Lysosome-associated protein transmembrane 4 α (LAPTM4 α) requires two tandemly arranged tyrosine-based signals for sorting to lysosomes

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Lysosome-associated protein transmembrane 4 α (LAPTM4 α) and homologues comprise a family of conserved proteins, which are found in mammals, insects and nematodes. LAPTM4 α functions to regulate the intracellular compartmentalization of amphipathic solutes and possibly the sensitivity of cells toward anthracyclines, antibiotics, ionophores, nucleobases and organic cations. This is similar to the multidrug-resistance phenotype exhibited by cells synthesizing high levels of P-glycoprotein. Accordingly, it is possible that LAPTM4 α may be a suitable target for development of novel chemotherapeutic agents. LAPTM4 α contains four putative membrane-spanning domains and a 55 amino acid C-terminal region that faces the cytoplasm. Localization of LAPTM4 α to endosomes and lysosomes appears to be tightly controlled as transient high-level expression of LAPTM4 α in cultured cells resulted in no detectable protein on

the cell surface. Mutagenic analysis of the C-terminus of LAPTM4 α indicated that two tandemly arranged tyrosine-containing motifs in the cytoplasmic domain are required for efficient localization of LAPTM4 α to vesicles containing the lysosomal marker lysosomal glycoprotein 120. Although a number of membrane proteins that localize to endosomes/lysosomes contain more than one independently functioning sorting signal, to our knowledge, LAPTM4 α is the first example of a membrane protein that requires two tandemly arranged tyrosine-based sorting signals for efficient localization in these compartments.

Key words: compartmentalization, late endosome, membrane protein.

INTRODUCTION

Lysosome-associated protein transmembrane 4 α (LAPTM4 α) is a four transmembrane-spanning protein that is relatively abundant in lysosome-enriched membranes isolated from rat liver, but whose endogenous levels of expression in various cultured cell lines are below the detection limits of immunofluorescence microscopy [1,2]. We recently demonstrated [1] LAPTM4 α to be a resident protein of lysosomes and late endosomes by using indirect immunofluorescence microscopic analysis of transiently expressed wild-type LAPTM4 α and N-terminal haemagglutinin epitope-tagged LAPTM4 α in cultured cells. Functional studies employing the heterologous expression of LAPTM4 α within the prevacuolar compartments of a drug-sensitive strain of *Saccharomyces cerevisiae* have demonstrated its ability to regulate cell sensitivity towards anthracyclines, antibiotics, dihydropyridines, ionophores, cytotoxic nucleobases and organic cations [2]. In addition, LAPTM4 α was able to alter intracellular steroid distribution in this yeast strain as well as in cultured mammalian cells (D. Hogue, unpublished work). The multidrug-resistant phenotype is similar to that exhibited by mammalian cells which overexpress P-glycoprotein, an ATP-binding cassette membrane protein that mediates the cellular efflux of xenobiotics and steroids [3]. Together, these studies indicate that LAPTM4 α functions to regulate the compartmentalization of amphiphilic solutes within lysosomes and late endosomes. Accordingly, it is possible that LAPTM4 α may be a suitable target for development of novel chemotherapeutic agents.

LAPTM4 α is one of the few type III membrane proteins currently identified to be a resident of lysosomes and late

endosomes, the others being lysosomal glycoprotein (lgp)85/lysosomal integral membrane protein (LIMP) II, CD63/LIMP I, natural resistance-associated macrophage protein 2 (NRAMP2)/divalent metal transporter 1 ('DMT1') and cystinosin [4–7]. The functions of lgp85/LIMP II and CD63/LIMP I are currently unknown, but NRAMP2 is thought to transport free bivalent metal ions from the lumen of late endosomes and lysosomes into the cytosol [8]. Cystinosin is a seven-transmembrane domain glycoprotein, which is predicted to transport cystine from the lysosome lumen to the cytosol.

Considerable effort has been dedicated to the study of sorting signals responsible for localization of type I membrane proteins to lysosomes and late endosomes [9–13]. For example, single tyrosine-based and/or di-leucine motifs present within the short (10–30 amino acid) cytoplasmic tails of lgp120/lysosome-associated membrane protein ('LAMP I') [14], tyrosinase [15,16] and endolyn [17] serve as sorting signals. The spacing of these tyrosine-based motifs relative to the transmembrane-spanning helix is important for optimal sorting of type I membrane proteins to lysosomes [13,18], as is the nature of the Φ residue within the tyrosine-based motif (YXX Φ , where X is any residue and Φ is a large hydrophobic residue) [19]. These features, as well as the presence of multiple potential sorting motifs on a single protein, complicate the analysis of these motifs with regard to their specific roles in sorting to lysosomes and late endosomes. Studies of sorting signals within type III membrane proteins that are responsible for their lysosomal/late-endosomal localization are restricted to those performed on lgp85/LIMP II and cystinosin. The cytoplasmically exposed C-terminus of lgp85/LIMP II contains a di-leucine-based motif necessary for its

