 cells/lane) or 9 % (v/v; **B** and **C**; equivalent of 30×10^6 cells/lane) gels and transferred for Western blot analysis. Additional PNGase F was added for maximal deglycosylation for the overnight (O/N) time points in (**B**) and (**C**), which were analysed on a separate Western blot. The precursor was detected with hp76Ab (**A**), the C-terminal fragment with Crp76Ab (**B**) and the N-terminal fragment with Nhp76Ab (**C**).

p76 is a Man6P protein

Although the specificity of the purification procedure has been demonstrated previously [6], some proteins might be indirectly trapped on the affinity column through their association with a Man6P protein, rather than being Man6P-labelled themselves. To address this question for hp76, we performed a biotinylated sCI-MPR overlay assay on 2DE-resolved Man6P proteins in the presence or absence of free Man6P competition. As the hp76 spot positions had been established both by MS (Figure 1A and Supplementary Table 2) and Western blotting (Figure 1B), we could identify hp76 among the proteins recognised by the biotinylated sCI-MPR (Figure 1C). Addition of free Man6P competed with this labelling effectively (Figure 1D), thus confirming the specificity of the MPR–hp76 interaction. Therefore, hp76 is a Man6P protein, which interacts directly with the sCI-MPR on the affinity column.

Human and mouse p76 co-localize with lysosomal markers

As the presence of Man6P sugars on p76 suggested that p76 might be a lysosomal protein, its localization was first studied by immunofluorescence. In HeLa cells overexpressing hp76, hp76 was observed in vesicles concentrated in a perinuclear area, as well as dispersed throughout the cytoplasm (Figure 4). Double-staining with a lysosomal membrane marker, LAMP1, or a luminal lysosomal marker, cathepsin D, showed that hp76 co-localized with both of them to a large extent (Figure 4). A similar immunofluorescence study was carried out on HeLa cells overexpressing mouse p76, which gave the same results. The intracellular distribution of mp76 also consisted in a vesicular staining mainly co-localizing with lysosomal markers, as determined by cathepsin D or LAMP1 co-labelling (results not shown).

Sub-cellular fractionation shows a lysosomal distribution for mp27, the 27 kDa C-terminal polypeptide fragment of maturated mp76

The sub-cellular localization of endogenous p76 was established by determining the distribution of mp27 (Figure 2D) in fractions obtained by sub-cellular fractionation of liver homogenates [29]. Experiments were performed on both rat and mouse samples. As we obtained similar results for both species, only the experiments carried out on mouse samples are presented here.

Differential centrifugation

Differential centrifugation of mouse liver homogenates was performed to separate nuclear (N), heavy mitochondrial (M), light

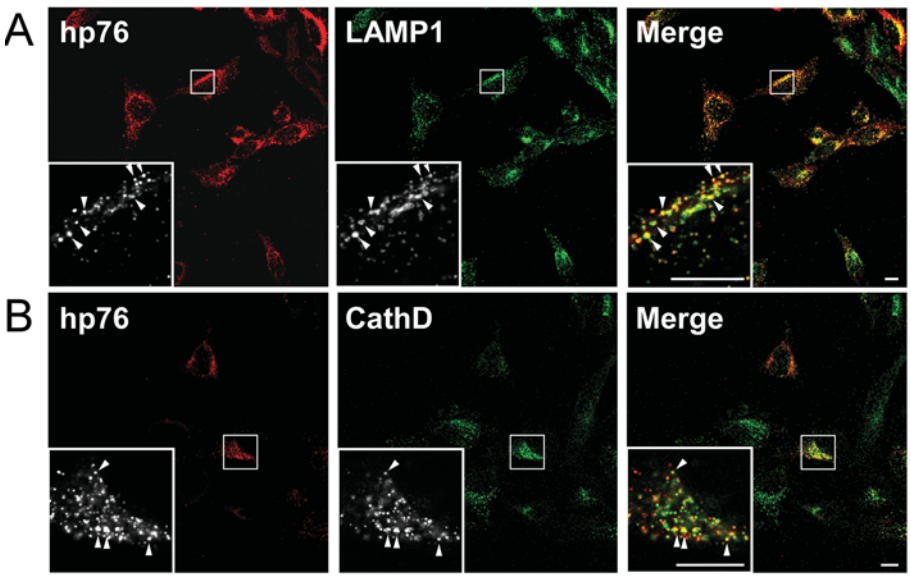

Figure 4 Co-localization of hp76 with lysosomal markers

HeLa cells were transfected with wild-type hp76 cDNA. At 72 h post-transfection, cells were fixed and double-immunolabelled for hp76 (with Crp76Ab; red) and (**A**) LAMP1 (green) or (**B**) cathepsin D (CathD; green). Arrowheads denote some vesicles that are positive for both types of staining. Scale bars, 10 μm.

