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Molecular characterization of the hypothetical 66.3-kDa protein in mouse: Lysosomal targeting, glycosylation, processing and tissue distribution

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Abstract **Recently, we and others identified the 66.3-kDa protein as one of several putative novel lysosomal matrix proteins by analyzing mannose 6-phosphate receptors binding proteins [Kollmann K., Mutenda K.E., Balleininger M., Eckermann E., von Figura K., Schmidt B., Lübke T. (2005) Identification of novel lysosomal matrix proteins by proteome analysis. Proteomics 5(15), 3966–3678, Sleat D.E., Lackland H., Wang Y., Sohar I., Xiao G., Li H., Lobel P. (2005) The human brain mannose 6-phosphate glycoproteome: a complex mixture composed of multiple isoforms of many soluble lysosomal proteins. Proteomics. 5(6), 1520–1532]. Here, we describe the expression of the mouse 66.3-kDa protein in HT1080 cells in which it is synthesized as a precursor of about 75 kDa and subsequently processed by limited proteolysis to mature polypeptides accumulating in the lysosomal compartment. The lysosomal localisation of the endogenous 66.3-kDa protein was verified by indirect immunofluorescence in mouse embryonic fibroblasts and by subcellular fractionation of tyloxapol-filled mouse liver lysosomes. Northern blot analysis reveals high transcriptional levels in testis, liver and kidney, whereas Western blot analysis shows high protein levels in brain, heart, lung and spleen. Interestingly, in mouse the endogenous 66.3-kDa protein is processed in a highly tissue-dependent manner to mature forms.**

Keywords: 66.3-kDa protein; Lysosomes; Mannose 6-phosphate; Lysosomal localisation; Lysosomal processing; Lysosomal storage diseases

1. Introduction

Recently, a number of proteomic studies have been performed on lysosomes and the lysosomal matrix [1–3]. They have been stimulated for at least two particular aspects: (i) Most of the approximately 50 known lysosomal matrix proteins are acid hydrolases that degrade macromolecules entering the lysosomes. Generally, loss-of-function mutations of lysosomal hydrolases manifest as lysosomal storage diseases (LSDs). Dependent on the affected protein and the severity of the deficit, the more than 40 known LSDs show a wide variety of different symptoms but share some common features like lysosomal storage of substrates of the hydrolases, a progressive clinical course, recessive mode of inheritance and – in many cases – neurodegenerative involvement [4]. The high likeliness that novel lysosomal proteins direct to LSDs of yet unknown etiology justifies the search for novel lysosomal proteins and their characterization. (ii) The lysosomal matrix proteins offer a particular advantage for an organelle proteome analysis as these proteins receive mannose 6-phosphate (M6P) residues as a unique lysosomal sorting signal. In vivo, M6P-tagged lysosomal matrix proteins bind to M6P-receptors (MPRs), which mediate their trafficking to the lysosomes. The M6P-tag can easily be exploited for affinity purification on immobilized MPRs. In former studies, we and other groups took advantage of this approach [1–3]. Beside a number of well-known lysosomal matrix proteins, we were able to identify at least three candidate proteins of the lysosomal matrix: mammalian ependymin-related protein-2 (MERP-2), retinoid-inducible serine carboxypeptidase (RISC) and the hypothetical 66.3-kDa protein (cDNA Accession No. BC038605; Protein Accession No. AAH38605). We verified that C-terminally tagged derivatives of the three candidate proteins bind in an M6P-dependent manner to immobilized MPRs and are internalized by MPR-mediated endocytosis. To characterize the hypothetical 66.3-kDa protein, we purified the protein and raised an antiserum against it. The antiserum allowed us to verify the lysosomal localisation of the endogenous 66.3-kDa protein in mouse and its tissue specific processing to multiple mature polypeptides. In HT1080 cells the putative protein is synthesized as a 75 kDa precursor, in which all five potential *N*-glycosylation sites are utilized. The precursor is processed into multiple discrete and stable fragments.

