Two dileucine motifs mediate late endosomal/lysosomal targeting of transmembrane protein 192 (TMEM192) and a C-terminal cysteine residue is responsible for disulfide bond formation in TMEM192 homodimers

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INTRODUCTION

Lysosomes are membrane-enclosed organelles of eukaryotic cells involved in macromolecule turnover. They are characterized by an acidic pH within their lumen and more than 50 different hydrolases capable of degrading various kinds of biological macromolecules [1]. Cargo reaches the lysosome from different sources and pathways: exogenous macromolecules are delivered via the endocytic and phagocytic pathways. In addition, cellular components such as dysfunctional organelles can be subjected to lysosomal degradation via autophagy, a mechanism also critically important to ensure a cell’s nutrient supply under conditions of starvation [1]. The importance of the lysosomal system is substantiated by the existence of more than 40 inborn diseases characterized by lysosomal dysfunction and accumulation of lysosomal storage material that are caused by mutations in lysosomal, but also non-lysosomal proteins [2]. Alterations of autophagy and lysosomal protein turnover have also been observed in common multifactorial diseases and are likely to contribute to the pathogenetic sequence [3].

In comparison with the extensively studied soluble lysosomal enzymes and hydrolases, the knowledge on the protein composition and functional protein networks in the lysosomal membrane is still fragmented [4,5]. Over the last few years proteomic approaches have addressed these questions and allowed the discovery of several novel lysosomal membrane proteins. Nevertheless a gap is still evident between the characterized functions of the lysosomal membrane (such as transport processes) and the identification of the proteins responsible [4,6].

Lysosomal membrane proteins require special sorting mechanisms in order to reach their destination within the cell [7]. Therefore the cytosolic portions of lysosomal membrane proteins contain distinct targeting motifs which are constituted by short stretches of amino acids following certain consensus sequences [7,8]. These motifs recruit different adaptor proteins that allow segregation of the cargo and finally their packaging into coated vesicles. There is a considerable overlap between lysosomal-targeting motifs facilitating direct segregation of lysosomal proteins at the TGN (trans-Golgi network) and internalization motifs found in plasma membrane proteins that are internalized by endocytosis.

Generally two major classes of motifs can be distinguished: tyrosine-based and dileucine-based sorting signals. Tyrosine-based motifs for sorting of lysosomal proteins follow the
consensus sequence YXXØ, with X being any amino acid and Ø an amino acid with a bulky hydrophilic side chain. Two different types of dileucine motifs exist exhibiting either a [DE]XXX[L,I] or a DXXLL pattern, with the square brackets indicating alternatives. Tyrosine-based sorting motifs, as well as dileucine motifs of the [DE]XXX[L,I] type, which function as lysosomal-targeting signals at the TGN, are usually found in close proximity to a transmembrane segment and are separated by six to nine residues from the membrane [7,8]. Whereas single-pass transmembrane proteins often rely on a single active targeting motif within their cytosolic tail, many multispanning transmembrane proteins depend on several motifs that act in synergy in order to mediate lysosomal targeting [8–12].

Canonical YXXØ and [DE]XXX[L,I] signals interact with heterotrimeric adaptor protein complexes AP1, AP2, AP3 or AP4 [8] that are capable of recruiting clathrin and thereby initiating the assembly and formation of coated vesicles. In contrast, DXXLL-type dileucine signals recruit GGA proteins (Golgilocalized, γ-ear-containing, Arf-binding proteins), a different class of monomeric clathrin adaptors functioning at the TGN [13]. Lysosomal membrane proteins can travel from the TGN via a direct route to endosomes and from there to lysosomes, or they can follow an indirect pathway via the plasma membrane and re-uptake by endocytosis [8]. The extent to which these routes are utilized and also the dependency on the different AP complexes (AP1–AP4) in the case of the YXXØ and [DE]XXX[L,I] signals has not been studied in detail for every lysosomal membrane protein; however, available data indicate that the pathway utilization and requirement of AP complexes for correct targeting vary considerably between different late endosomal/lysosomal membrane proteins [8].

TMEM192 (transmembrane protein 192) has been previously identified as a novel component of late endosomal/lysosomal membranes by means of organellar proteomics [14]. Late endosomal/lysosomal localization was confirmed for overexpressed, as well as endogenous, TMEM192 which was detected with a specific antiserum [15]. Furthermore, the formation of TMEM192 homodimers, covalently stabilized by one or more disulphide bridges, has been reported [15]. In the present study, the sequence determinants mediating late endosomal/lysosomal targeting of TMEM192 are explored using CD4 chimaeric constructs and mutagenesis of putative targeting motifs. In addition, the contributions of different cysteine residues to the formation of disulphide bridges between TMEM192 monomers are investigated by mutagenesis. Finally, the topology of TMEM192 in the late endosomal/lysosomal membrane is clarified.