mitochondrial (L), microsomal (P) and soluble (S) fractions [24]. As shown in Figure 5(A), the distribution of mp27, LAMP1, mitochondrial ATPase F_0 subunit and CD98 were determined by Western blotting or by enzymatic assays for β-galactosidase and β-hexosaminidase. For Western blot analysis, the same quantity of proteins was loaded onto each lane of the gel and thus the intensity of the signal revealed after immunoblotting corresponds to the relative enrichment of the protein in each fraction. For enzymatic assays, β-galactosidase and β-hexosaminidase activities are represented as proposed by de Duve et al. [24]: bar height (specific activity) is indicative of the lysosome purification in the nuclear, heavy mitochondrial, light mitochondrial, microsomal and soluble fractions. As shown in Figure 5(A), a strong mp27 signal was detected in the total mitochondrial fraction (heavy and light), with the strongest signal in the light fraction. This pattern is highly indicative of liver lysosomes, as shown by the distribution of LAMP1 and lysosomal enzymes. It should be noted that a 29 kDa fragment was also detected with Crp76Ab in all fractions (Figure 5A). Although this signal is not detected by the non-immune serum (results not shown), we assume that it is not related to mp76 because, (i) Irp76Ab does not reveal this 29 kDa band in those fractions (results not shown), and (ii) Crp76Ab and Irp76Ab do not detect this band in either HeLa cells overexpressing mp76 or in mouse brain purified Man6P proteins (Figure 2C). The distribution of the mitochondrial ATPase F_0 subunit (strongest signal in the mitochondrial heavy fraction) and the plasma membrane protein CD98 (in nuclear and microsomal fractions) agreed with the distribution of these sub-cellular compartments from liver samples reported previously [24]. These patterns were clearly distinct from those of the lysosomal markers.

Density gradient fractionation

An ML fraction was prepared and analysed further by isopycnic centrifugation on a linear sucrose-density gradient. Lysosomes, represented by β-galactosidase and β-hexosaminidase activity, equilibrated in the heavy fractions of the gradient (Figure 5B). mp27 was detected in the same fractions as these lysosomal markers. However, to assess the significance of this co-distribution, lysosomes were submitted to a density shift experiment. We injected mice with a non-haemolytic detergent, Triton WR 1339, that specifically accumulates in lysosomes and strongly reduces their density [30]. As shown in Figure 5(C), mp27 accompanied the shift of the lysosomes toward the low densities of the gradient after this treatment, providing strong evidence for mp27 residence in lysosomes.

Osmotic release of mp27

We studied the sensitivity of mp27-containing vesicles to a hypo-osmotic treatment [26] and compared the release of mp27 with that of known lysosomal markers. Aliquots of an ML fraction were incubated in decreasing sucrose concentration. As shown in Figure 6, the osmotic release curve of the luminal lysosomal hydrolases β-galactosidase, β-hexosaminidase and β-glucuronidase was similar to that of mp27. This result is therefore consistent with mp27 being a soluble polypeptide present in the lysosome. Conversely, the non-specific 29 kDa signal mentioned above (see the Differential centrifugation subsection) was never released from the membrane fraction.