Abbreviations used: AP, adaptor protein complex; CHX, cycloheximide; EST, expressed sequence tag; LAPTM4 α , lysosome-associated protein transmembrane 4 α ; lgp, lysosomal glycoprotein; LIMP, lysosomal integral membrane protein; VL motif, valine-leucine motif; wt, wild type.

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proper localization [20] and a di-acidic amino acid motif, which contributes towards its steady state-distribution between lysosomes, late endosomes and early endosomes [21]. Localization of cystinosin to lysosomes requires the presence of two tyrosine-based motifs, which are present on different cytoplasmically exposed regions of the protein [7]. Presently, nothing is known about how LAPTM4 α is targeted to endosomes/lysosomes.

In the present study, our goal was to identify regions within LAPTM4 α that serve as sorting signals for its trafficking to lysosomes and late endosomes. Two tandemly arranged tyrosine-based motifs within the C-terminus of LAPTM4 α have been identified as being essential for proper lysosomal/late endosomal localization. Identification of additional mammalian, insect and nematode members of the LAPTM family has also allowed for a more detailed examination of these sorting signals and other features within the C-termini of these proteins.

MATERIALS AND METHODS

Generation of LAPTM4 α expression vectors

The open reading frame of murine LAPTM4 α (GenBank® accession number NM_008640) was PCR amplified (primer A, 5'-GCAGGATCCATGGTGTCCATGACTTTC-3', where the *Bam*HI site is italicized, *Nco*I site is underlined and the translation initiation codon is in bold; and primer B, 5'-GCAGGTACCTCAGGCAGGCAGGTAAGGA-3', where the *Kpn*I site is underlined) and cloned into the *Bam*HI and *Kpn*I sites of pBlueScript II. Expand polymerase (Roche Molecular Biochemicals, Laval, QC, Canada) was used for all PCR reactions. Two complementary oligonucleotides (5'-CATGGAGGAGCAAAAGCTTATTTCTGAAGAGGACTTGCT-3' and 5'-CATGAGCAAGTCCTCTCCAGAAATAAGCTTTTGCTCCTC-3') encoding the c-myc epitope (MEEQKLISEEDLL, where single-letter amino-acid notation has been used and the epitope is underlined) were ligated into the unique *Nco*I site. The c-myc-LAPTM4 α open reading frame was cloned as a *Bam*HI-*Kpn*I fragment into pcDNA3 to obtain pcDNA3-myc-LAPTM4 α . An identical approach was employed to generate pcDNA3-myc-LAPTM4 α ΔCT, except that primer C (5'-GCAGGTACCTCAAGCCTTAAAAATGATGAATAC-3', where the translation stop codon is in bold and the *Kpn*I site is underlined) was substituted for primer B.

Generation of CD8-C-terminus (CD8-CT) expression vectors

A combination of PCR and overlap extension was used to fuse the coding regions for the extracellular and transmembrane domains of CD8 to the cytoplasmic domain of LAPTM4 α . Briefly, the LAPTM4 α cDNA was used as template in a PCR reaction with the following primers: forward primer 1, 5'-CCCTTTACTGCAACCACCTAATCAATTGTGTTTGGAAAC-3'; and reverse primer 2, 5'-ACCGTCGACTCAGGCAGGCAGGTAAGG-3'. The underlined bases in forward primer 1 correspond to the 3' region of the CD8 transmembrane domain. Reverse primer 2 contains a *Sal*I site (bold and italic). The resulting 194 bp PCR product was purified and used as the reverse primer in a second PCR reaction using a human CD8 cDNA [22] as the template. The forward primer for the second PCR was a vector-specific oligonucleotide (5'-TACGGTGGGAGGTCTATATAGC-3'). The second PCR product CD8-CT_{wt} (wild type), which encodes the CD8 extracellular and transmembrane domains of CD8 fused to the cytoplasmic domain of LAPTM4 α , was digested with *Eco*RI and *Sal*I and then ligated

into the mammalian expression vector pCMV5 [23]. The authenticity of the CD8-CT cDNA was confirmed by DNA sequencing.