**EXPERIMENTAL**

**Antibodies**

A monoclonal antibody against the HA (haemagglutinin) epitope tag (3F10) was purchased from Roche. A polyclonal rabbit antisera against cathepsin D has been described previously [15,16], as well as a monoclonal antibody against h-LAMP2 (human lysosomal-associated membrane protein 2) [17]. Anti-transferrin receptor antibody (clone H68.4) was from Invitrogen. Monoclonal anti-GFP (green fluorescent protein) used for detection on Western blots was purchased from Santa Cruz Biotechnology, anti-(human CD4) was from BD Pharmingen and anti-β-tubulin clone E7 was from Developmental Studies Hybridoma Bank. Fluorochrome-conjugated secondary antibodies (anti-rat IgG coupled to Alexa Fluor® 488, and anti-rabbit and anti-mouse IgG coupled to Alexa Fluor® 594) were purchased from Molecular Probes. Secondary antibodies coupled to HRP (horseradish peroxidase) were used for immunoblotting (Dianova). Rabbit anti-GFP ( Fitzgerald Industries International, RDI-GRNFp4abr) and mouse anti-LAMP1 (Developmental Studies Hybridoma Bank, H4A3) were used for immuno-EM (immunno-electron microscopy), followed by goat anti-rabbit IgG coupled to 15 nm gold particles and goat anti-mouse IgG coupled to 6 nm gold particles (British BioCell).

**Cloning of TMEM192 mutants and the CD4 chimaera**

Cloning of the human TMEM192 cDNA and expression constructs with a C-terminally appended GFP and HA tag respectively (TMEM192-peGFP-N1, TMEM192-HA-pcDNA3.1/Hygro+) have been described previously [15,18]. Mutations of putative targeting signals and cysteine residues within the TMEM192 open reading frame were introduced by overlap-extension PCR using the flanking primers TMEM192-HindIII-Fw (5’-TGCCAAGTTACCGCACCAGGCGGGGGAGGCGAGGAT-3’) and TMEM192-HA-Xhol-Rv (5’-GATCCTCGAGTTAAGCGTAGTCTGGAGCAGCTATGGAATCGT-TCATCTGCGTACGC-3’) in combination with appropriate internal primers. Fusion PCR products were subcloned into pcDNA3.1/Hygro+ after restriction digestion with HindIII and Xhol.

Human CD4 cDNA (clone IRATp970c1044D) was obtained from ImaGenes. For the generation of the CD4-TMEM192 chimaera, a fragment corresponding to amino acid residues 1–420 of CD4 was amplified using CD4-HindIII-Fw (5’-GTCATCTCA-AGCTTGCCACCATGAGGAGGAGGATCCCTTATGAGC-3’) and CD4-Xhol-Rv (5’-GACAGAAGAAGAAGTCCTAG-3’) as forward and reverse primers respectively. The respective segments of TMEM192 (N-terminus, 2–47; Loop-II, 115–138; C-terminus, 191–271) were amplified separately [TMEM192_2–47_CD4-Fw (5’-CTAGGACATCTTTCTTCTTGCTTGCCGGCGGGGGGAGGATGGAG-3’), TMEM192_2–47_Xhol-Rv (5’-GATCCTCGAGTTAAGCGTAGTCTGGAGCAGCTATGGAATCGTTCATCTGCGTACG-3’), TMEM192_115–138_CD4-Fw (5’-CTAGGGCATCTTTCTTCTTGCTTGCCGGCGGGGGGAGGATGGAG-3’), TMEM192_115–138_Xhol-Rv (5’-GATCCTCGAGTTAAGCGTAGTCTGGAGCAGCTATGGAATCGTTCATCTGCGTACG-3’)] and fused to the CD4 fragment by overlap-extension PCR. Fusion PCR products were subcloned into pcDNA3.1/Hygro+ after restriction digestion with HindIII and Xhol.

For the generation of the CD4–Nterm192 chimaera, the amino acid sequence of residues 2–47 of TMEM192 was inverted to 47–2. This sequence was reverse-translated in silico in order to obtain a corresponding nucleotide sequence. This nucleotide sequence was generated and attached to the 3’ end of the CD4-(1–420) fragment by repetitive rounds of PCR amplification using the CD4 cDNA as template with CD4-HindIII-Fw as the forward primer and consecutively TMEM192_47–2_part1-Rv (5’-TGTGAAGCTTAACTCTGGGTCGGAGAATGAGAAGAGGTGGTGATCAACAGAGAGATGGGACTAG-3’), TMEM192_47–2_part2-Rv (5’-TGGACAGATCTGCAGGCTAAGAGGTTGTTGATTAATGGAGGCGTAGTAACTCTGGGTC-3’), TMEM192_47–2_part3-Rv
analysed either 24 h or 48 h after transfection.