DISCUSSION

During the course of our proteomic analyses of Man6P proteins [6,7], a novel protein was identified in two human cell lines, U937 and MCF7. This protein migrated mainly at an apparent molecular mass of 76 kDa, which led us to name it p76. While we were carrying out the experiments of the present paper, hp76 appeared in the NCBI protein database as the hypothetical protein LOC196463, and the human and murine p76 proteins were identified in proteomic analyses of similarly affinity-purified Man6P proteins from human brain extracts and from mouse cell culture supernatants respectively [8,14]. In the latter study, murine p76 was named '66.3 kDa protein'. p76 was also identified in a proteomic analysis of purified neuromelanin granules from human brain

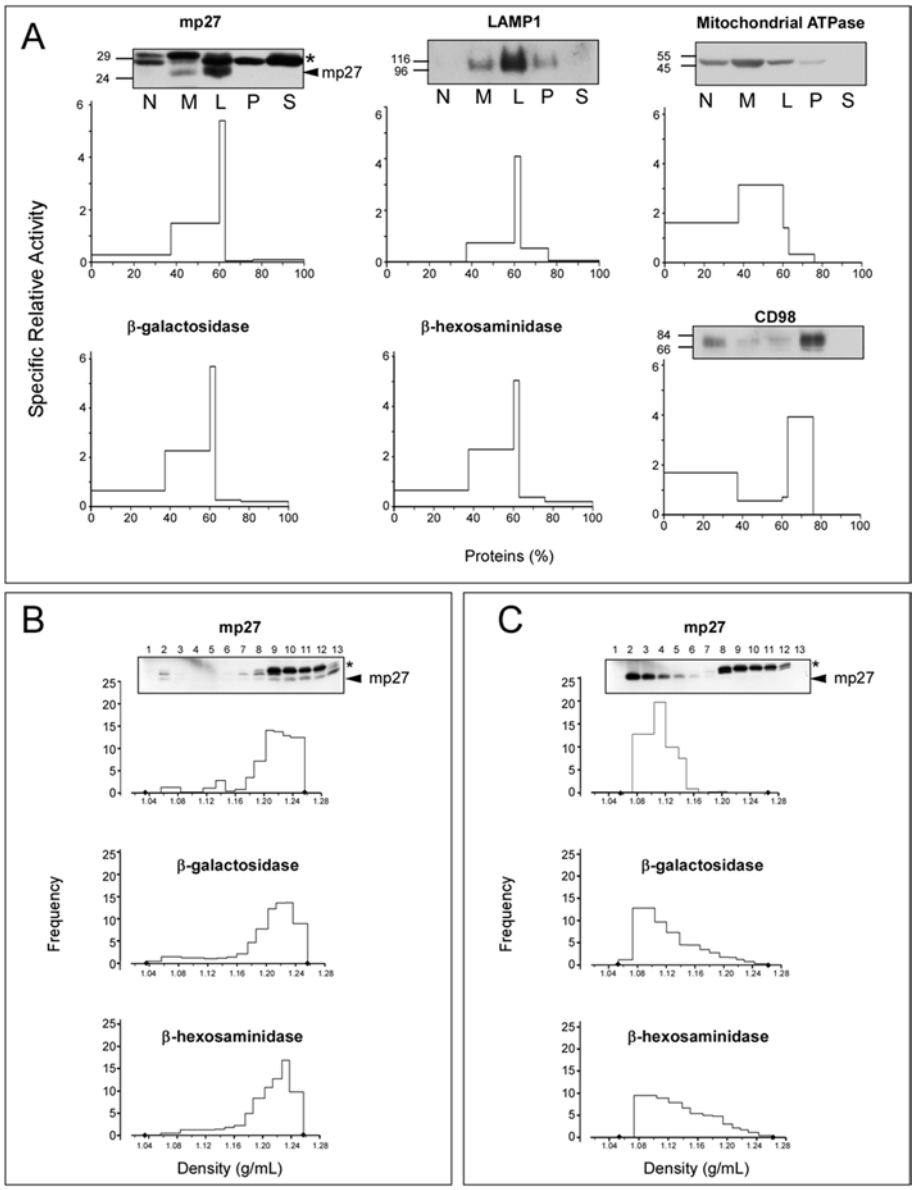

Figure 5 Distribution of mp27 in mouse liver sub-cellular fractions

(**A**) Differential centrifugation of mouse liver. The distribution of various markers and mp27 was analysed either by Western blotting (mp27, LAMP1, mitochondrial ATPase and CD98) and quantified using the NIH (National Institutes of Health) Image software or by enzymatic activity measurement (β-galactosidase and β-hexosaminidase). Fractions correspond to nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and soluble (S) fractions. Equal amounts of proteins (40 μg) were loaded for each fraction onto an SDS/10 % (v/v) PAGE gel and transferred for Western blot analysis. mp27 was detected by Crp76Ab. For all graphs, the ordinate is the relative specific activity or intensity and the abscissa is the relative protein content plotted cumulatively from left to right. Asterisk (*), non-specific band. (**B** and **C**) Distribution of mp27, β-galactosidase and β-hexosaminidase after isopycnic centrifugation in a linear sucrose density gradient. ML fractions were prepared from liver homogenates of non-treated (**B**) or Triton WR-1339-injected (**C**) mice. The sucrose density gradient was cut into 13 fractions from low to high density. Equal volumes of each fraction were loaded onto an SDS/12 % (v/v) PAGE gel and transferred for Western blot analysis of mp27 with Crp76Ab. Distributions of β-galactosidase and β-hexosaminidase were determined by enzymatic assays on each fraction. Histograms represent the frequency (percentage of activity or intensity divided by the increment of density) as a function of the density. Asterisk (*), non-specific band.