Site-directed mutagenesis of the CD8-CT_{wt} expression vector and pcDNA3-myc-LAPTM4 α was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) and pairs of complementary mutagenic oligonucleotides. DNA sequences of myc-LAPTM4 α , myc-LAPTM4 α ΔCT, CD8-CT_{wt} and identity of all nucleotide mutations were confirmed by DNA sequencing.

Identification of LAPTM4 β , *Bombyx mori* LAPTM, *Caenorhabditis elegans* LAPTM and *Drosophila melanogaster* LAPTM cDNAs

The amino acid sequence of LAPTM4 α was compared with expressed sequence tag (EST) nucleotide databases from which a human EST cDNA (GenBank® accession number AA995835) and murine EST cDNA (GenBank® accession number AA168317) were identified, obtained from a commercial source and subjected to DNA sequencing. Anchor PCR amplification was performed using three different human cDNA libraries as independent templates to confirm the identity of the 5' terminus of human LAPTM4 β . The full-length cDNA sequences of human and murine LAPTM4 β have been assigned the GenBank® accession numbers AF317417 and AF317418 respectively.

Searching of non-mammalian EST nucleotide databases was performed using regions of the mammalian LAPTM protein sequences. The *B. mori* cDNA clone e40437 (GenBank® accession number AU000351) was provided by Dr K. Mita (Genome Research Group, Tokyo, Japan), the *Caenorhabditis elegans* cDNA clone yk643e7 (GenBank® accession number AV195585) was provided by Dr Y. Kohara (National Institute of Genetics, Mishima, Japan), and the *D. melanogaster* cDNA clone GH27451 (GenBank® accession number AI516866) was obtained from a commercial source (ResGen, Burlington, ON, Canada). Clones were subjected to DNA sequencing and all were found to encode full-length proteins. The cDNA sequences of the LAPTM orthologues from *B. mori*, *D. melanogaster* and *C. elegans* have been assigned GenBank® accession numbers AF317420, AF317419 and AF317421 respectively. It should be noted that the coding regions of the *D. melanogaster* and *C. elegans* LAPTM homologues are incorrectly predicted and annotated in their respective genomic databases.

Cell culture

COS-1 cells obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.) were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum and plated at 1.3×10^5 cells/well in 35-mm wells containing 12-mm glass coverslips. Transfections were performed 24 h later by co-incubation with 2 μ g of expression vector and 5 μ l of FuGene 6 (Roche Molecular Biochemicals). At 24–40 h post-transfection, cells were processed for indirect immunofluorescence staining. Where indicated, protein synthesis was inhibited by the incubation of cells in 500 μ M cycloheximide (CHX) for 3 h prior to processing for staining.

Indirect immunofluorescence microscopy

Cells were fixed with methanol at -20°C and then processed for indirect immunofluorescence using mouse anti-myc (9E10),

mouse anti-CD8 (OKT8) and rabbit anti-(lgp120) antibodies, as described previously [24]. Cells were examined using a Zeiss Axioskope2 microscope or, where indicated, a Zeiss 510 confocal microscope. Multiple optical sections ($0.5\ \mu\text{m}$) were collected from each sample and representative optical sections (X–Y orientation) are shown in the Figures.

Figure 1 A schematic representation of murine LAPTM4 α

The full-length protein is depicted at the top, with orientation of the predicted four transmembrane-spanning helices (TM) relative to the N- and C-termini. The amino acid sequence of the 55 amino acids comprising the hydrophilic carboxyl terminus of LAPTM4 α is shown at the bottom. Potential tyrosine-based sorting motifs (bold) are indicated and numbered inwards from the C-terminus. The VL motif is underlined.

RESULTS

The C-terminus of LAPTM4 α is necessary for its sorting to lysosomes and late endosomes

The hydrophilic C-terminus of LAPTM4 α contains four tyrosine-based motifs (YXX Φ) and a single valine–leucine (VL) motif that could potentially function as sorting signals (Figure 1); therefore the role of this entire region in sorting was investigated. We employed LAPTM4 α constructs that contained a myc-epitope tag at its N-terminus in these experiments, a location which has been shown previously [1] to tolerate the presence of a small epitope tag without altering the intracellular localization of the protein. Accordingly, the myc-tagged LAPTM4 α distributed to a population of intracellular vesicles many of which were positive for lgp120, a resident membrane protein of lysosomes and late endosomes (Figures 2A–2C). Transfected cells were treated with CHX to reduce the pool of newly synthesized LAPTM4 α in the perinuclear region, thus allowing us to examine more clearly the compartments in which this protein stably resides. CHX treatment did not alter the co-localization of LAPTM4 α and lgp120 in vesicles (Figures 2D–2F), suggesting that LAPTM4 α is a stable resident protein of lysosomes/late endosomes.