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Abbreviations: MPR, mannose 6-phosphate receptor; M6P, mannose 6-phosphate; MEFs, mouse embryonic fibroblasts; LSDs, lysosomal storage diseases

2. Materials and methods

2.1. Cell lines and cell culture

If not stated different cell lines were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, GIBCO Life Technologies) containing 10% FCS (PAN Biotech GmbH).

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2.2. Antibodies

Monoclonal antibodies were purchased as followed: α-RGS-His6-Tag (Qiagen), α-Hsp60 and α-Porin (Calbiochem), α-LAMP-1 (1D4B, Developmental Studies Hybridoma Bank). HRP-conjugated secondary antibodies were supplied by Dianova.

2.3. Cloning, transfection and protein expression of the 66.3-kDa protein

We subcloned the 66.3-kDa cDNA [1] by add-on PCR using a RGS-His6 containing reverse primer into the pcDNA3.1/Hygro(+) vector (Invitrogen). HT1080 cells were transfected with Lipofectamine 2000 following the protocol recommended by Invitrogen. After selection with hygromycin (200–600 μg/ml, Calbiochem), colonies were picked and grown with 600 μg/ml hygromycin.

2.4. Purification of recombinant 66.3-kDa protein

66.3-kDA expressing HT1080 cells were shifted to 0.05% FCS in DMEM. Medium including cell debris was collected three times every 48 h and subjected to ammonium sulfate precipitation. After dialyzation to PBS, the 66.3-kDa protein-His6 was purified by Ni-NTA agarose and eluted as recommended by Qiagen. The eluate was dialyzed to PBS and subjected to HPLC anion exchange chromatography (BiocadVision, Applied Biosystems) by applying a step-wise gradient up to 500 mM NaCl in PBS. Purification was monitored by silver staining and Western blotting.

2.5. Mass spectrometry and Edman degradation

Peptide mass fingerprint analysis was performed according to [1]. For N-terminal sequencing the samples were subjected to Edman degradation on a Procise cLC sequenator (Applied Biosystems) [5].

2.6. Deglycosylation experiments

For deglycosylation with PNGase F, cell lysates from HT1080 cells stably expressing the 66.3-kDa protein were treated with PNGase F (Roche) according to [5].

2.7. Indirect immunofluorescence microscopy

To detect endogenous expression of 66.3-kDa protein and colocalization studies, methanol-fixed cells were incubated with a 66.3-kDa protein antiserum and α-LAMP-1 and visualized by secondary antibodies as described in [1].

2.8. Lysosome (tritosomes) isolation

The isolation procedure was essentially performed as described before by [6] but was adopted from rat to mouse by injecting 0.75 mg/g body weight tyloxapol four days prior to the subcellular fractionation. Marker enzyme activity determinations were performed according to [6].

2.9. Metabolic labeling of cells with [^{35}S] methionine/cysteine

(Hartmann Analytic) followed by immunoprecipitation with the 66.3-kDa protein antiserum, SDS–PAGE and autoradiography was performed as described earlier for cathepsin D [7].

2.10. Other methods

Bioinformatic analysis has been performed as described in [1] using NCBI BLAST and Vector NTI software (Invitrogen). Northern blot analysis was carried out as described [8].

3. Results

3.1. Heterologous expression and purification of 66.3-kDa protein from HT1080 cells

The expression of the formerly described V5-His6-double tagged version of the 66.3-kDa protein [1] resulted only in low protein levels. The murine 66.3-kDa protein cDNA could be stably expressed at high levels in HT1080 cells as a C-terminally His6-tagged derivative. The 66.3-kDa protein was purified from the secretions of these cells by a combination of Ni-NTA affinity and anion exchange chromatography.