[31]. hp76 is a 589-residue protein with a putative 41-amino-acid signal peptide and six potential glycosylation sites.

All potential glycosylation sites of hp76 are glycosylated, and some of them are effectively mannose-6-phosphorylated

The extent of hp76 glycosylations was determined on U937 Man6P proteins and we unambiguously showed that all six potential hp76 glycosylation sites were occupied. Some glycosylation heterogeneity was observed, residing on any one of the residues at the following three positions 436, 465 or 515. Such heterogeneous glycosylation events are common among Man6P proteins [32]. However, in a recent global proteomic study, which aimed at listing the mannose-6-phosphorylated glycopeptides of purified brain Man6P proteins from both human and mouse origin, Sleat et al. [15] did not observe heterogeneous glycosylation at any of these three positions. This discrepancy might be due to the respective p76 sources, i.e. brain Man6P proteins compared with Man6P proteins secreted from a monocytic cell line.

Since hp76 could have been be purified on the MPR affinity column through association with a true Man6P-bearing protein,

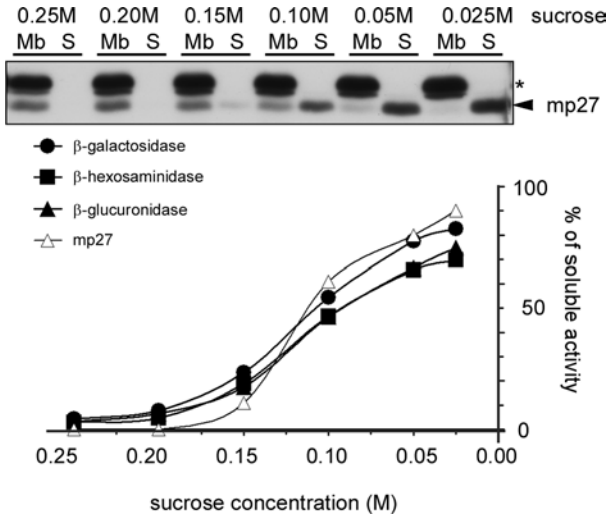

Figure 6 Osmotic release of mp27

Aliquots from a freshly prepared ML fraction were incubated for 15 min at 4 °C in sucrose solutions ranging from 0.25 M to 0.025 M. Broken membranes and intact organelles were separated from released soluble proteins by ultracentrifugation. Pellets (Mb) and supernatant (S) proteins were separated on an SDS/12 % (v/v) PAGE gel and transferred for Western blot analysis of mp27 with Crp76Ab. Enzymatic activities of lysosomal hydrolases β-galactosidase, β-hexosaminidase and β-glucuronidase were measured in each fraction. The ordinate (percentage of soluble activity or intensity) represents the ratio of the activity or intensity measured in the soluble fraction to the sum of the activities or intensities measured in the soluble and the membrane fractions. Asterisk (*), non-specific band.