The deletion of the entire hydrophilic C-terminus from LAPTM4 α led to a near-complete loss of its co-localization with

Figure 2 C-terminus of LAPTM4 α is essential for its sorting to lysosomes and late endosomes

COS cells were transfected with expression vectors encoding myc-tagged LAPTM4 α (LAPTM4 α) (A–F) or myc-tagged LAPTM4 α lacking the C-terminus (LAPTM4 α Δ CT) (G–I). (D–F) CHX (500 μM) treatment was performed prior to cell fixation and staining with rabbit anti-(lgp120) and mouse anti-myc antibodies. Primary antibodies were visualized with FITC-conjugated anti-(rabbit IgG) (A, D and G) and Texas Red-conjugated anti-(mouse IgG) (B, E and H) antibodies. (C, F and I) Merged confocal images, where co-localization of lgp120 and myc-tagged LAPTM4 α is observed as yellow signals. Arrows denote vesicles that contain both lgp120 and LAPTM4 α . Magnification, $\times 85$.

Figure 3 C-terminus of LAPT $M4\alpha$ contains tyrosine-based signals necessary for sorting to lysosomes and late endosomes

COS cells were transfected with expression vectors encoding CD8-CT_{wt} (A–C), CD8-CT_{4Y>4A} (D–F), CD8-CT_{VL>AA} (G–I) or CD8-CT_{6A} (J–L). Cells were treated with CHX (500 μ M) for 3 h, fixed, and then stained with rabbit anti-(lgp120) and mouse anti-CD8 antibodies. Primary antibodies were visualized with FITC-conjugated anti-(rabbit IgG) (A, D, G and J) and Texas Red-conjugated anti-(mouse IgG) (B, E, H and K) antibodies. (C, F, I and L) Merged confocal images, where co-localization of lgp120 and CD8 fusion proteins is observed as yellow signals. Arrows denote vesicles that contain both lgp120 and CD8. Magnification, $\times 65$.

lgp120 (Figures 2G–2I). The truncated protein, designated LAPT $M4\alpha\Delta$ CT, localized to juxtanuclear regions of transfected cells as well as the endoplasmic reticulum. Subsequent experiments revealed that the juxtanuclear pattern of LAPT $M4\alpha\Delta$ CT overlapped significantly with the resident Golgi membrane protein giantin (results not shown). This indicated that the hydrophilic C-terminus is necessary for the proper sorting of LAPT $M4\alpha$ to endocytic compartments.

Identification of individual sorting signals in the C-terminus of LAPT $M4\alpha$

The results presented above indicated that the C-terminus of LAPT $M4\alpha$ was required for sorting to lysosomes, but did not reveal whether it was sufficient for sorting to these compartments. Moreover, the fact that LAPT $M4\alpha\Delta$ CT was arrested in a

juxtanuclear compartment, which is proximal to lysosomes, raises the concern that the mutant protein is either misfolded/improperly assembled or that the C-terminal domain of LAPT $M4\alpha$ is required for efficient transport beyond the juxtanuclear region. Accordingly, we elected to address whether the C-terminal domain of LAPT $M4\alpha$ was able to re-direct the localization of a plasma-membrane protein to lysosomes. The type I plasma membrane protein CD8 has been used by us [25] and others [26] to analyse intracellular sorting signals. We have demonstrated previously [25] that wild-type CD8 protein expressed from the vector pCMV5 in cultured cells localizes to the plasma membrane. A chimaeric construct consisting of the luminal and transmembrane domains of CD8 fused to the C-terminal 55 amino acid residues of wild-type LAPT $M4\alpha$ (CD8-CT_{wt}) was expressed in transfected cells and examined by indirect immunofluorescence. The distribution of CD8-CT_{wt} was primarily restricted to a vesicle population, many of which were positive for lgp120 (Figures 3A–3C), thereby confirming that the

Figure 4 Mutation of tyrosine motifs 2 or 3 in the C-terminus of LAPTM4 α leads to a partial loss of lysosomal localization

COS cells were transfected with expression vectors encoding CD8-CT_{Y1>A} (**A–C**), CD8-CT_{Y2>A} (**D–F**), CD8-CT_{Y3>A} (**G–I**) or CD8-CT_{Y4>A} (**J–L**). Cells were treated with CHX (500 μ M) for 3 h, fixed, and then stained with rabbit anti-(lgp120) and mouse anti-CD8 antibodies. Primary antibodies were visualized with FITC-conjugated anti-(rabbit IgG) (**A, D, G and J**) and Texas Red-conjugated anti-(mouse IgG) (**B, E, H and K**) antibodies. (**C, F, I and L**) Merged confocal images, where co-localization of lgp120 and CD8 fusion proteins is observed as yellow signals. Arrows denote vesicles that contain both lgp120 and CD8. Magnification, $\times 65$.

