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Biochemical characterisation and lysosomal localisation of the mannose-6-phosphate protein p76 (hypothetical protein LOC196463)

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Short title: Lysosomal localisation of p76

Key words: mannose-6-phosphate, lysosome, proteomics, sub-cellular fractionation, immunofluorescence, post-translational modifications.

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Abbreviations used: 2DE, 2-dimensional electrophoresis; HRP: horseradish peroxidase; LAMP, lysosomal-associated membrane protein; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF, matrix assisted laser desorption ionisation time of flight; Man6P, mannose-6-phosphate; MPR, mannose-6-phosphate receptor; PNGase F, peptide-N-glycosidase F; sCI-MPR, soluble cation-independent mannose-6-phosphate receptor; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide-gel electrophoresis; TBS, Tris buffered saline.

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ABSTRACT

Most soluble lysosomal proteins carry mannose-6-phosphate (Man6P), a specific carbohydrate marker that enables their binding to cellular Man6P receptors (MPRs) 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 this 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 using SDS-PAGE. We named this protein "p76" and it appeared later in the NCBI protein database as the "hypothetical protein LOC196463". In the present report, we describe p76 identification by mass spectrometry and we analyse several of its biochemical characteristics. The presence of Man6P sugars was confirmed by an MPR overlay experiment, showing the direct and Man6P dependent interaction between p76 and the MPR. The presence of 6 N-glycosylation sites was validated by progressive peptide-N-glycosidase F (PNGase F) deglycosylation. Experiments using N-terminus and C-terminus directed anti-p76 antibodies provided insights into p76 maturation. Most importantly, we were able to demonstrate the lysosomal localisation of this protein, initially suggested by its Man6P tags, by both immunofluorescence and sub-cellular fractionation of mouse liver homogenates.

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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 are not yet completely elucidated, lysosomes have been shown to be involved in many other cellular processes, 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 mannose-6-phosphate (Man6P) marker, which is recognised by two Man6P receptors (MPRs), 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 aiming at identifying soluble lysosomal proteins have been performed on Man6P proteins, purified by affinity on immobilised 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], and mainly on brain homogenates [13-15]. Indeed, as lysosomal Man6P protein dephosphorylation is less efficient in the brain than in other organs [13, 16], brain is the most appropriate organ for purification of mature lysosomal proteins through their Man6P sugar.

Our own proteomic study 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 report, we describe its identification as the hypothetical protein LOC196463 and its biochemical characterisation and finally, we effectively demonstrate its lysosomal localisation.

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EXPERIMENTALS

Mass spectrometry analysis and protein N-terminal sequence determination

Proteins were identified by MALDI-TOF peptide fingerprinting or LC-MS/MS analysis as previously described [7, 17]. For N-terminal sequence analysis, Edman degradation of electroblotted proteins [6] was performed by the Laboratoire d'Enzymologie Moléculaire (IBS, Grenoble, France) using an Applied Biosystems gas-phase sequencer (model 492) and an Applied Biosystems Model 140C 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 cDNA was initially obtained by RT-PCR on U937 mRNA with the F1/R1 primers (see *Supplemental data, Table1*). The vector pFLAG-hp76 was generated by inserting the sequence encoding hp76 from which both signal sequence and stop codon had been removed (F2/R2 primers), within 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), and inserted into the pCEP4 expression vector (Invitrogen), generating pCEP4/hp76-Myc-His. An expression vector encoding hp76 without tag was built, by ligation into the pcDNA3.1 vector (Invitrogen) 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).

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

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

Cell lines and transfections

HeLa, U937 and Rat2 cells were from the American Type Culture Collection (ATCC), and 293-EBNA cells were from Invitrogen. Murine JR11 fibroblasts [19] are a kind gift from Dr B. Hoflack (Technical University of Dresden, Germany). They constitutively secrete Man6P proteins because of their deficiency in both MPRs.

HeLa, 293-EBNA, Rat2 and JR11 cells were grown in DMEM-GlutamaxI supplemented with 10% FBS, while U937 cells were maintained in RPMI-GlutamaxI medium supplemented with 10% 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 hygromycin B (0.3 mg/ml, Invitrogen).

Expression and purification of recombinant proteins

The FLAG-p76 recombinant protein was expressed and purified as inclusion bodies from transformed *Escherichia coli* BL21 bacteria as described [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 were collected 3 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 [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 synthesised with an additional cysteine residue (NeoMPS, Strasbourg, France) and coupled to activated maleimide ovalbumin (Pierce). The lyophilised antigens were injected into rabbits (Charles River Laboratories or NeoMPS; [21]) to raise respectively the Irp76Ab, Crp76Ab and Nhp76Ab antisera.