without being mannose-6-phosphorylated itself, we checked the Man6P status of hp76. We showed, unambiguously, a direct and Man6P-dependent interaction between hp76 and sCI-MPR, indicating that hp76 is a true Man6P protein. Our overlay results confirm the proteomic analysis published by Sleat et al. [15], who detected the presence of Man6P on five glycopeptides of brain hp76. This high number of Man6P glycopeptides in hp76 is interesting with regard to other known lysosomal Man6P proteins. Indeed, for the 43 known lysosomal proteins that were identified through their Man6P-glycopeptides, an average of two Man6P-glycopeptides was identified per protein. Only two hydrolases displayed five or more Man6P-glycopeptides: the acid ceramidase (five peptides) and the N-acetylglucosamine-6-sulfatase (nine peptides) [15]. The high number of Man6P in hp76 might have implications for the specificity of its recognition by the MPR and for its potential lysosomal targeting efficiency. Besides, this result might suggest that although the six hp76 glycosylation sites are occupied (as presented here), only five of them are mannose-6-phosphorylated. The *N*-glycosylation of residue 110 of hp76 could be of the complex type and therefore absent from the Man6P glycopeptides identified by Sleat et al. [15]. Differences in the sugar types borne by lysosomal proteins do exist, such as for human β-glucuronidase and human β-hexosaminidase B, which both have only two mannose-6-phosphorylated *N*-glycosylations out of their four *N*-glycosylated sites [33,34].

Human and mouse p76 are subject to maturation

In secreted human Man6P proteins, we observed an N-terminal fragment of 32 kDa as well as a 45 kDa C-terminal doublet, both specific for hp76. The corresponding fragments were also observed in the secreted recombinant hp76–Myc protein. Their N-terminal sequencing identified the signal peptide cleavage site, between positions 41 and 42, in addition to an internal cleavage site between position 243 and 244. In human brain Man6P proteins [14], hp76 was found in numerous spots in the 42–30 kDa molecular mass range. An N-terminal sequence starting at position 291 of hp76 was determined, indicating the presence of at least a C-terminal fragment in brain. Nonetheless, the 32 kDa N-terminal chain might also be present in these spots. The two different N-terminal sequences obtained for the C-terminal fragment might be due to different proteolytic events occurring for the respective proteins, i.e. the recombinant protein secreted from cells in culture (the present paper) compared with the intracellular proteins from brain [14].

In Man6P proteins purified from mouse cell secretions, our antibodies allowed us to detect a 40 kDa mp76 C-terminal fragment. Intracellularly, this 40 kDa fragment appeared to be further processed, since an extra 27 kDa C-terminal fragment (mp27) was detected in mp76 cDNA transfected cell lysates, in the enriched ML fraction from mouse liver, and in mouse brain Man6P proteins. As the 40 kDa mp76 fragment was detected in cell secretions, the first proteolytic mp76 cleavage is likely to occur before segregation of lysosomal enzymes from the secretory pathway. The cleavage event responsible for mp27 formation would happen once the 40 kDa fragment is engaged in the endocytic pathway. In a proteomic study of secreted mouse Man6P proteins [8], mp76 was found on a 2DE gel in a series of three spots, migrating at molecular masses of 66 kDa, 40 kDa and 30 kDa. These species might correspond to the precursor, the C-terminal and the N-terminal fragments respectively. The difference in the apparent molecular masses of the p76 precursor (76 kDa compared with 66 kDa) in the present paper and in the paper by Kollmann et al. [8] might be due to lower mp76 glycosylation or differences in the respective electrophoresis gels and standards.

Maturation events of this type are not uncommon for lysosomal proteins. The human 53 kDa cathepsin D or the 85 kDa β-galactosidase precursors are processed proteolytically so that the mature forms are heterodimers composed of a heavy and a light chain in lysosomes [35,36]. Conversely, human β-glucuronidase loses a small C-terminal peptide upon maturation [37]. In conclusion, we propose that the 76 kDa p76 precursor is cleaved into a 32/30 kDa N-terminal polypeptide and a 45/40 kDa C-terminal polypeptide (human compared with mouse; the present study and [8]). Intracellularly, a further cleavage would occur in the C-terminal chain, at least for the rat and mouse proteins, generating mp27.