C-terminus of LAPTM4 α is sufficient for sorting to lysosomes/late endosomes.

The next objective was to identify amino acid motifs within the C-terminus of LAPTM4 α that are responsible for its sorting to lysosomes. To study the potential sorting roles of the tyrosine-based motifs in the C-terminus of LAPTM4 α (see Figure 1), all four tyrosine residues were mutated to alanine residues (CD8-CT_{4Y>4A}). Alanine substitution is often utilized in mutagenesis studies, because its small neutral side group of minimizes the introduction of spatial or electrostatic constraints. CD8-CT_{4Y>4A} was localized primarily to the plasma membrane and exhibited very little overlap with lgp120-positive vesicles (Figures 3D–3F), revealing that one or more of the four tyrosine-based motifs was necessary for sorting to lysosomes. This prediction was supported by the observation that myc-tagged LAPTM4 α containing alanine substitutions at all four tyrosine residues also failed to co-localize with lgp120-positive vesicles, but instead exhibited a juxtanuclear localization that

partially overlapped with the Golgi-resident protein giantin (results not shown).

Di-leucine and leucine-based motifs also constitute important signals for the sorting of integral membrane proteins to lysosomes and late endosomes [27]. Although the C-terminus of LAPTM4 α does not contain a di-leucine motif, the VL motif present may serve as a variant of the di-leucine motif, and for this reason we decided to determine if it functioned in sorting to endosomes/lysosomes (Figure 1). CD8-CT_{VL>AA} was generated by alanine substitution of this VL motif, and its sub-cellular distribution was subsequently assessed by indirect immunofluorescence. Interestingly, CD8-CT_{VL>AA} was distributed within lgp120-positive vesicles and the juxtanuclear region in addition to the plasma membrane (Figures 3G–3I). When alanine substitution of the four tyrosine residues and the VL motif was performed, the resulting protein (CD8-CT_{6A}) exhibited a pattern of localization very similar to that exhibited by CD8-CT_{4Y>4A} (see Figures 3D–3F). The majority of CD8-CT_{6A} was distributed to the

Figure 5 Mutation of tyrosine motifs 2 and 3 in the C-terminus of LAPT M4 α leads to complete loss of lysosomal localization

COS cells were transfected with expression vectors encoding CD8-CT_{Y1,4>A} (A–C) and CD8-CT_{Y2,3>A} (D–F). Cells were treated with CHX (500 μ M) for 3 h, fixed, and then stained with rabbit anti-(lgp120) and mouse anti-CD8 antibodies. Primary antibodies were visualized with FITC-conjugated anti-(rabbit IgG) (A and D) and Texas Red-conjugated anti-(mouse IgG) (B and E) antibodies. (C and F) Merged confocal images, where co-localization of lgp120 and CD8 fusion proteins is observed as yellow signals. Arrows denote vesicles that contain both lgp120 and CD8. Magnification, $\times 65$.

plasma membrane, with very little being observed in lgp120-positive vesicles or the juxtanuclear region (Figures 3J–3L). This suggested that the VL motif may either play a role directly in LAPT M4 α trafficking to endosomes/lysosomes or that mutagenesis of this motif perturbed the ability of adjacent tyrosine-based motifs to mediate proper sorting to these compartments. Indeed, alteration of amino acids adjacent to tyrosine-based sorting motifs can affect trafficking of membrane proteins possibly by regulating exposure of the signal to their cognate adaptor complexes [28,29].

The contribution of each tyrosine-based motif towards the sorting of CD8-CT_{wt} into lysosomes and late endosomes was examined next. Alanine substitutions were independently performed on the tyrosine residues within each of the four motifs, which are numbered inwards from the C-terminus of LAPT M4 α (see Figure 1). CD8-CT_{Y1>A}, which harbours a substitution in motif 1, was localized to a lgp120-containing vesicle population that was indistinguishable from that exhibited by CD8-CT_{wt} (Figures 4A–4C). In contrast, substitutions of tyrosine residues in motifs 2 (CD8-CT_{Y2>A}) or 3 (CD8-CT_{Y3>A}) resulted in a significant loss of association with vesicular structures and consequent localization to the plasma membrane (Figures 4D–4I). Substitution in motif 4 (CD8-CT_{Y4>A}) did not significantly alter the transport of the chimaeric protein to lgp120-positive vesicles (Figures 4J–4L).