The culture medium of transfected cells was removed and replenished between 6 and 10 h post-transfection in order to respectively. The final PCR product was subcloned into pcDNA3.1/Hygrom after restriction digestion with Hind III and XhoI.

An expression construct comprising GFP fused to the N-terminus of TMEM192 was generated in peGFP-C1 vector after amplification of the TMEM192 open reading frame with 47-2_part4-XhoI-Rv (5'-GATCCATCTCGGCCGCTCCCTCACTCGTCAACGGAGAAAATCGATTG-3') as reverse primers. The final PCR product was subcloned into pcDNA3.1/Hygrom after restriction digestion with Hind III and XhoI.

**Cell culture and transfection**

HeLa cells (DSMZ) were cultured in DMEM (Dulbecco’s modified Eagle’s medium; PAA) supplemented with 10% (v/v) FBS (fetal bovine serum; PAA), 100 units/ml penicillin (PAA) and 100 μg/ml streptomycin (PAA) in a humidified 5% CO2/air atmosphere at 37°C. Cells were seeded 24 h prior to transfection. For immunofluorescence analysis, coverslips were included in the culture dishes. Expression plasmids were transfected using Turbofect (Fermentas) according to the manufacturer’s protocol with 0.5 and 2.5 μg of DNA per 3.5 and 10 cm culture dish respectively. The culture medium of transfected cells was removed and replenished between 6 and 10 h post-transfection in order to minimize cytotoxic effects by the transfection reagent. Cells were analysed either 24 h or 48 h after transfection.

**Protein extraction and immunoblotting**

The cell monolayer was washed with ice-cold PBS three times and then scraped off into 1 ml of PBS with a rubber policeman. Cells were recovered by centrifugation (1000 g, 5 min) and extracts in lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1.0% (w/v) Triton X-100, 0.1% SDS and 4 mM EDTA] supplemented with Complete protease inhibitor cocktail (Roche), 4 mM Pefabloc® (Roth) and 1 μg/ml pepstatin A (Sigma–Aldrich). After sonication (level 4 for 20 s using a Branson Sonifier 450) and incubation on ice for 1 h, samples were centrifuged (15000 g, 10 min) and total lysates were recovered. The lysate protein concentration was measured using a Branson Sonifier 450 and incubation on ice for 1 h, samples were centrifuged (15000 g, 10 min) and total lysates were recovered. The lysate protein concentration was measured using a Branson Sonifier 450 and incubation on ice for 1 h, samples were centrifuged (15000 g, 10 min) and total lysates were recovered. The lysate protein concentration was measured using a Branson Sonifier 450 and incubation on ice for 1 h, samples were centrifuged (15000 g, 10 min) and total lysates were recovered.

**Proteinase protection assay**

SDS/PAGE was performed as described in [19]. Samples were adjusted to final concentrations of 1% (w/v) SDS and 100 mM DTT (dithiothreitol) and denatured at 95°C for 5 min. Semi-dry transfer to nitrocellulose and immunodetection was conducted as described previously [15].

**Indirect immunofluorescence**

Immunocytochemical stainings were performed as described previously [15]. In brief, HeLa cells grown on coverslips were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.2% saponin in PBS. Non-specific binding sites were blocked by a pre-incubation step in 10% (v/v) FBS in PBS which was also used as diluent for primary and secondary antibodies. Primary antibodies (anti-CD4, anti-cathepsin D, anti-HA and anti-LAMP2) were applied overnight at 4°C, followed by fluorescently labelled secondary antibodies (Alexa Fluor® 488- and 594-conjugated goat anti-mouse IgG, Alexa Fluor® 594-conjugated goat anti-rabbit and Alexa Fluor® 488-conjugated goat anti-rat) for 1 h on the following day. Nuclei were visualized with DAPI (4',6-diamidino-2-phenylindole; Sigma–Aldrich) added to the embedding medium. Photographs were acquired with an Axiosvert 200M fluorescence microscope (Zeiss) equipped with an Apotome for optical sectioning.

**Surface biotinylation and streptavidin pulldown of biotinylated proteins**

Surface biotinylation was performed with the membrane impermeable, cleavable reagent sulfo-NHS-SS-biotin [sulfo succinimidyl-1-(biotinamido)ethyl-1,3-dithiopropionate; Thermo Scientific]. HeLa cells were washed three times with PBS-CM (PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2). Subsequently, sulfo-NHS-SS-biotin dissolved at a concentration of 1 mg/ml in PBS-CM was added to the cell monolayer and incubated for 30 min on ice. Unbound biotin was removed by incubating the cells in 50 mM Tris/HCl (pH 8), in PBS-CM for a further 10 min on ice and washing three times with PBS-CM. Subsequently, cells were harvested and lysed as described above.