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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 nor the specificity of the antisera, these were used without purification. Non-immune sera did not reveal any significant protein species.

Electrophoresis, Western blot and MPR overlay

SDS-PAGE separation of the reduced proteins and 2DE were performed as described [7, 22]. Proteins were stained by silver nitrate or colloidal blue (Bio-Safe, Bio-Rad).

Western blot analyses [21] were performed with the following primary antibodies: i) rabbit polyclonal antibodies: hp76Ab, 1/1000; Nhp76Ab, 1/500; Crp76Ab, 1/1000; Irp76Ab, 1/400; anti-mitochondrial ATPase (anti-F_0 subunit), 1/3000 (generous gift of Drs Dupuis and Lunardi); ii) mouse monoclonal anti-LAMP1, 1/500 (1D4B, Developmental Studies Hybridoma Bank); iii) goat polyclonal anti-mouse CD98, 1/200 (M-20, Santa Cruz). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit (1/50000, Pierce), goat anti-mouse (1/3000, Bio-Rad), or mouse anti-goat (1/10000, Sigma-Aldrich). Signal detection was performed using the SuperSignal West Pico chemiluminescent substrate (Pierce) and photographic revelation.

MPR overlay assays were adapted from Valenzano *et al.* [23]. Soluble CI-MPR (sCI-MPR, purified as described in [6]) was biotinylated using the Biotin Protein Labeling Kit (Roche), according to the manufacturer protocol. 2DE-resolved Man6P proteins were electro-transferred onto nitrocellulose membranes (Bio-Rad), that were blocked in TBS, 0.1% Tween-20, 3% (w/v) BSA, incubated with biotinylated sCI-MPR in TBS, 0.1% Tween-20, 1% BSA with or without Man6P (Sigma-Aldrich), and then with neutravidin-HRP (1/75000; Pierce) in TBS, 0.1% Tween-20, 1% BSA. Bound biotinylated sCI-MPR was revealed using the SuperSignal West Pico chemiluminescent substrate (Pierce) and photographic revelation.

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 immobilised on Epoxy membranes (Sartorius).

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

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Carbohydrate modification analysis

Purified U937 Man6P proteins were deglycosylated by PNGase F (E.C. 3.5.1.52, Roche), according to the manufacturer's instructions. The reaction was stopped by 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, in methanol:acetone (50:50) previously chilled 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); Cy3-conjugated goat anti-rabbit, 1/1500 (Jackson); Alexa 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 (20g) that received, when specified, an intravenous injection of 17 mg 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 as described [25].

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

Enzymatic assays

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

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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 [6, 7]. One of these novel proteins, which was not mentioned in these papers, was initially identified on a 2DE gel of a U937 sample, in a series of spots of neutral to alkaline pI (approximately pH 7-8) and at a molecular mass of 70 kDa by a unique MS/MS peptide sequence (LASDGATWADIFK, residues 355-367 of LOC196463). These spots were hardly detected by colloidal blue staining (Figure 1A) and were not visible on MCF7 2DE gels. The same protein was also identified from a series of spots of more acidic pI (approximately pH 5) and migrating at a slightly higher molecular mass of 76 kDa, in both U937 and MCF7 samples (Figure 1A and *Supplemental data, Table 2*). We named it "hp76", due to its apparent molecular mass. In the U937 sample, 2 peptides were identified by LC-MS/MS. In the MCF7 sample, 11 peptides of hp76 were identified by MALDI-TOF mass 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 7 tryptic peptides of hp76 in the U937 sample, and 9 peptides in the MCF7 one (respective coverages, 13 and 17%; *Supplemental data, Table 2*).

This novel protein, reported in the NCBI protein database as the "hypothetical protein LOC196463", is 589 amino acids long with a predicted signal peptide of 41 amino acids (*Supplemental data, Figure 1*). Since 6 potential N-glycosylation sites are predicted, 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 analogy are found in several mammals and other species, namely *Caenorhabditis elegans*, *Dictyostelium discoideum* and *Trypanosoma brucei* (*Supplemental data, Table 3*). However, no similar protein is found in yeast. All these proteins are members of a family, based on the presence of a domain named "Laminin A domain". This name likely 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 (*Supplemental data, Figure 1*). The mouse hypothetical protein LOC71772 is 594 amino acids long and shares 82% identity and 91% similarity with hp76 over a 558 amino acids alignment, whereas the rat hypothetical

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protein LOC246120 (Ensembl peptide ID ENSRNOP00000001872), is 585 amino acids long and shares 82% identity and 92% similarity with hp76 over a 547 amino acid alignment. The similar protein expressed in the amoeba *Dictyostelium discoideum* is of particular interest, because it is the only one for which a function (phospholipase B) has been demonstrated *in vitro* so far [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, a 1767 bp ORF and a 789 bp 3'-UTR containing a polyadenylation signal and a short poly(A) stretch.