Tyrosine-based motifs 2 and 3 within CD8-CT_{wt} each appeared to significantly contribute towards its localization to lysosomes and late endosomes, whereas tyrosine-based motifs 1 and 4 did not. To assess any co-operative function of these two pairs of motifs, simultaneous alanine substitutions were performed. CD8-CT_{Y1,4>A}, which contained substitutions in both motif 1 and 4, localized to vesicles that contained lgp120 (Figures 5A–5C). This pattern of localization was indistinguishable from that of CD8-CT_{wt}, thereby confirming that motifs 1 and 4 do not appear to fulfil any sorting roles. Conversely, simultaneous substitutions in motifs 2 and 3 led to an apparent complete loss

of CD8-CT_{Y2,3>A} from lgp120-positive vesicles and re-distribution to the plasma membrane (Figures 5D–5F). These results suggest that in the context of the CD8 chimaeras, tyrosine motifs 2 and 3 of the LAPT M4 α C-terminal domain act co-operatively to maintain the proteins in lysosomes and late endosomes, since substitution of either motif alone did not totally eliminate localization to lgp120-positive vesicles (Figures 4D–4I).

We also analysed the effect of single tyrosine to alanine substitutions on lysosomal targeting in the context of the myc-LAPT M4 α protein (Figure 6). The results from these experiments support the conclusion that motifs 1 and 4 do not play significant roles in targeting of LAPT M4 α to lysosomes (Figures 6A–6C and 6J–6L). Conversely, mutation of the tyrosine residues in motifs 2 or 3 resulted in accumulation of myc-LAPT M4 α in the perinuclear region and concomitant loss of co-localization with lgp120-containing vesicles. Interestingly, unlike in the context of the CD8 chimaeras containing the LAPT M4 α C-terminal domain, mutation of the tyrosines in either motif 2 or 3 resulted in complete loss of lysosomal-associated LAPT M4 α (Figures 6D–6I). However, the results still support the previous data indicating that both motifs are required for efficient localization to lysosomes. In the context of LAPT M4 α though, it appears that motifs 2 and 3 are also required to mediate efficient anterograde transport at the level of the endoplasmic reticulum and/or Golgi complex. Similarly, a recent study [30] showed that a YXX Φ motif, which was previously known to function in basolateral sorting of a viral membrane protein, is also required for efficient anterograde transport at the level of the endoplasmic reticulum and/or Golgi complex.

Conservation of sorting signals between members of the LAPT M family

The characterization of the tyrosine-based sorting motifs present within the C-terminus of LAPT M4 α has been

Figure 6 Tyrosine motifs 1 and 4 are not required for lysosomal targeting of LAPTMs

COS cells were transfected with expression vectors encoding LAPTMs $\alpha_{Y1>A}$ (A–C), LAPTMs $\alpha_{Y2>A}$ (D–F), LAPTMs $\alpha_{Y3>A}$ (G–I) or LAPTMs $\alpha_{Y4>A}$ (J–L). Cells were treated with CHX (500 μ M) for 3 h, fixed, and then stained with rabbit anti-(lgp120) and mouse anti-myc antibodies. Primary antibodies were visualized with FITC-conjugated anti-(rabbit IgG) (A, D, G and J) and Texas Red-conjugated anti-(mouse IgG) (B, E, H and K). (C, F, I and L) Merged images, where co-localization of lgp120 and LAPTMs α proteins is observed as yellow signals. Note the images were acquired using a Zeiss Axioskope2 microscope equipped with a SPOTTM digital camera. Magnification, $\times 65$.

complemented by our identification and cloning of mammalian LAPTMs β as well as LAPTM orthologues from *B. mori* (silkworm), *C. elegans* (nematode) and *D. melanogaster* (fruitfly). Examination of completed genomic databases of *D. melanogaster* and *C. elegans* revealed that only a single LAPTM homologue is encoded within each of these species genomes. The mammalian LAPTM family currently consists of LAPTMs α , LAPTMs β and LAPTMs γ , which are classified according to their number of predicted transmembrane-spanning helices [1,31]. These three proteins exhibit 21–43% overall identity and each protein is highly conserved between mammalian species [1]. The insect homologues exhibit clusters of amino acid sequence conserved with the mammalian LAPTM proteins, whereas the *C. elegans* homologue has undergone significantly greater evolutionary divergence.