Lysates (340 μg of protein) were diluted in lysis buffer supplemented with Complete™ protease inhibitor cocktail (Roche). Subsequently, 50 μl of high-capacity streptavidin agarose resin (Thermo Scientific) equilibrated in lysis buffer with Complete™ protease inhibitor cocktail was added to each sample. Pulldown was performed for 1 h at 4°C under continuous end-over-end rotation. The beads were washed five times with 1 ml of lysis buffer and finally the biotinylated proteins were eluted by incubating the beads in 100 μl of reducing SDS/PAGE sample buffer for 10 min at 95°C followed by 15 min at 37°C. For Western blot analysis, 20 μl of the eluate was subjected to SDS/PAGE. Aliquots from total cell lysates (20 μg of protein) were analysed in parallel in order to account for differences in transfection efficiency and expression level between different constructs.

**Immuno-EM**

Cells were fixed in 4% (w/v) paraformaldehyde in 0.2 M Heps, pH 7.4 at room temperature (22°C) for 2 h, and then stored in 2% (w/v) paraformaldehyde in the same buffer at 4°C. The cells were embedded in gelatin, infiltrated in a mixture of polyvinylpyrrolidone and sucrose, frozen in liquid nitrogen and cryosectioned at −100°C as described previously [20]. The sections were immunogold-labelled using anti-GFP or both anti-GFP and anti-LAMP1, followed by gold-conjugated secondary antibodies. Topology of the anti-GFP label, in relation to the limiting membranes of late endosomal and lysosomal compartments, was quantified from two consecutive immunogold-labelling experiments. The gold particles associated with the limiting membranes were scored as locating inside the compartment, outside the compartment or on top of the cross-sectioned membrane.

**Proteinase protection assay**

HeLa cells were washed twice in PBS and homogenization buffer (250 mM sucrose, 10 mM Heps/NaOH and 1 mM EDTA). Cells were harvested by scraping in homogenization buffer, sedimented (500 g, 5 min) and resuspended in fresh homogenization buffer. Cells were homogenized by 24 passages through a 27-gauge cannula connected to a syringe. The homogenate was centrifuged for 10 min at 1000 g and the postnuclear supernatant was recovered. A late endosome/lysosome-enriched fraction was prepared by centrifugation at 15000 g for 15 min. The sediment was resuspended in homogenization buffer and the
protein concentration was determined as described above. For the proteinase protection assay, the lysosome-enriched fractions were diluted in homogenization buffer at a protein concentration of 0.5 μg/μl and incubated either in the absence or presence of 150 μg/ml Proteinase K (Fermentas) and 1% (w/v) Triton X-100 respectively, for 30 min at 37°C. The reaction was terminated by adding PMSF to a final concentration of 5 mM and incubation on ice for 5 min. Subsequently, an equivalent volume of preheated 2-fold reducing SDS sample buffer was added and protein in the samples was denatured by incubation at 95°C for 10 min. For Western blot analysis, 40 μl of the denatured sample corresponding to 10 μg of cellular protein were subjected to SDS/PAGE.

RESULTS

TMEM192 contains several putative lysosomal-targeting signals

Lysosomal localization of overexpressed and endogenous TMEM192 has been demonstrated previously by several approaches [15]. Available data also indicate residence of TMEM192 in late endosomal compartments, e.g. demonstrated by co-localization with the late-endosomally enriched lipid BMP [bis(monoacylglycerol)phosphate] [15]. The membrane topology of TMEM192 according to bioinformatic prediction (Figure 1A) is characterized by four transmembrane segments and cytosolic localization of both the N- and C-termini. In the predicted cytosolic segments three motifs fully matching the consensus sequences of established lysosomal/endosomal targeting signals are present (Figure 1A, bold boxes).

The N-terminal section contains two potential dileucine motifs localized in direct vicinity to each other: E19DDPLL23 and D25AQLL29. As pointed out, two different types of dileucine motifs can be distinguished that differ in their consensus sequence as well as the interactions they mediate. Whereas the proximal dileucine motif (residues 19–24) fits either the [DE]XXXL[LI] or the DXXLL pattern, the distal motif (residues 25–29) is only compatible with the DXXLL consensus. Figure 4(B) shows an alignment between several characterised dileucine motifs of the DXXLL type and the respective section of TMEM192.
Lysosomal targeting and disulfide bond formation of TMEM192 protein

Figure 2 Surface localization of CD4—TMEM192 chimaeras in HeLa cells

HeLa cells were transiently transfected with CD4 wild-type (wt) (residues 1–458) (A–C), CD4—Nterm (D–F), CD4—Nterminv (G–I), CD4—Loop-II (K–M) or CD4—Cterm (N–P). Cells were fixed 48 h after transfection. Distribution of CD4-fusion proteins and the lysosomal marker protein cathepsin D were visualized by indirect immunofluorescence using a monoclonal anti-CD4 antibody and a polyclonal antiserum against human cathepsin D respectively, followed by appropriate fluorescently labelled secondary antibodies. Scale bars = 10 μm.