A Northern blot experiment (*Supplemental data, Figure 2*) showed that a transcript migrating at 2.4 kb, consistent with hp76 cDNA length, is found in all tissues studied, with a 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 2 additional transcripts is unknown.

Characterisation 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 either mock-transfected 293-EBNA cells (not shown) or 293/hp76-myc cells showed that 3 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 (*Supplemental data, Table 4*). Edman analysis of the 32 kDa species identified position 42 at its N-terminus, indicating that this fragment is N-terminal (Figure 2B, and *Supplemental data, Table 4*). As for the 50 kDa fragment, it starts at position 244 of the preprotein (Figure 2B, and *Supplemental data, 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

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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 revealed a strong band of 76 kDa as well as a faint 32 kDa one (Figure 2A, lane 4), while Crp76Ab detected the same 76 kDa band and a 45 kDa doublet (Figure 2A, lane 7).

Therefore, in both our models, 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 identically produced from hp76 or hp76-myc, whereas the C-terminal fragment appears as a doublet of 45 kDa in 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 *versus* 80 kDa) and between the C-terminal polypeptides (45 kDa *versus* 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 mouse p76 (mp76) allowed us to visualise 3 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 (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 of a rat cell line, Rat2 (not shown), or of a mouse cell line, JR11 [19], only the 76 kDa and 40 kDa polypeptides were revealed (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 inside the cell only. 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

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major intracellular 27 kDa fragment. This 27 kDa fragment will now be referred to as "mp27" (Figure 2D).

Human p76 precursor glycosylation

All potential glycosylation sites are glycosylated

To determine the actual number of occupied N-glycosylations sites, endogenous Man6P proteins purified from U937 cells were subjected to a time-course deglycosylation by PNGase F and subsequently analysed by western blot, using 3 anti-p76 antibodies: the previously mentioned hp76Ab and Crp76Ab antibodies, and Nhp76Ab, an antibody raised against the N-terminal peptide of hp76. Several bands of decreasing molecular masses ranging from 76 kDa to approximately 60 kDa were progressively produced (Figure 3A). Progressive deglycosylation of the 45 kDa C-terminal doublet gave rise to 2 additional bands of lower molecular masses, with the fully deglycosylated polypeptide migrating at 38 kDa (Figure 3B). Therefore, the initial doublet likely represents di-glycosylated and tri-glycosylated forms of the 38 kDa fragment, present in approximately equivalent amounts among U937 Man6P proteins. Similarly, deglycosylation of the 32 kDa N-terminal fragment produced 3 additional species of decreasing molecular masses, indicating the presence of 3 glycosylations on the 22 kDa polypeptide backbone (Figure 3C). Taken together, these data indicate that all 6 hp76 predicted N-glycosylation sites are actually glycosylated.

p76 is a Man6P protein

Although the specificity of the purification procedure was previously demonstrated [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 mass spectrometry (Figure 1A and *Supplemental data, Table 2*) and western blot (Figure 1B), we could identify hp76 among the proteins recognised by the biotinylated sCI-MPR (Figure 1C). Addition of free Man6P effectively competed this labelling (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.

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Human and mouse p76 co-localise with lysosomal markers

As the presence of Man6P sugars on p76 suggested that p76 might be a lysosomal protein, its localisation 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-localised with both of them to a large extent (Figure 4).

A similar immunofluorescence study was carried out on HeLa cells overexpressing mouse p76, leading to the same results. The intracellular distribution of mp76 also consisted in a vesicular staining mainly co-localising with lysosomal markers, as determined by cathepsin D or LAMP1 co-labelling (not shown).

Sub-cellular fractionation shows a lysosomal distribution for mp27, the 27 kDa C-terminal polypeptide fragment of maturated mouse p76

The sub-cellular localisation of endogenous p76 was established by determining the distribution of mp27, the 27 kDa C-terminal polypeptide fragment of mouse p76 (Figure 2D), in fractions obtained by sub-cellular fractionation of liver homogenates [29]. In the following study, experiments were performed on both rat and mouse samples. As we obtained similar results for both species, experiments carried out on mouse samples only are presented here.