SDS–PAGE separated the purified protein into six polypeptides ranging from 75 kDa to ~14 kDa (Fig. 1A, lane 1). To confirm that these polypeptides correspond to the 66.3-kDa protein, the polypeptides visualized by Coomassie staining were analyzed by mass spectrometry peptide mass fingerprint (MS PMF) or blotted onto a membrane, stained with Coomassie (Fig. 1A, lane1) and analyzed by Edman degradation. N-terminal sequencing (Edman degradation) of the major 75 kDa polypeptide identified a sequence predicted for the 66.3-kDa protein starting with leucine at position 47. Edman analysis of the 66 kDa polypeptide yielded the same N-terminal sequence starting with Leu47. PMF identified 13 peptides of the 66.3-kDa protein, covering 33% of the entire protein sequence. The N-terminus of the 40 kDa polypeptide started with Cys249 of the 66.3-kDa protein. PMF identified eight peptides of the 66.3-kDa protein all located C-terminal of Cys249. Edman analysis for the 28 kDa polypeptide identified Leu47 at the N-terminus while the N-terminus of the 15/14 kDa polypeptides corresponded to Ser514 of the 66.3-kDa protein.

The purified protein (Fig. 1A) was used to generate a rabbit polyclonal antiserum against the full length 66.3-kDa protein. Western blot analyses of purified protein with the 66.3-kDa antiserum showed high immunoreactivity of the serum against the 75, 66 and 28 kDa form but only weak reactivity with the 40 and 15 kDa forms (Fig. 1A, lane 2). Western blot analyses with an antibody directed against the C-terminal His6-tag of the 66.3-kDa protein detected the 75, 40 and the 15 kDa forms (Fig. 1A, lane 3) indicating that these polypeptides carry the C-terminus of the 66.3-kDa protein. The 66 kDa polypeptide was not detected by the anti-His6 antibody suggesting that this form is C-terminally truncated.

We were interested to see whether the seven cysteines located between position 147 and 562 of the 66.3-kDa protein contribute to a covalent linking of the different polypeptides of the purified protein. Under non-reducing conditions, we observed an aggregation of the protein that prevented electrophoretic separation. This aggregation was not observed in cell lysates of HT1080 cells stably expressing the 66.3-kDa protein. In HT1080 cells, the 66.3-kDa protein was represented by 75, 40, 28 and 15/14 kDa polypeptides (Fig. 1B, lane 2). Their electrophoretic mobility was not or only slightly affected (Fig. 1B, lane 3) when reducing agents were omitted from the electrophoresis buffers indicating that the different polypeptides are not linked to each other by disulfide bonds.

3.2. N-Glycosylation of 66.3-kDa protein derived from HT1080 cells

Cell lysates of HT1080 cells expressing the 66.3-kDa protein were treated with PNGase F for up to 6 h, separated by SDS–PAGE and analyzed by Western blotting using the 66.3-kDa antiserum. After treatment with PNGase for 30 min and 1 h, the 75 kDa form became partially deglycosylated and after 3 h only the ~66 kDa fully deglycosylated form remained detectable (Fig. 1C). The 40 kDa form was deglycosylated via an intermediate to a ~35 kDa form indicating that it carries two N-linked glycans. The 28 kDa form was deglycosylated via three intermediates to a ~19 kDa form indicating that it carries 3N-linked glycans (Fig. 1C). The glycosylation of the different polypeptides and their position relative to the N- and C-terminus of the precursor of the 66.3-kDa protein are summarized in Fig. 1D.