p76 is a lysosomal protein

Proteins bearing Man6P glycosylations are mainly soluble lysosomal proteins, despite the known existence of a few non-lysosomal Man6P proteins [38–43]. Nonetheless, the recent identification of numerous non-lysosomal Man6P proteins in plasma weakened this idea [11]. This finding reinforces the necessity to experimentally assess the sub-cellular localization of any newly identified Man6P protein. Accordingly, Sleat et al. [11] defined three criteria to select new candidate lysosomal proteins: (i) enrichment of proteins by MPR purification cycles; (ii) low abundance in plasma; and (iii) predicted or known function compatible with a lysosomal localization. Hence, of the 44 Man6P proteins that were identified in the cytoplasm and that were not known to be lysosomal, nine candidates were selected, among which was hp76 [11]. On the other hand, the identification of hp76 in a proteomic analysis of neuromelanin granules from human brain also supported the hypothesis that p76 could be a lysosomal protein [31]. Indeed, it was demonstrated that neuromelanin granules belonged to the lysosome-like organelles family [31]. Moreover, Kollmann et al. [8] have shown that purified recombinant

mp76 was internalized via MPR-mediated endocytosis, providing another argument in favour of a lysosomal localization of p76.

To determine p76 sub-cellular localization, we first used immunofluorescence on cells overexpressing either human or mouse p76, and showed a clear co-localization of p76 with the lysosomal markers LAMP1 and cathepsin D. To analyse the sub-cellular localization of endogenous p76, we performed biochemical studies on rat and mouse liver samples, in which p76 was detected as the mp27 maturated polypeptide. The similar behaviour of mp27 and lysosomal marker proteins in sub-cellular fractionation experiments (differential centrifugation, isopycnic centrifugation of ML fractions on linear sucrose density gradients, with or without a specific reduction of the lysosome density), and in osmotic release experiments strongly indicates residence of p76 in lysosomes. Taken together, these results give very strong evidence for p76 being a lysosomal protein.

Concluding remarks

Now that the lysosomal localization of p76 has been clearly assessed, the next step will be to determine its function. As the only protein displaying significant sequence similarity with p76 and for which a function has been experimentally demonstrated is a *D. discoideum* phospholipase B [28], we are setting up functional assays for the analysis of the putative phospholipid degradation by p76. As little is known about phospholipid catabolism, the identification of p76 as a novel lysosomal phospholipase and the analysis of the possible effects of a deficiency in this protein would be an important contribution to lysosome comprehension.

We are grateful to Dr D. Grünwald [CEA (Commissariat à l'Energie Atomique)-Grenoble, Grenoble, France] for help and guidance with confocal microscopy, Professor B. Hoflack (Technical University of Dresden, Germany) for the kind gift of JR11 cells, Dr A. Dupuis for helpful discussions and Dr M. Gallagher-Gambarelli (both from CEA-Grenoble, Grenoble, France) for reading of the manuscript. This work was supported by the INSERM, the Life Sciences Division of the CEA, the Joseph Fourier University and the Fonds de la Recherche Fondamentale Collective (Contract no. 2.4505.01). A. G. J. and M. C. are recipients of fellowships from the CEA.

Note: While the present paper was under revision, a study has been published on the mouse p76 (66.3 kDa protein; [44]). The experimental data presented in this publication complement our own work and strongly support our conclusions.