A comparison between the C-termini of these mammalian and non-mammalian LAPTM orthologues revealed that three of the four tyrosine-based motifs are conserved between all proteins, except for that of *C. elegans*. Disregarding the divergent *C. elegans* homologue, each of these motifs exists within a larger evolutionarily conserved domain of amino acid sequence

(Figure 7). Tyrosine-based motifs 1 and 4 are not involved in sorting of LAPTMs α , but exist within the domains of PPPYXXA/V and VW/YXCXYK/RXI/L respectively (where single-letter amino-acid notation has been used; the motifs are underlined and X represents any amino acid). These domains may be responsible for interacting with other binding proteins that regulate the function or stability of LAPTMs α . For example, we have shown ([32] and D. Hogue, unpublished work) that the 55 amino acid cytoplasmic domain of LAPTMs α binds to the ubiquitin ligase Nedd4. Although we have not mapped the regions of LAPTMs α that interact with Nedd4, the latter is known to interact with proline-rich tyrosine-containing motifs [33]. Of the two motifs important for the lysosomal/late-endosomal sorting of LAPTMs α , motif 2 (PX $\underline{\text{YE}}$ /DXA, where the motif is underlined and X represents any amino acid) is conserved between all LAPTM orthologues, and motif 3 (VYXXE/ $\underline{\text{S}}$, where the motif is underlined and X represents any amino acid) exists only in LAPTMs α and LAPTMs β . Alanine substitution of residues present at the –3 and –4 position (VL > AA) of motif 2 within LAPTMs α perturbed lysosomal sorting to a noticeable degree, indicating that this region may comprise an important

Figure 7 C-termini of LAPTMs proteins contain conserved domains that encompass the tyrosine-based sorting motifs

A ClustalW alignment of the amino acid sequences of the hydrophilic C-termini of human and murine orthologues of LAPTMs4 α (GenBank® accession numbers NM_014713 and NM_008640 respectively), LAPTMs4 β (GenBank® accession numbers AF317417 and AF317418 respectively) and LAPTMs5 (GenBank® accession numbers NM_006762 and NM_010686 respectively) as well as homologues from *B. mori* LAPTMs (BmLAP, GenBank® accession number AF317420), *C. elegans* LAPTMs (CeLAP, GenBank® accession number AF317421) and *D. melanogaster* LAPTMs (DmLAP, GenBank® accession number AF317419) is shown. Shading indicates regions of identity (dark) or similarity (light). Consensus amino acid sequences of domains encompassing each of the four tyrosine-based motifs are shown.

feature of the sorting motif. However, the fact that tyrosine motif 2 is the most highly conserved among species, and mutation of this domain results in the most significant loss of targeting to lysosomes/endosomes, indicates that it may be the most important sorting determinant in the LAPTMs family.

DISCUSSION

Membrane proteins destined to reside within, or traffic through, endosomes and lysosomes contain specific sorting signals within their hydrophilic cytoplasmic termini. Moreover, the presence of multiple sorting signals in the cytoplasmic domains of lysosome-associated integral membrane proteins is not altogether uncommon. Examples include Igpb85/LIMP II, which contains di-leucine and di-acidic amino acid sorting motifs [20,21], tyrosinase, which utilizes di-leucine and tyrosine-based motifs for lysosomal targeting [15,16], cystinosin, which requires two tyrosine-based signals for efficient localization to lysosomes [7], and now LAPTMs4 α (the present study). The presence of multiple tyrosine-based sorting signals may be greatly beneficial for a protein whose cellular localization must be tightly restricted. For example, the tyrosine-based motifs may act as redundant sorting signals recognizable in multiple cellular compartments and thereby restrict the distribution of protein within the cell. In this respect, the two tandemly arranged tyrosine-based sorting signals present in LAPTMs4 α and LAPTMs4 β may function to tightly restrict the distribution of these proteins to lysosomes and late endosomes [1].

To our knowledge at least, LAPTMs family members and cystinosin are the only membrane proteins known to contain multiple tyrosine-based motifs that co-function as signals for targeting to endosomes/lysosomes. A potentially significant difference between these two groups of proteins is that LAPTMs