A potential tyrosine-based sorting motif is present in the central cytosolic loop of TMEM192: Y126NLI129. Finally, several leucine pairs (L251L252, L256L257) or leucine—isoleucine combinations (L128I129) are scattered throughout the cytosolic segments of TMEM192, which may in principle form the core of dileucine motifs (Figure 1A, boxes). These lack the characteristic acidic residues known to precede the hydrophobic leucines in dileucine-based sorting motifs and therefore are considered as inferior candidates for representing active dileucine motifs involved in late endosomal/lysosomal sorting of TMEM192.

Cytosolic segments of TMEM192 are not capable of targeting CD4 chimaera to late endosomes/lysosomes

In a systematic unbiased approach, the predicted cytosolic segments of TMEM192 (the N-terminal segment, residues 2–47; the central loop, residues 115–138; and the C-terminal segment, residues 191–271) were fused to the extracellular part and transmembrane segment of the plasma membrane protein CD4 as a reporter (Figure 1B). Subcellular distribution of these CD4—TMEM192 chimaera [CD4—Nterm (chimaera of CD4 and the N-terminus of TMEM192), CD4—Loop-II and CD4—Cterm (chimaera of CD4 and the C-terminus of TMEM192)] was analysed upon transient overexpression in HeLa cells (Figures 2D–2P) in comparison with wild-type full-length CD4 (Figures 2A–2C). All CD4—TMEM192 fusion constructs were located at the plasma membrane, similar to wild-type CD4. Small amounts of all expressed proteins, both CD4 wild-type and CD4—TMEM192 chimaera, were also observed intracellularly with a vesicular distribution. However, no co-localization with lysosomal/late endosomal marker proteins could be detected, as shown here for cathepsin D (Figure 2), indicating that these vesicles are of different origin.

According to the predicted topology of TMEM192 as depicted in Figure 1, the N-terminal segment is inserted into the late endosomal/lysosomal membrane in a type II orientation. In contrast, CD4 is a type I transmembrane protein with an extracellular/luminal N-terminus and a cytosolic C-terminus. Therefore the N-terminal part of TMEM192 is present with an inverted topology in the CD4—Nterm chimaera as compared with its assumed natural orientation. This may alter the distance between targeting motifs and the membrane, their accessibility for adaptor proteins and thereby their functionality. In order to simulate the natural position of the N-terminal segment, an additional CD4—TMEM192 chimaera was generated in which...
Figure 3  Disruption of single putative sorting motifs does not prevent late endosomal/lysosomal targeting of TMEM192

HeLa cells were transiently transfected with TMEM192-HA (A–C), TMEM192-LL23/24AA—HA (D–F), TMEM192-LL28/29AA—HA (G–I), TMEM192-Y126A—HA (K–M), TMEM192-LL128/129AA—HA (N–P) or TMEM192-LL251/252AA—HA (Q–S). Cells were fixed 48 h after transfection. Late endosomal/lysosomal localization of overexpressed HA-tagged wild-type (wt) or mutant TMEM192 protein was demonstrated by indirect immunofluorescence using monoclonal antibodies directed against the HA epitope tag (3F10) and the late endosomal/lysosomal membrane protein LAMP2 (2D5) in conjunction with appropriate fluorescently labelled secondary antibodies. Scale bars = 10 μm.

the 46 amino acid residues of the TMEM192 N-terminal section were fused to CD4 in reverse order (47–2): CD4—Nterm<sub>TMEM192</sub> (Figure 1B). However, this chimaeric protein was also detected at the plasma membrane of transiently transfected HeLa cells. In conclusion, none of the created CD4—TMEM192 chimaera was targeted to late endosomes/lysosomes, which may indicate that none of the cytosolically exposed sequence stretches of TMEM192 that were fused to CD4 contain active sorting motifs.

Disruption of single putative targeting signals does not prevent late endosomal/lysosomal targeting of TMEM192

In an alternative approach it was evaluated whether disruption of any of the putative targeting motifs may prevent or inhibit late endosomal/lysosomal targeting of TMEM192. The following mutations were introduced into TMEM192: LL23/24AA, LL28/29AA, Y126A, LL128/129AA and LL251/252AA. All constructs were prepared with a C-terminally appended HA epitope tag allowing specific detection of the overexpressed mutated TMEM192. Subcellular distribution of mutant, as well as wild-type, TMEM192 protein was analysed after transient overexpression in HeLa cells (Figure 3). All mutants of TMEM192 containing a single disrupted putative targeting motif (Figures 3D–3S) were present in LAMP2-positive compartments with a distribution not distinguishable from that of wild-type TMEM192 (Figures 3A–3C).