REFERENCES

1 Futerman, A. H. and van Meer, G. (2004) The cell biology of lysosomal storage disorders. Nat. Rev. Mol. Cell Biol. **5**, 554–565
2 Li, P., Gregg, J. L., Wang, N., Zhou, D., O'Donnell, P., Blum, J. S. and Crotzer, V. L. (2005) Compartmentalization of class II antigen presentation: contribution of cytoplasmic and endosomal processing. Immunol. Rev. **207**, 206–217
3 Kroemer, G. and Jäättelä, M. (2005) Lysosomes and autophagy in cell death control. Nat. Rev. Cancer **5**, 886–897
4 Hille-Rehfeld, A. (1995) Mannose 6-phosphate receptors in sorting and transport of lysosomal enzymes. Biochim. Biophys. Acta **1241**, 177–194
5 Einstein, R. and Gabel, C. A. (1989) Serum factors alter the extent of dephosphorylation of ligands endocytosed via the mannose 6-phosphate/insulin-like growth factor II receptor. J. Cell Biol. **109**, 1037–1046
6 Journet, A., Chapel, A., Kieffer, S., Louwagie, M., Luche, S. and Garin, J. (2000) Towards a human repertoire of monocytic lysosomal proteins. Electrophoresis **21**, 3411–3419
7 Journet, A., Chapel, A., Kieffer, S., Roux, F. and Garin, J. (2002) Proteomic analysis of human lysosomes: application to monocytic and breast cancer cells. Proteomics **2**, 1026–1040
8 Kollmann, K., Mutenda, K. E., Balleininger, M., Eckermann, E., von Figura, K., Schmidt, B. and Lubke, T. (2005) Identification of novel lysosomal matrix proteins by proteome analysis. Proteomics **5**, 3966–3978
9 Czupalla, C., Mansukoski, H., Riedl, T., Thiel, D., Krause, E. and Hoflack, B. (2006) Proteomic analysis of lysosomal acid hydrolases secreted by osteoclasts: implications for lytic enzyme transport and bone metabolism. Mol. Cell. Proteomics **5**, 134–143
10 Sleat, D. E., Kraus, S. R., Sohar, I., Lackland, H. and Lobel, P. (1997) α-Glucosidase and *N*-acetylglucosamine-6-sulphatase are the major mannose-6-phosphate glycoproteins in human urine. Biochem. J. **324**, 33–39
11 Sleat, D. E., Wang, Y., Sohar, I., Lackland, H., Li, Y., Li, H., Zheng, H. and Lobel, P. (2006) Identification and validation of mannose 6-phosphate glycoproteins in human plasma reveal a wide range of lysosomal and non-lysosomal proteins. Mol. Cell. Proteomics **5**, 1942–1956
12 Sleat, D. E., Chen, T. L., Raska, Jr, K. and Lobel, P. (1995) Increased levels of glycoproteins containing mannose 6-phosphate in human breast carcinomas. Cancer Res. **55**, 3424–3430
13 Sleat, D. E., Sohar, I., Lackland, H., Majercak, J. and Lobel, P. (1996) Rat brain contains high levels of mannose-6-phosphorylated glycoproteins including lysosomal enzymes and palmitoyl-protein thioesterase, an enzyme implicated in infantile neuronal lipofuscinosis. J. Biol. Chem. **271**, 19191–19198
14 Sleat, D. E., Lackland, H., Wang, Y., Sohar, I., Xiao, G., Li, H. and Lobel, P. (2005) The human brain mannose 6-phosphate glycoproteome: a complex mixture composed of multiple isoforms of many soluble lysosomal proteins. Proteomics **5**, 1520–1532
15 Sleat, D. E., Zheng, H., Qian, M. and Lobel, P. (2006) Identification of sites of mannose 6-phosphorylation on lysosomal proteins. Mol. Cell. Proteomics **5**, 686–701
16 Jadot, M., Lin, L., Sleat, D. E., Sohar, I., Hsu, M. S., Pintar, J., Dubois, F., Wattiaux-De Coninck, S., Wattiaux, R. and Lobel, P. (1999) Subcellular localization of mannose 6-phosphate glycoproteins in rat brain. J. Biol. Chem. **274**, 21104–21113
17 Saksouk, N., Bhatti, M. M., Kieffer, S., Smith, A. T., Musset, K., Garin, J., Sullivan, Jr, W. J., Cesbron-Delauw, M. F. and Hakimi, M. A. (2005) Histone-modifying complexes regulate gene expression pertinent to the differentiation of the protozoan parasite *Toxoplasma gondii*. Mol. Cell Biol. **25**, 10301–10314
18 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
19 Ludwig, T., Munier-Lehmann, H., Bauer, U., Hollinshead, M., Ovitt, C., Lobel, P. and Hoflack, B. (1994) Differential sorting of lysosomal enzymes in mannose 6-phosphate receptor-deficient fibroblasts. EMBO J. **13**, 3430–3437
20 Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell Biol. **7**, 2745–2752
21 Harlow, E. and Lane, D. (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685
23 Valenzano, K. J., Kallay, L. M. and Lobel, P. (1993) An assay to detect glycoproteins that contain mannose 6-phosphate. Anal. Biochem. **209**, 156–162
24 de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem. J. **60**, 604–617
25 Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. and de Duve, C. (1964) Tissue fractionation studies. 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. Biochem. J. **92**, 184–205
26 Appelmans, F. and de Duve, C. (1955) Tissue fractionation studies. 3. Further observations on the binding of acid phosphatase by rat-liver particles. Biochem. J. **59**, 426–433
27 Peters, T. J., Muller, M. and de Duve, C. (1972) Lysosomes of the arterial wall. I. Isolation and subcellular fractionation of cells from normal rabbit aorta. J. Exp. Med. **136**, 1117–1139
28 Morgan, C. P., Insall, R., Haynes, L. and Cockcroft, S. (2004) Identification of phospholipase B from *Dictyostelium discoideum* reveals a new lipase family present in mammals, flies and nematodes, but not yeast. Biochem. J. **382**, 441–449
29 Beaufay, H. (1972) In Lysosomes: A Laboratory Handbook. (Dingle, J. T., ed.), pp. 1–44, North-Holland, Amsterdam
30 Wattiaux, R. and Wattiaux-de Coninck, S. (1967) Influence of the injection of "Triton WR-1339" on lysosomes of a rat transplantable hepatoma. Nature **216**, 1132–1133
31 Tribl, F., Gerlach, M., Marcus, K., Asan, E., Tatschner, T., Arzberger, T., Meyer, H. E., Bringmann, G. and Riederer, P. (2005) Subcellular proteomics of neuromelanin granules isolated from the human brain. Mol. Cell. Proteomics **4**, 945–957
32 von Figura, K. and Hasilik, A. (1986) Lysosomal enzymes and their receptors. Annu. Rev. Biochem. **55**, 167–193
33 Sonderfeld-Fresko, S. and Proia, R. L. (1989) Analysis of the glycosylation and phosphorylation of the lysosomal enzyme, β-hexosaminidase B, by site-directed mutagenesis. J. Biol. Chem. **264**, 7692–7697
34 Shipley, J. M., Grubb, J. H. and Sly, W. S. (1993) The role of glycosylation and phosphorylation in the expression of active human β-glucuronidase. J. Biol. Chem. **268**, 12193–12198
35 Gieselmann, V., Pohlmann, R., Hasilik, A. and von Figura, K. (1983) Biosynthesis and transport of cathepsin D in cultured human fibroblasts. J. Cell Biol. **97**, 1–5