Differential centrifugation

Differential centrifugation of mouse liver homogenates was performed to separate nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and soluble (S) fractions [24]. As shown in Figure 5A, the distribution of mp27, LAMP1, mitochondrial ATPase F_0 subunit, and CD98 were determined by western blot analysis, 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 [24]: bar height (specific activity) is indicative of the lysosome purification in the N, M, L, P and S fractions. As shown in Figure 5A, a strong mp27 signal was detected in the total mitochondrial fraction (M and L) with the strongest signal in the L fraction. This pattern is highly indicative of liver lysosomes, as shown by the distributions of LAMP1 and lysosomal enzymes. It should be noted that a 29 kDa fragment was also detected

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with Crp76Ab in all fractions (Figure 5A). Although this signal is not detected by the non-immune serum (not shown), we assume that it is not related to mp76 because i) Irp76Ab does not reveal this 29 kDa band in those fractions (not shown) and ii) Crp76Ab and Irp76Ab detect this band neither in HeLa cells overexpressing mp76 nor in mouse brain purified Man6P proteins (Figure 2C). The distribution of the mitochondrial ATPase F_0 subunit (strongest signal in the M fraction) and the plasma membrane protein CD98 (in N and P fractions) agreed with the previously reported distribution of these sub-cellular compartments from liver samples [24]. These patterns were clearly distinct from that of lysosomal markers.

Density gradient fractionation

An ML fraction was prepared and further analysed by isopycnic centrifugation on a linear sucrose density gradient. Lysosomes, represented by β-galactosidase and β-hexosaminidase, 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 5C, 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 an hypo-osmotic treatment [26] and compared the release of mp27 to 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 "differential centrifugation" section) was never released from the membrane fraction.

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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 present study, human p76 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 [31]. Human p76 is a 589 residue protein with a putative 41 amino acid signal peptide and 6 potential glycosylation sites.

All potential glycosylations sites of human p76 are glycosylated, and some of them are effectively mannose-6-phosphorylated

The extent of human p76 glycosylations was determined on U937 Man6P proteins, and we unambiguously showed that all 6 potential hp76 glycosylation sites were occupied. Some glycosylation heterogeneity was observed, residing on either one of the 3 positions 436, 465, or 515. Such heterogeneous glycosylations 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 [15], Sleat *et al.* did not observe heterogeneous glycosylation at any of these 3 positions. This discrepancy might be due to the respective p76 sources, *i.e.* brain Man6P proteins *versus* 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, without being mannose-6-phosphorylated itself, we checked hp76 Man6P status. 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 Lobel's group, which detects the presence of Man6P on 5 glycopeptides of brain hp76 [15]. 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 2 Man6P-glycopeptides was identified per protein. Only 2 hydrolases displayed 5 or more Man6P-glycopeptides: the acid ceramidase (5 peptides) and the N-acetylglucosamine-6-

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sulfatase (9 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 6 human p76 glycosylation sites are occupied (our study), only 5 of them are mannose-6-phosphorylated. The N-glycosylation in position 110 of hp76 could be of the complex type and therefore absent from the Man6P glycopeptides identified by Sleat *et al.*. 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 2 mannose-6-phosphorylated N-glycosylations out of their 4 N-glycosylated sites [33, 34].

Human and mouse p76 are subject to maturation

In human secreted 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 on 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 2 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 (this study) *versus* the intracellular protein 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], murine p76 was found on a 2DE gel in 3 series of 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

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difference in the apparent molecular masses of the p76 precursor (76 kDa *versus* 66 kDa) in our study and in Kollmann's one 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 proteolytically processed so that the mature forms are hetero-dimers 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 *versus* mouse; this 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 localisation of any newly identified Man6P protein. Accordingly, Sleat and collaborators defined 3 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 localisation. Hence, among the 44 Man6P proteins that were identified in plasma and were not known to be lysosomal, 9 candidates were selected, among which hp76 [11]. Besides, the identification of hp76 in a proteomic analysis of neuromelanin granules from human brain also brought support to the hypothesis that p76 could be a lysosomal protein [31]. Indeed, the authors demonstrated that neuromelanin granules belonged to the lysosome-like organelles family. Moreover, Kollmann *et al.* have shown that purified recombinant mp76 was internalized via MPR-mediated endocytosis [8], giving another argument in favor of a lysosomal localisation of p76.