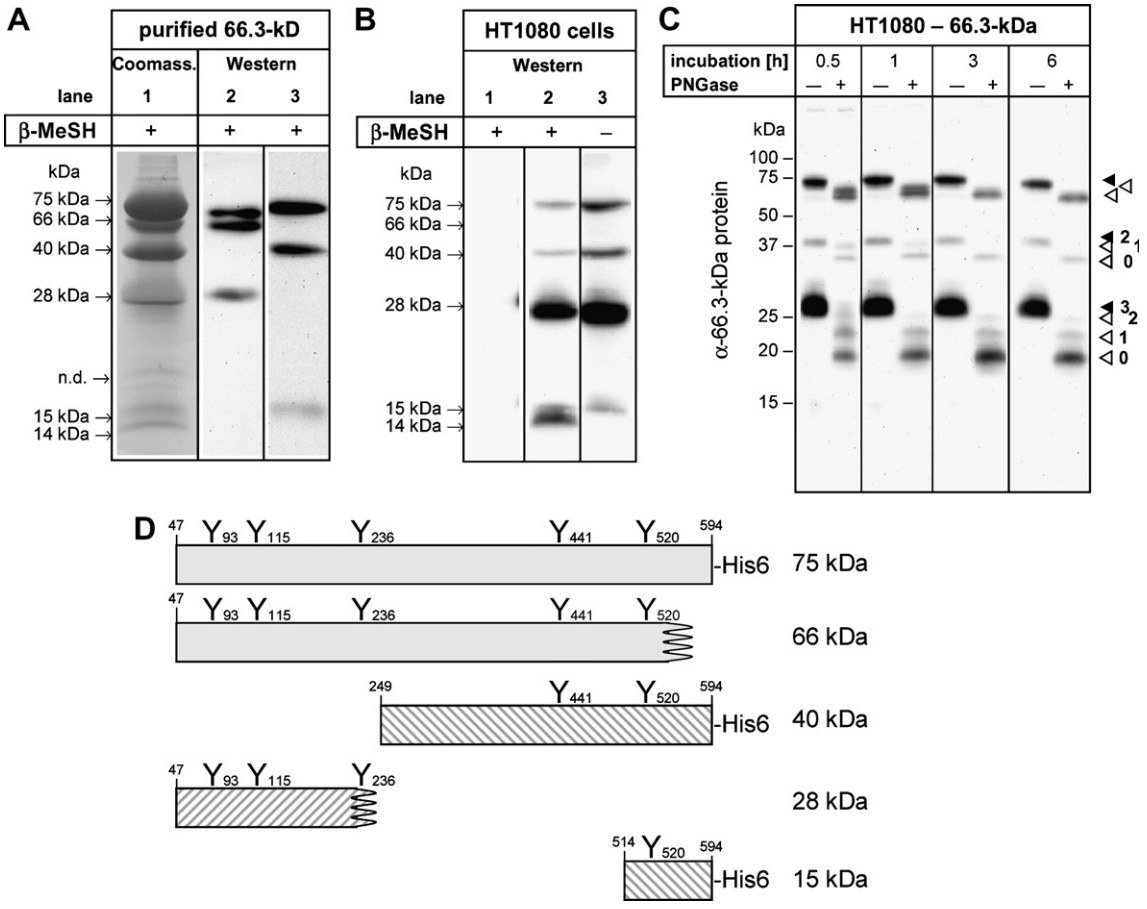

Fig. 1. Molecular forms of 66.3-kDa protein in HT1080 cells. (A) Murine 66.3-kDa protein with a C-terminal His6-tag stably expressed in HT1080 cells was purified using Ni-NTA affinity and anion exchange chromatography, separated on SDS–PAGE under reducing conditions, blotted onto a membrane and stained by Coomassie (lane 1) or analyzed by Western blot using either the 66.3-kDa antiserum (lane 2) or a monoclonal antibody against the C-terminal His6-tag (lane 3). (B) 50 μg of cell extracts of non-transfected HT1080 cells (lane 1) and HT1080 cells stably expressing the His6-tagged 66.3-kDa protein (lanes 2 and 3) were separated by SDS–PAGE under reducing (lanes 1 and 2) or non-reducing (lane 3) conditions and analyzed by Western blotting using the 66.3-kDa protein antiserum. (C) 50 μg of HT1080 cells stably expressing 66.3-kDa protein were treated with PNGase for up to 6 h, separated by SDS–PAGE and analyzed by Western blotting using the 66.3-kDa protein antiserum. The filled arrowheads (◀) point to the different glycosylated forms with the number of their *N*-glycans. The open arrowheads (◁) point to deglycosylated 66.3-kDA forms. (D) Scheme of the polypeptides seen in the purified 66.3-kDa protein fraction and their *N*-glycosylation sites.

3.3. Localisation of endogenous 66.3-kDa protein

To localize endogenous 66.3-kDa protein, mouse embryonic fibroblasts were analyzed by indirect immunofluorescence with the antiserum against the murine 66.3-kDa protein. The 66.3-kDa protein perfectly colocalizes with LAMP-1 (Fig. 2A), a lysosomal membrane protein. In the confocal images, the LAMP-1 signal appears as a ring-like structure surrounding the lysosomal matrix protein 66.3-kDa protein.