contain tandemly arranged motifs located within a contiguous 18 amino acid region (Figure 1), whereas the motifs in cystinosin are present on different cytoplasmic domains of the protein [7]. Accordingly, the LAPTMs motifs may be part of a larger high-affinity binding site for proteins that ensures highly efficient sorting to endosomes and lysosomes at the level of the Golgi complex. It is tempting to speculate that the tyrosine-based motifs may also function to expedite anterograde transport of LAPTMs4 α at the level of the endoplasmic reticulum and/or Golgi complex by facilitating interactions with COPII complexes [34]. Indeed, evidence is emerging that, in addition to their well-recognized functions as post-Golgi sorting signals, tyrosine-based motifs play important roles in sorting at the level of the endoplasmic reticulum [30,35]. Since LAPTMs4 α regulates the compartmentalization of amphipathic molecules, its localization to endocytic membranes may be imperative, and mislocalization to other membrane compartments may be detrimental to cell physiology. For example, if LAPTMs4 α were mislocalized to the endoplasmic reticulum or the plasma membrane rather than endosomes/lysosomes, the associated transport activity of this protein could result in inappropriate transport of substrates or toxic metabolites into the endoplasmic reticulum or out of the cell, rather than into lysosomes where they would be degraded and the products recycled.

The conservation of the two tyrosine-based sorting signals between LAPTMs4 α and LAPTMs4 β , but not LAPTMs5, suggests the cellular trafficking pattern of the latter protein may be subtly different. However, this may be significant in light of the fact that LAPTMs5 expression is generally restricted to tissues and cells of haematopoietic origin [31], whereas LAPTMs4 α and LAPTMs4 β are expressed in many different types of tissues [36]. Thus the lack of one tyrosine-based sorting signal may enable LAPTMs5 to fulfil a haematopoietic-specific role in lysosomes or a specialized

lysosome-derived compartment, such as secretory lysosomes [37]. Interestingly, none of the four YXX Φ motifs is preceded by a glycine residue, which is common feature of these motifs in lysosomal membrane proteins [38]. In addition, only one of the two required tyrosine-containing motifs (motif 3) conforms well to the classical YXX Φ , where Φ is a bulky hydrophobic amino acid, yet alteration of motif 2 had the greatest effect upon trafficking of LPTM4 α . This is similar to the atypical tyrosine motif YFPQA required for localization of cystinosin [7].

Tyrosine- and di-leucine-based motif recognition by adaptor complexes is mediated through their binding by different adaptor subunits, with tyrosine-based motifs being bound to the μ subunits and di-leucine motifs by the β subunits [39]. The association of a given adaptor complex with a tyrosine-based motif present in a membrane protein regulates the trafficking of the protein between different subcellular compartments. The adaptor protein complex (AP)-1 associates with clathrin-coated vesicles derived from the *trans*-Golgi network and endosomes, but its role in trafficking of lysosomal enzymes between endosomes and lysosomes has recently come into question [40]. AP-2 functions at the level of the plasma membrane to facilitate endocytosis of lysosomal enzymes and other proteins into clathrin-coated vesicles [39,41]. AP-3 is involved in the trafficking of certain lysosomal membrane proteins between the *trans*-Golgi network and lysosomes, but it is not clear if binding to the tyrosine-based sorting signals occurs at the level of the *trans*-Golgi network and/or endosomes [42–44]. Finally, AP-4 can interact with tyrosine-based motifs on certain lysosomal membrane proteins, but a direct role for this adaptor complex in transport of proteins to lysosomes has yet to be demonstrated [45]. Adaptor complexes, such as AP-1 and AP-3, also bind to the di-leucine-based sorting motifs of lysosomal/late-endosomal membrane proteins [11,44,46]. Members of the adaptor complex family exhibit different binding affinities for overlapping sets of tyrosine-based sorting motifs [47,48]. A recent study [49] indicates that amino acids that occupy positions +2 and +3 (relative to the tyrosine residue) are critical for differential binding to adaptor complexes; however, the authors suggest that the identities of amino acids at these positions cannot be used to generally predict which type of adaptor complex will bind to a given sorting signal. In the present study, however, we were unable to detect interactions between the μ 1, μ 2, μ 3A or μ 3B subunits with the hydrophilic C-terminus of LPTM4 α (results not shown).

In conclusion, the present study has demonstrated that the hydrophilic C-terminus of the type III membrane protein LPTM4 α is both necessary and sufficient for its proper localization to the lysosomal-endosomal compartment of mammalian cells. Deletion or mutagenesis of this C-terminal region induced an accumulation of LPTM4 α within the endoplasmic reticulum and Golgi, suggesting the C-terminus may also fulfil a role in facilitating the anterograde transport and/or complex assembly of LPTM4 α . Two tandemly arranged tyrosine-based motifs present in this C-terminal region have been identified as the major sorting signals responsible for the lysosomal localization of LPTM4 α . The fact that two inter-dependent sorting signals are present on LPTM4 α indicates that its localization to endosomes and lysosomes must be tightly regulated.

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