To determine p76 sub-cellular localisation, we firstly used immunofluorescence on cells overexpressing either human or mouse p76, and showed a clear co-localisation of p76 with the lysosomal markers LAMP1 and Cathepsin D. To analyse the sub-cellular localisation 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

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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 localisation 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 *Dictyostelium 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.

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Note: While the present report 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.

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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) alpha-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

Copyright 2006 Biochemical Society

12 Sleat, D. E., Chen, T. L., Raska, K., Jr. 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, W. J., Jr., 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, NY

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

Copyright 2006 Biochemical Society

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, beta-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 beta-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

Copyright 2006 Biochemical Society

36 van der Spoel, A., Bonten, E. and d'Azzo, A. (2000) Processing of lysosomal beta-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 beta-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

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FIGURE LEGENDS

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, 10% SDS-PAGE gel (20×20 cm) before colloidal blue staining (modified from [7] with permission). (B to D) Man6P proteins purified from 200×10^6 U937 cells were resolved by 2DE over a 4-8 linear pH range, 10% SDS-PAGE minigels (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. White oval: main series of hp76 spots; black oval: position of the minor series of hp76-containing spots which could only be detected with high protein amounts.

Figure 2: Human and mouse p76 maturation

(A) Characterisation of human p76-derived polypeptides. Protein samples were loaded onto a 12% SDS-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. Lane 4 is overexposed compared to the other lanes.

(B) Hypothetical maturation scheme for human p76. hp76Ab targets epitopes in the N-terminal part of hp76 (light grey). Crp76Ab targets the C-terminal peptide of hp76 (black).

(C) Characterisation of mouse p76-derived polypeptides. Protein samples were loaded onto a 12% SDS-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 mouse p76. Crp76Ab targets the C-terminal peptide of rat and mouse p76 (black). Irp76Ab targets an internal peptide of rat and mouse p76 (dark grey).

Figure 3: hp76 N-glycosylations

U937 Man6P proteins were subjected to deglycosylation by PNGase F for the indicated times, loaded onto 7.5% (A; equivalent of 6×10^6 cells/lane) or 9% (B, C; equivalent of 30×10^6 cells/lane) SDS-PAGE gels and transferred for western blot analysis. Additional PNGase F

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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 revealed with hp76Ab (A), the C-terminal fragment with Crp76Ab (B) and the N-terminal fragment with Nhp76Ab (C).

Figure 4: Co-localisation 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, in red) and LAMP1 (A) or cathepsin D (B) (in green). Arrowheads denote some vesicles that are positive for both stainings. Scale bars, 10 μm.

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, 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 a 10% SDS-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 a 12% SDS-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.

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Figure 6: Osmotic release of mp27

Aliquots from a freshly prepared ML fraction were incubated 15 minutes 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 supernatants (S) proteins were separated on a 12% SDS-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.