3.4. Localisation of 66.3-kDa protein in tyloxapol-filled lysosomes

Exposing cells to tyloxapol (Triton WR1339) leads to uptake of tyloxapol into lysosomes and lowering of the density of lysosomes [9]. Tyloxapol-loaded rat liver lysosomes can be easily separated from other organelles by a combination of differential centrifugation and a single discontinuous sucrose-density gradient [6]. We adopted this method to mouse liver and typically obtained in the final fraction a 40–60-fold enrichment of lysosomal β-glucuronidase and β-hexosaminidase, with only traces of the mitochondrial marker succinate dehydrogenase and the ER marker glucose-6-phosphatase (see Supplementary Material Table 1, fraction F2). Western blot analysis of the fractions obtained after sucrose density gradient centrifugation revealed two major signals at 30 kDa and 15 kDa in the lysosomal fraction F2. The lysosomal marker LAMP-1 was markedly enriched in the lysosomal fraction F2, whereas the mitochondrial marker porin and Hsp60 are absent from F2 (Fig. 2B). These results confirm the lysosomal localisation of the 66.3-kDa protein.

3.5. Tissue distribution of the 66.3-kDa protein

Transcripts for the 66.3-kDa protein in mouse multiple tissue Northern blots were examined with two different probes (full-length and 3′-probe). Three RNA species ranging between 2 kb to ~4 kb, expressed in variable relative amounts were detected with the highest levels in testis, liver and kidney (see Supplementary Material Fig. 1). The two probes detected identical transcript patterns in the different tissues (not shown).

Western blot analysis in adult mouse tissues showed high expression of the 66.3-kDa protein in spleen, lung and brain and intermediate levels in heart. In testis, liver and kidney