Either none of the motifs tested are involved in late endosomal/lysosomal targeting or several motifs act in synergy, meaning that one or more of the remaining motifs in the single mutants are able to compensate for the loss of any individual
Lysosomal targeting and disulfide bond formation of TMEM192 protein

Figure 4  Disruption of two N-terminal dileucine motifs redirects mutant TMEM192 to the plasma membrane

(A) HeLa cells were transiently transfected with TMEM192-LL23/24AA-Y126A−HA, TMEM192-LL23/24AA-LL28/29AA−HA or TMEM192-LL23/24AA-LL28/29AA-Y126A−HA. Cells were fixed 48 h after transfection. Subcellular localization of overexpressed HA-tagged mutant TMEM192 protein was examined by indirect immunofluorescence with monoclonal antibodies directed against the HA epitope tag (3F10) and the late endosomal/lysosomal membrane protein LAMP2 (2D5) in combination with appropriate fluorescently labelled secondary antibodies. Scale bars = 10 μm.

(B) Alignment of residues 14–31 of human TMEM192 with the C-terminally localized DXXL-type sorting motifs of the CD-M6PR and CI-M6PR, sortilin, LRP9 and BACE. The critical aspartate residue, as well as the dileucine characterizing DXXLL-type dileucine motifs, are shaded in grey and printed in bold.

The dileucine motifs LL23/24AA and LL28/29AA mediate late endosomal/lysosomal targeting of TMEM192

Two double mutants (LL23/24AA+Y126A and LL23/24AA+LL28/29AA) and a triple mutant (LL23/24AA+LL28/29AA+Y126A) were created and analysed regarding their subcellular distribution and delivery to late endosomes/lysosomes (Figure 4). Whereas the LL23/24AA+Y126A mutant was still correctly targeted and present in late endosomes/lysosomes (Figure 4A), the two mutants with disruption of both N-terminal dileucine motifs (LL23/24AA+LL28/29AA and LL23/24AA+LL28/29AA+Y126A) demonstrated impaired transport to late endosomes/lysosomes (Figure 4A). Major portions of these two TMEM192 mutants were located at the plasma membrane. Minor amounts of both proteins were detected in LAMP2-positive compartments. Apparently, the two dileucine motifs LL23/24 and LL28/29 are critical for late endosomal/lysosomal delivery of TMEM192 as their combined disruption significantly impairs targeting of TMEM192. Each of these motifs alone is sufficient to ensure late endosomal/lysosomal sorting of TMEM192 in the case where the other motif is disrupted, as was demonstrated above by analysis of the single mutants LL23/24AA and LL28/29AA (Figures 3D–3F and 3G–3I). However, if both motifs are disrupted, correct targeting is severely reduced and the protein is found in significant amounts at the plasma membrane.

Small amounts of the dileucine double mutant LL23/24AA+LL28/29AA are still found in late endosomal/lysosomal compartments. This is also not abolished in the triple mutant after additional disruption of the putative tyrosine motif, which may indicate that additional determinants and motifs contribute to late endosomal/lysosomal targeting of TMEM192. Considering that TMEM192 forms dimers that are linked by disulfides [15], another possible explanation is that overexpressed mutant TMEM192 is carried to late endosomes/lysosomes after formation of heterodimers with endogenous wild-type TMEM192 possessing intact sorting motifs that may be sufficient to initiate targeting of the whole complex.
Figure 5  Mutation of two N-terminal dileucine motifs increases cell-surface expression of TMEM192

HeLa cells transiently expressing TMEM192–HA (lane 2), TMEM192-LL23/24AA–HA (lane 3), TMEM192-LL28/29AA–HA (lane 4), TMEM192-Y126A–HA (lane 5), TMEM192-LI128/129AA–HA (lane 6), TMEM192-LL25/252AA–HA (lane 7), TMEM192-LL23/24AA-Y126A–HA (lane 8), TMEM192-LL23/24AA-LL28/29AA–HA (lane 9) or TMEM192-LL23/24AA-LL28/29AA-Y126A–HA (lane 10) or transfected with empty vector (lane 1) were surface biotinylated 24 h after transfection with the membrane-impermeable reagent sulfo-NHS-SS-biotin for 30 min on ice. Biotinylated proteins were isolated from total cell lysates using streptavidin–agarose. Equal volumes of eluted biotinylated proteins (Bound) were subjected to SDS/PAGE and Western blot analysis. In order to detect differences in expression level between the different mutants, aliquots of total cell lysates (20 μg of protein) were analysed in parallel (Total). As indicated, membranes were probed with antibodies against the HA epitope detecting overexpressed TMEM192, transferrin receptor (TFR) or the intracellular enzyme GAPDH, followed by suitable peroxidase-conjugated secondary antibodies. The molecular mass in kDa is indicated on the right-hand side. wt, wild-type.