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FIGURES

Figure 1

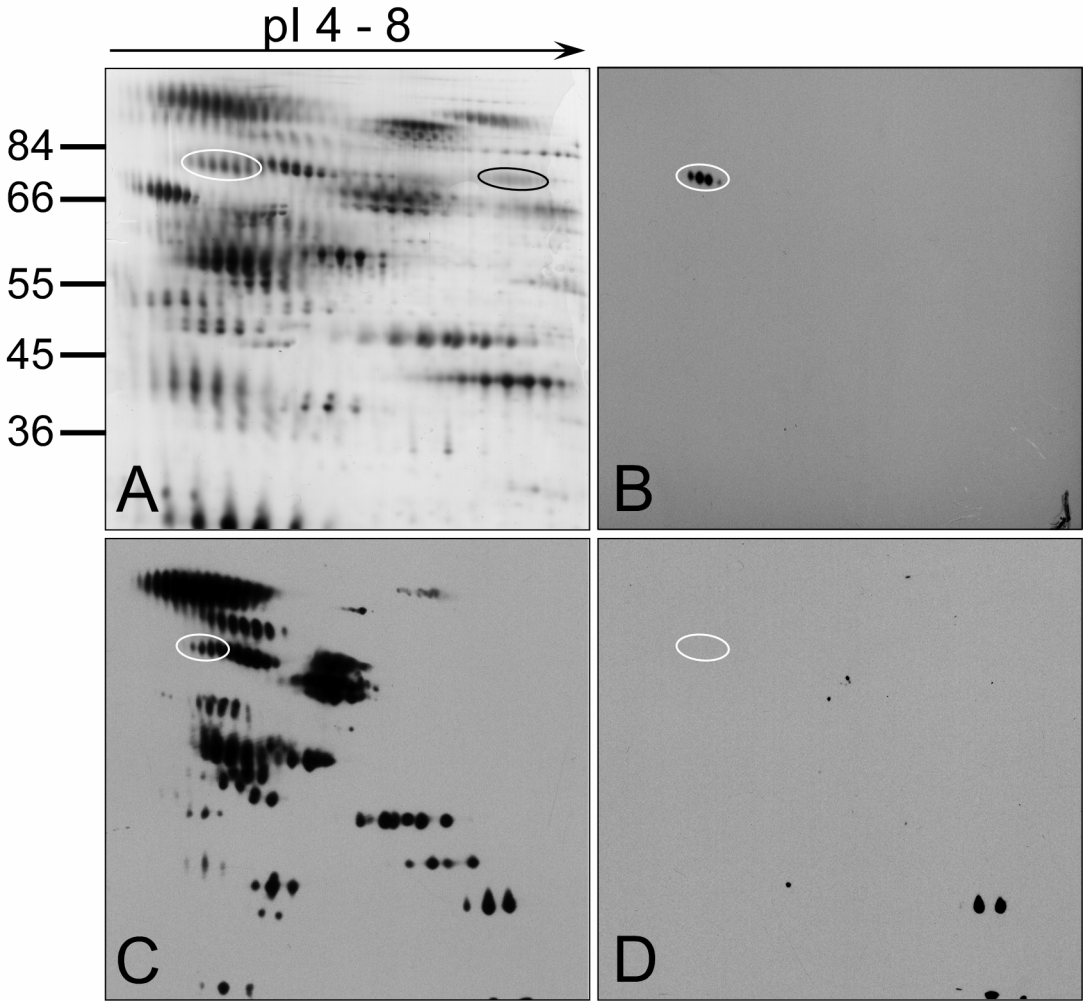

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Figure 2

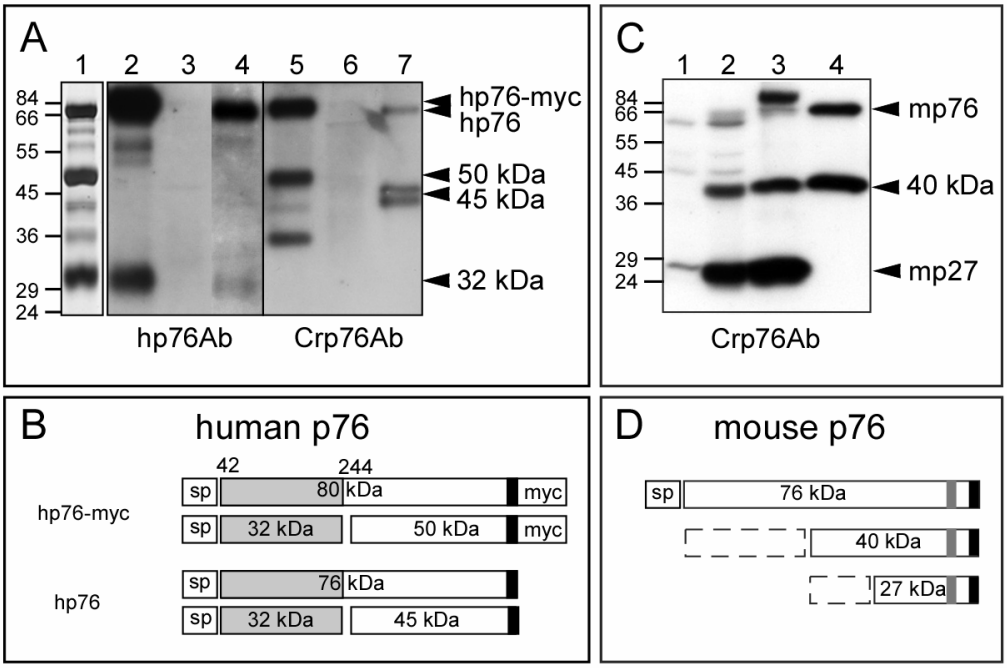