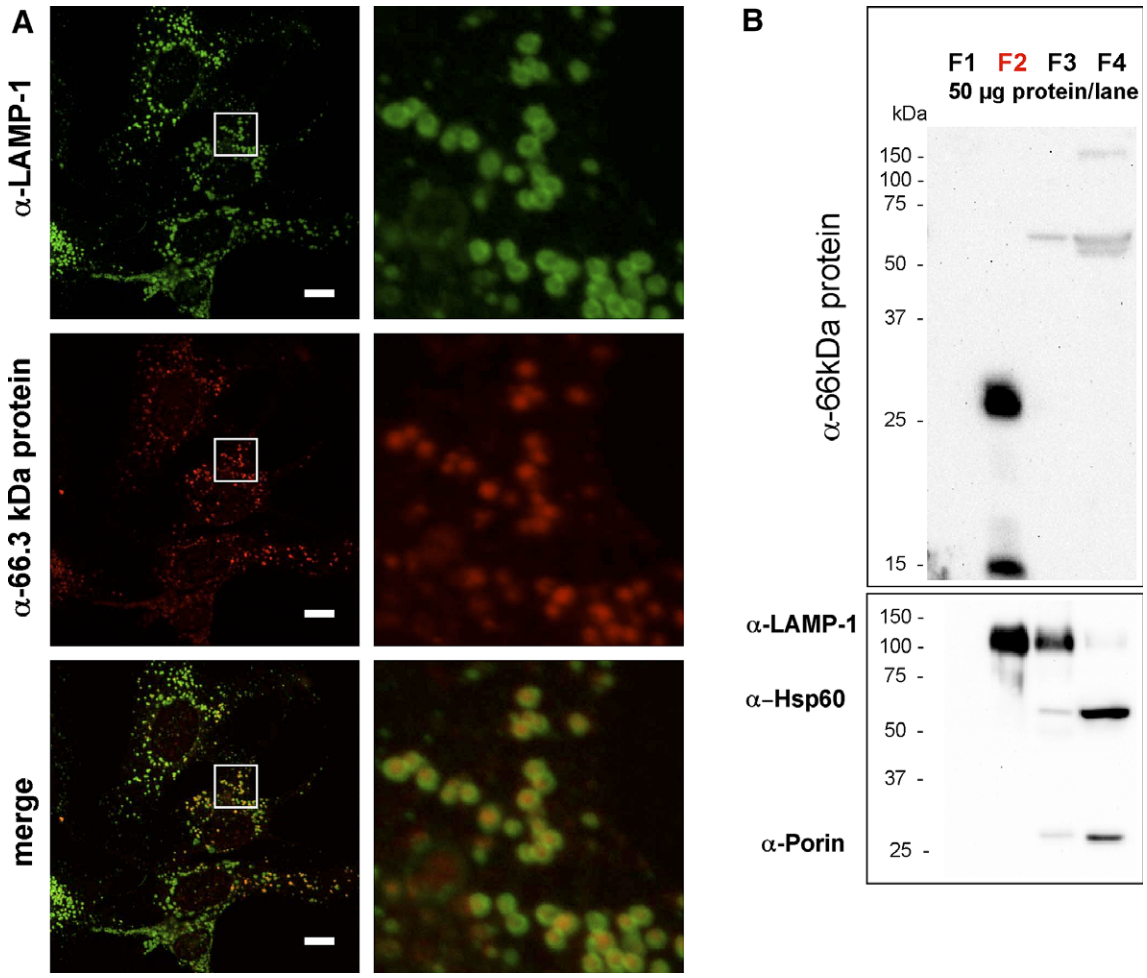

Fig. 2. Localisation of endogenous 66.3-kDa protein. (A) Endogenous 66.3-kDa protein was visualized in mouse embryonic fibroblasts by indirect immunofluorescence using 66.3-kDa protein antiserum and LAMP-1 as a lysosomal marker. Bars 20 μm. (B) A 28 kDa form of the 66.3-kDa protein localizes to Tyloxapol-filled lysosomes from mouse liver. Protein (50 μg/lane) from all fractions of the sucrose gradient of the lysosomal preparation were separated by SDS–PAGE, blotted and probed with the antibodies indicated. F2 represents the fraction enriched in lysosomes as indicated by the LAMP-1 signal (bottom panel) and β-hexosaminidase and β-glucuronidase activity (see Suppl. Material Table 1).

immunoreactive material was barely detectable (Fig. 3). Most interestingly, spleen, lung, brain and heart showed highly distinct molecular forms of the protein.

Brain showed a prominent ~50 kDa polypeptide and weak signals at 100 and 34 kDa. In lung the pattern was almost reverse with a dominant 34 kDa but only a faint 50 kDa form. In addition a minor 24 kDa form was seen. In spleen, the 34 kDa polypeptide was a minor form whereas the 24 kDa form was prominent. In heart, a 32 kDa was the major form accompanied by minor 50 and 15 kDa forms. In kidney, a very faint 32 kDa signal was detectable. In mouse embryonic fibroblasts we observed several weak signals ranging from 66 to 75 kDa and two major bands at 34 kDa and 28 kDa (Fig. 3). The faint 40 kDa band seen in brain, heart, liver and kidney was detectable also with the preimmune serum of the rabbit used for raising the antiserum and is therefore considered to be unrelated to the 66.3-kDa protein.

3.6. Biosynthesis and processing of the 66.3-kDa protein

HT1080 cells overexpressing the His-tagged 66.3-kDa protein and vector transfected HT1080 cells were pulse-labelled with [^{35}S] methionine/cysteine for 30 min and harvested after various chase periods. The 66.3-kDa protein was immunoprecipitated, separated by SDS–PAGE and visualized by autoradiography (Fig. 4). The 66.3-kDa protein was synthesized as a ~75 kDa precursor (Fig. 4). The precursor was processed to a 40 kDa form (first seen after a chase for 1 h) and to a 32 kDa intermediate (seen after 1 and 4 h of chase). After four hours of chase a 28 kDa form became detectable at the expense of the 32 kDa intermediate form while a 15 kDa form appeared first after 12 h of chase. A fraction of the 75 kDa precursor was detectable even after three days of chase along with the 40, 28 and 15 kDa forms.