Disruption of both dileucine motifs results in increased cell-surface expression of TMEM192

In order to verify the surface expression of the TMEM192 mutants biochemically and to compare the amount of surface expression quantitatively, HeLa cells transiently transfected with wild-type or mutant TMEM192 were surface-biotinylated with the membrane impermeable reagent sulfo-NHS-SS-biotin. Total cell lysates, as well as biotinylated proteins isolated from total lysates by pulldown on streptavidin–agarose, were analysed by Western blotting (Figure 5).

With the exception of TMEM192-LL25/252AA which was expressed at a slightly higher level, comparable expression levels of wild-type and mutant TMEM192 were achieved, as detected in the total cell lysates. Therefore a signal intensity obtained for TMEM192 in the streptavidin-bound fractions provides a direct measure for comparing the degree of surface expression between different constructs.

The detection of biotinylated wild-type TMEM192 indicates plasma membrane localization of minor amounts of the overexpressed wild-type protein (Figure 5, lane 2) that are not detectable by indirect immunofluorescence (Figures 3A–3C) [15]. This may either represent a small, but stable, pool of plasma membrane localized TMEM192, or may result from TMEM192 on its way to late endosomes/lysosomes, following an indirect trafficking pathway via the plasma membrane just transiently associated with the cell surface.

Regarding the analysis of the TMEM192-targeting mutants, the surface biotinylation approach confirmed the observation made by immunocytochemistry. As a measure of relative surface expression, the B/T (Bound/Total) signal ratio was quantified by densitometry and normalized to wild-type TMEM192 (B/T = 1).

The level of surface expression of the double dileucine mutant (TMEM192-LL23/24AA-LL28/29AA, lane 9, B/T = 5.3) and the triple mutant (TMEM192-LL23/24AA-LL28/29AA-Y126A, lane 10, B/T = 5.1) was considerably higher than that of wild-type TMEM192 (Figure 5, lane 2). Since the increase for both mutants was very similar, an additional role of the putative tyrosine motif could not be substantiated. The comparably strong signal obtained for TMEM192-LL25/252AA in the ‘Bound’ fraction (Figure 5, lane 7) could be largely attributed to the overall higher expression level of this construct. However, densitometric quantification of the B/T ratio (B/T = 1.7) seemed to indicate a slightly increased surface expression of this mutant as compared with wild-type TMEM192 that had not been detected by immunofluorescence.

Cys266 is critical for disulfide-dependent dimerization

The presence of TMEM192 homodimers linked by one or more disulfide bridges was deduced from appropriate differences in the apparent molecular mass and co-immunoprecipitation experiments, as described previously [15]. In the present work the cysteine residue(s) responsible for this covalent stabilization of the dimer were identified in order to gain important information on the topology and structure of TMEM192.

Several of the seven cysteine residues (Cys72, Cys82, Cys112, Cys156, Cys182, Cys186 and Cys266) were mutated to alanine residues and the apparent molecular mass of the respective TMEM192 mutant proteins was analysed by Western blotting under reducing and non-reducing conditions (Figure 6). Three of the seven
section preceding the first putative transmembrane segment may be seen as an indirect indication regarding the orientation of the N-terminus. In order to recruit adaptor protein complexes and mediate sorting at the TGN, an exposure of these sorting motifs towards the cytosol is required, and therefore cytosolic localization of the N-terminus can be assumed.

The topology of TMEM192 in the late endosomal/lysosomal membrane

Two strategies were chosen to clarify the orientation of the N- and C-termini of TMEM192. In both approaches, N- or C-terminally tagged GFP-fusion proteins (GFP–TMEM192, TMEM192–GFP) were utilized owing to their good detectability. Correct sorting of these fusion proteins was validated by indirect immunofluorescence (results not shown) [14]. In the first approach, TMEM192-fusion proteins were visualized in HeLa cells transiently expressing GFP–TMEM192 or TMEM192–GFP with immunogold labelling of the GFP tag. In order to investigate the subcellular localization of TMEM192 at high resolution, co-labelling was performed with the late endosomal/lysosomal marker protein LAMP1. Co-localization of TMEM192 and LAMP1 was observed in late endosomal and lysosomal, as well as late autophagic, compartments (Figures 7A and 7B, and results not shown). This indicates that, at the ultrastructural level also, TMEM192 is localized in the limiting membrane of late endosomal and lysosomal compartments. In addition, some gold labelling for GFP was also detected in the lumen of late endosomal/lysosomal compartments (Figures 7A and 7B). A similar labelling pattern for GFP was also detected upon transient overexpression of GFP-tagged Rab7 (results not shown). This is most likely due to small amounts of the tagged protein entering autophagic compartments, which then fuse with late endosomal and lysosomal vesicles. GFP is relatively resistant to lysosomal proteolysis and thus remains detectable by anti-GFP for some time.