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Figure 3

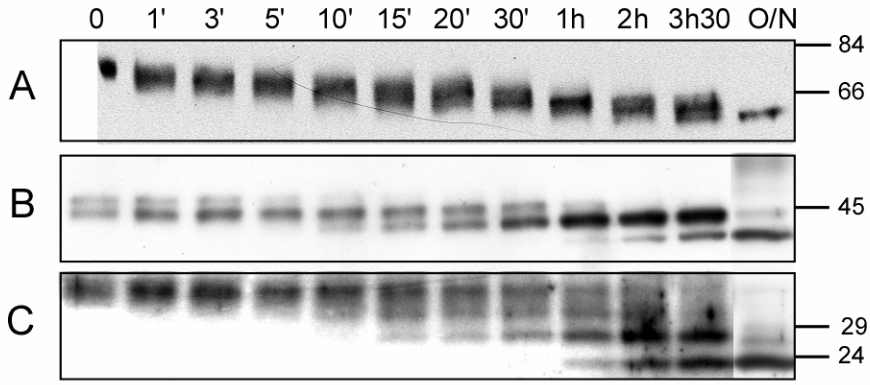

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Figure 4

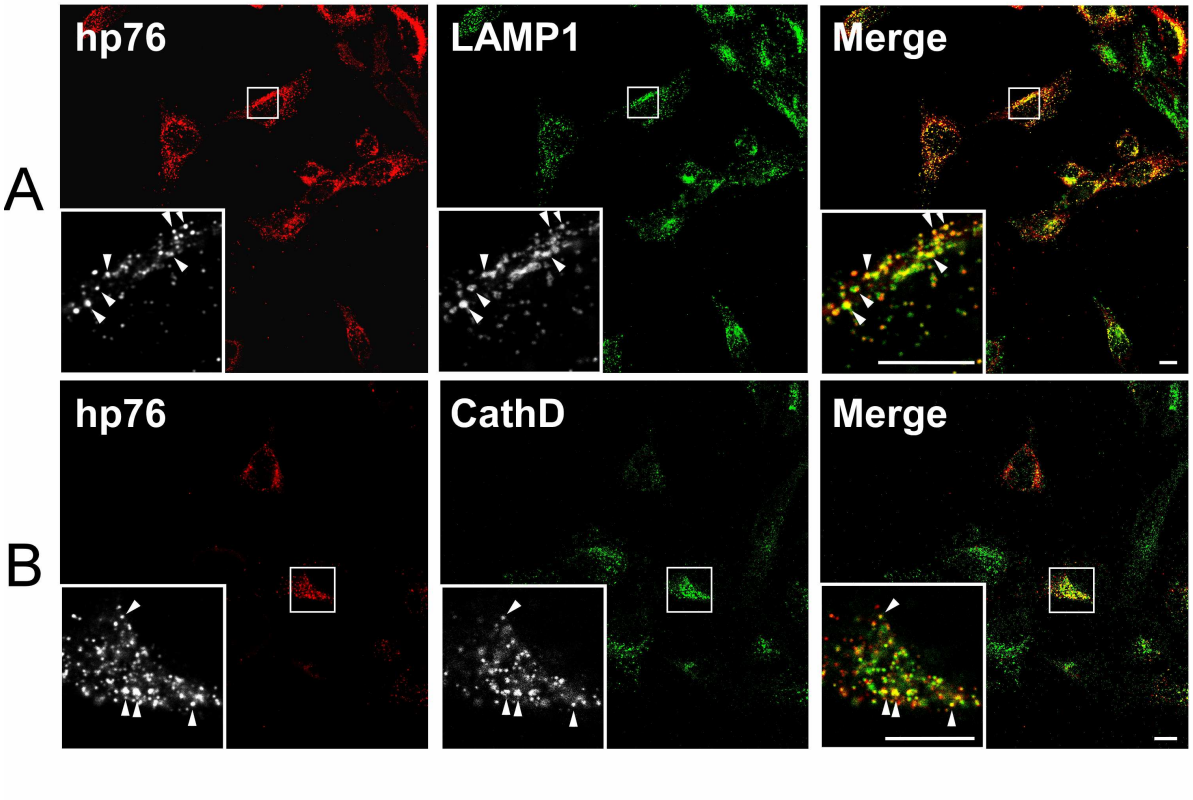

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Figure 5