4. Discussion

This study demonstrates that the 66.3-kDa protein is a lysosomal matrix protein. The endogenous 66.3-kDa protein colocalizes with LAMP-1 and it cofractionates with lysosomal markers like β-hexosaminidase, β-glucuronidase and LAMP-1 upon subcellular fractionation of mouse liver. These data confirm our earlier assumption on the lysosomal nature of the 66.3-kDa protein which was based on the MPR-dependent

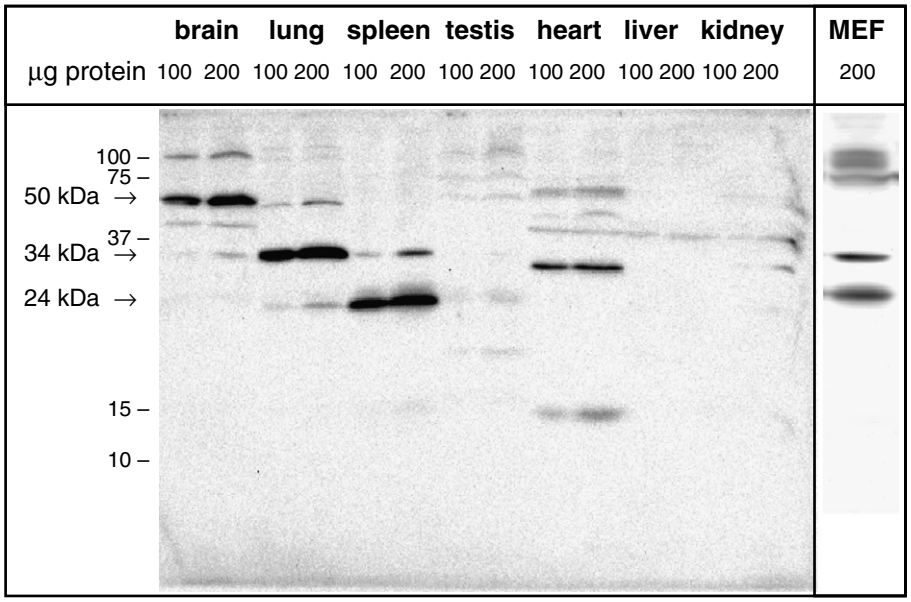

Fig. 3. Distribution of 66.3-kDa protein in adult mouse tissue. 100 and 200 µg of total protein from various tissue lysates and mouse embryonic fibroblasts (MEF) were separated on SDS–PAGE, transferred onto PVDF membrane and detected with the 66.3-kDa protein antiserum. Brain (50 kDa), lung (34 kDa), spleen (24 kDa) and heart (32 kDa) show immunoreactive bands of different sizes whereas in testis, liver and kidney signals were not or barely detectable.