36 van der Spoel, A., Bonten, E. and d'Azzo, A. (2000) Processing of lysosomal β-galactosidase. The C-terminal precursor fragment is an essential domain of the mature enzyme. J. Biol. Chem. **275**, 10035–10040
37 Erickson, A. H. and Blobel, G. (1983) Carboxyl-terminal proteolytic processing during biosynthesis of the lysosomal enzymes β-glucuronidase and cathepsin D. Biochemistry **22**, 5201–5205
38 Faust, P. L., Chirgwin, J. M. and Kornfeld, S. (1987) Renin, a secretory glycoprotein, acquires phosphomannosyl residues. J. Cell Biol. **105**, 1947–1955
39 Herzog, V., Neumüller, W. and Holzmann, B. (1987) Thyroglobulin, the major and obligatory exportable protein of thyroid follicle cells, carries the lysosomal recognition marker mannose 6-phosphate. EMBO J. **6**, 555–560
40 Lee, S. J. and Nathans, D. (1988) Proliferin secreted by cultured cells binds to mannose 6-phosphate receptors. J. Biol. Chem. **263**, 3521–3527
41 Purchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., Kovacina, K. S., Roth, R. A. and Marquardt, H. (1988) Identification of mannose 6-phosphate in two asparagine-linked sugar chains of recombinant transforming growth factor-β1 precursor. J. Biol. Chem. **263**, 14211–14215
42 Todderud, G. and Carpenter, G. (1988) Presence of mannose phosphate on the epidermal growth factor receptor in A-431 cells. J. Biol. Chem. **263**, 17893–17896
43 Cacia, J., Quan, C. P., Pai, R. and Frenz, J. (1998) Human DNase I contains mannose 6-phosphate and binds the cation-independent mannose 6-phosphate receptor. Biochemistry **37**, 15154–15161
44 Deuschl, F., Kollmann, K., von Figura, K. and Lubke, T. (2006) Molecular characterization of the hypothetical 66.3-kDa protein in mouse: lysosomal targeting, glycosylation, processing and tissue distribution. FEBS Lett **580**, 5747–5752

Received 7 August 2006/8 November 2006; accepted 15 November 2006
Published as BJ Immediate Publication 15 November 2006, doi:10.1042/BJ20061205