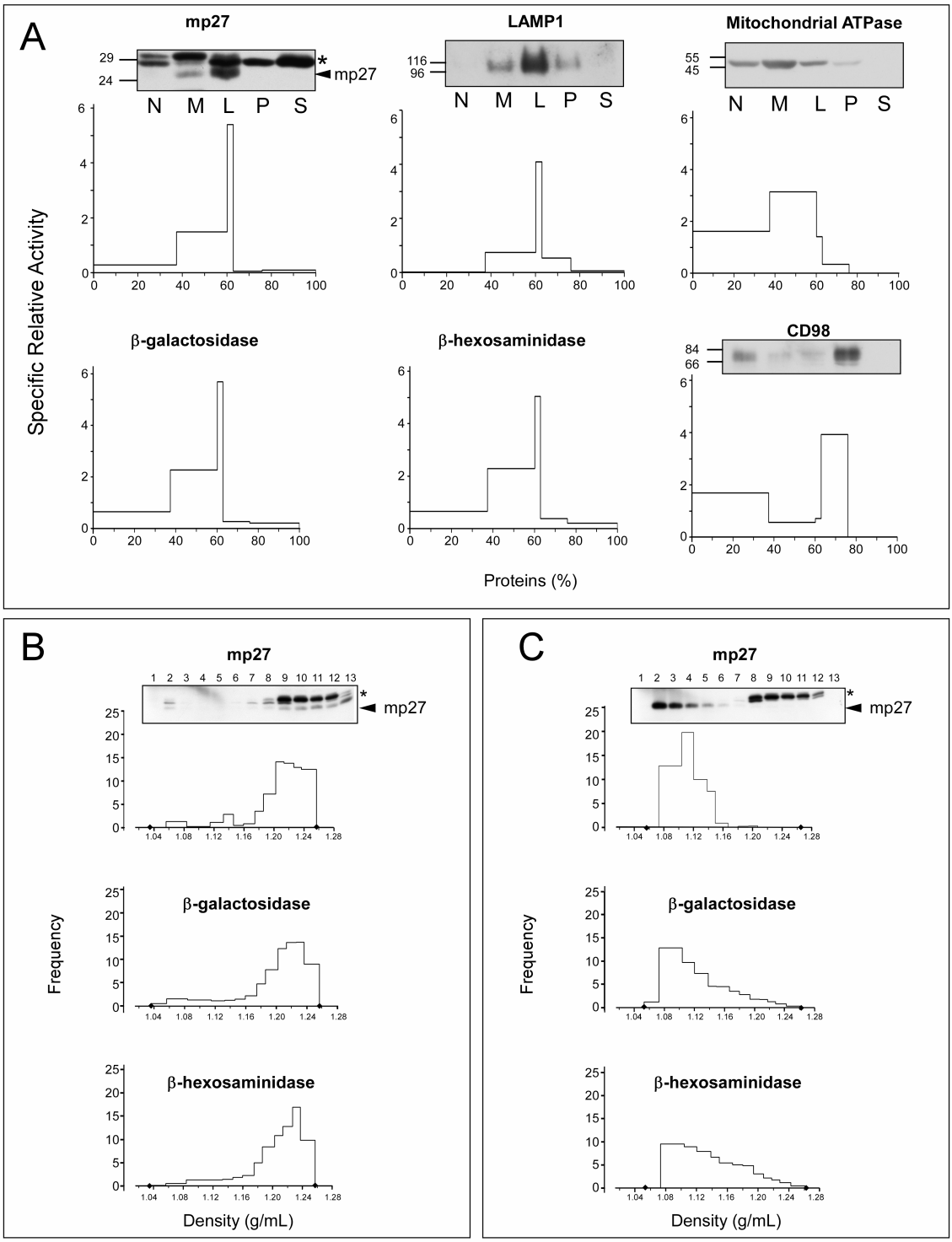

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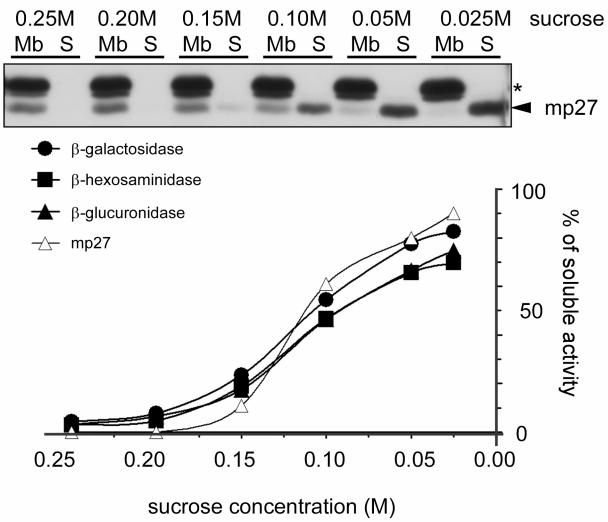
Figure 6



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