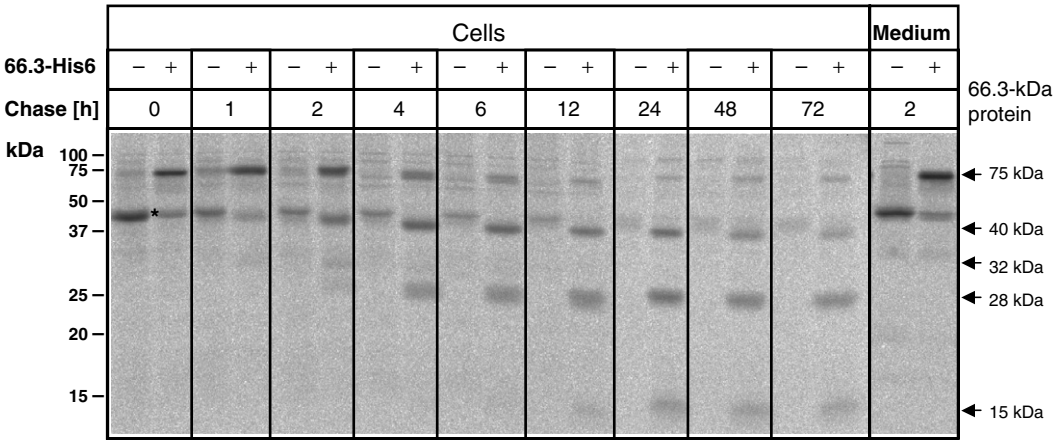

Fig. 4. Processing of the 66.3-kDa protein in normal and stably expressing 66.3-kDa protein HT1080 cells. HT1080 cells, non-transfected (−) or stably expressing C-terminally His6-tagged 66.3-kDa protein (+) were metabolically labelled for 30 min with 100 µCi of [^{35}S] methionine/cysteine and chased for the indicated times. The 66.3-kDa protein was immunoisolated from cell extracts and media, separated by SDS–PAGE and visualized by autoradiography. The polypeptides of the 66.3-kDa protein are marked by arrows together with their apparent molecular size. The 32 kDa form is an intermediate from that is seen only between one and four hours of chase. The ~45 kDa signal (marked with an asterisk at chase time 0) was immunoisolated also from the cell extracts and media of non-transfected cells and is therefore considered to be unrelated to the 66.3-kDa protein.

internalization and transfer to lysosomes of a C-terminally tagged variant of the 66.3-kDa protein [1].

Our studies in HT1080 cells stably expressing 66.3-kDa protein revealed that the 66.3-kDa protein is synthesized as 75 kDa precursor that is subject to limited proteolysis at multiple sites yielding fragments of 66 kDa, 40 kDa, 28 kDa and 15 kDa. Analysis of the different fragments revealed that the N-terminus of the mature protein and of the N-terminal 28 kDa fragment starts with leucine 47 rather than with serine 41 as predicted by the classical algorithms for signal peptide cleavage sites [1]. The 40 kDa form (starting with cysteine 249) and the 15/14 kDa form, starting with serine 514, carry the C-terminus of the 66.3-kDa protein. The 66 kDa form lacks the C-terminal His6-tag and therefore results from C-terminal truncation of the precursor.

A fraction of the 75 kDa precursor escapes targeting to lysosomes and is secreted (Fig. 4). As typical for lysosomal matrix proteins, presence of NH_4Cl impaired the intracellular processing and increased the secretion of the precursor (not shown).

The lack of covalent disulfide bridges between the proteolytic fragments points to non-covalent interactions between the fragments, which coelute as an approximately ~70 kDa complex upon gel filtration of purified 66.3-kDa protein (not shown).

Limited deglycosylation of the precursor and processed fragments by PNGase F treatment demonstrated that all five

putative N-glycosylation sites in the murine precursor are utilized in HT1080 cells. This finding is in line with observations of the 66.3-kDa homologue from human brain for which in a proteome wide analysis of all glycopeptides carrying M6P-residues, five of the six peptides of the 66.3-kDa peptides carrying N-glycosylation sites were detected [10].

Most interestingly, a Western blot analysis of different mouse tissues with our 66.3-kDa antiserum revealed a complex pattern of 66.3-kDa-protein-derived immunoreactive forms with apparent sizes of 75 kDa down to 15 kDa. Some forms are present in several tissues but varying expression levels, as been shown for the 50 and 34 kDa forms, whereas other forms are limited to specific tissues like the prominent 32 kDa form in heart. The tissue specific processing to the extent as we observed for the 66.3-kDa protein and the profound tissue specific expression are features that distinguish the 66.3-kDa protein from most other lysosomal matrix proteins. This may point to particular and tissue specific roles of the 66.3-kDa protein, the function of which is yet completely unknown. A BLAST analysis (blastp) of the 66.3-kDa protein revealed a number of orthologs from human (81% identity) to p67 from *Trypanosoma brucei* (27%). The latter has been formerly shown to be an integral lysosomal membrane protein [11] and has been extensively discussed before in the context of the identification of the 66.3-kDa protein [1]. In order to get insights into the function of the 66.3-kDa protein, we are generating a knock-out model in mouse for the 66.3-kDa protein. Provided the mouse model exhibits a phenotype, this might help to address a number of questions regarding its function and its relation to LSD-like diseases of unknown etiology. Such a reverse genetical approach has already helped to identify the loss of the integral lysosomal membrane protein LAMP-2 as the cause of Danon disease, also known as ''glycogen storage disease with normal acid maltase'' [12,13].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006. 09. 029.

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