In order to obtain information on the membrane topology of TMEM192, the distribution of the anti-GFP label was quantitatively assessed with respect to the limiting membranes of late endosomal/lysosomal compartments, as an indicator of the position of the GFP tag and thus the N- or C-termini of TMEM192. The anti-GFP label associated with the limiting membranes of late endosomes/lysosomes was scored into one of three classes: on the cytosolic side of the limiting membrane, directly on top of the cross-sectioned membrane, or on the luminal side of the membrane. The majority of the gold particles detecting the GFP in both GFP–TMEM192 and TMEM192–GFP were located on the cytosolic side of the limiting membrane (66.8% and 69.3% of the gold label respectively, Figure 7C). This suggests that both termini of TMEM192 are localized on the cytosolic side of the membrane, as indicated by the very similar distribution of the gold label, in agreement with the predicted model (Figure 1A).

This finding was further substantiated by performing a protease-protection assay (Figure 8). A late endosome/lysosome-enriched organelle fraction was obtained by differential centrifugation from a postnuclear supernatant of HeLa cells transiently expressing TMEM192–GFP, GFP–TMEM192 or Rab7–GFP, a small GTPase involved in lysosomal biogenesis with confirmed localization in the cytosol or on the cytosolic face of the lysosomal membrane [14].

Organelles were incubated in the absence or presence of proteinase K and/or detergent. Susceptibility to proteinolysis as an indicator of cytosolic or luminal/intralysosomal localization of the respective GFP tag was assessed by Western blotting.

cysteine residues are conserved among all species possessing an orthologue of TMEM192 (Cys72, Cys82 and Cys186) and were therefore considered as primary candidates for being involved in the disulfide bridge formation. However, none of the mutations of one or several of these three cysteine residues (Figure 6, lanes 4–7) affected the formation and stability of the disulfide-linked dimer visible under non-reducing (−DTT) and reducing (+DTT) conditions and protein transferred to nitrocellulose. Overexpressed wild-type (wt) and mutant TMEM192 was detected with a monoclonal antibody against the HA epitope (3F10) and a peroxidase-conjugated secondary antibody. Open and closed arrowheads indicate positions of TMEM192 monomer and dimer respectively. The molecular mass in kDa is indicated on the right-hand side.

Figure 6 Disulfide-dependent dimerization of TMEM192 is mediated by Cys266

HeLa cells were transiently transfected with TMEM192−HA (lane 2), TMEM192-C266A−HA (lane 3), TMEM192-C72A-C82A−HA (lane 4), TMEM192-C186A−HA (lane 5), TMEM192-C72A-C82A-C186A−HA (lane 6), TMEM192-C182A-C186A−HA (lane 7) or empty pcDNA3.1/Hygro+ (lane 1). Cells were harvested 2 days after transfection. Total lysates (20 μg of protein) were subjected to SDS/PAGE under non-reducing (−DTT) and reducing (+DTT) conditions and protein transferred to nitrocellulose. Antibody. Open and closed arrowheads indicate positions of TMEM192 monomer and dimer respectively. The molecular mass in kDa is indicated on the right-hand side.
Protection of the lysosomal enzyme \( \beta \)-glucocerebrosidase from proteinolytic degradation in the absence of detergent confirmed the integrity of the late endosomal/lysosomal membrane in the analysed material. GFP fused to either end of TMEM192 was susceptible to proteinolysis by added proteinase K in a similar way to cytosolically exposed Rab7 protein. In all cases the bands representing the fusion proteins nearly disappeared after proteinase treatment. The small amount of proteinase-resistant GFP-fusion protein detected with all three constructs is likely to be due to the internalized protein also detected in immuno-EM analysis (see above, and Figures 7A and 7B). Instead, a prominent cleavage product with an apparent molecular mass of 25 kDa appeared. However, this band was also observed when a cytosolic fraction of cells expressing GFP alone was subjected to a similar assay (results not shown), suggesting that this band represents a proteinolytic fragment of GFP resistant to further digestion under the conditions chosen.

Based on the observations that a fragment of 25 kDa was also obtained from GFP alone and there was no difference in molecular mass of the final fragment in the absence or presence of detergent, we conclude that in both TMEM192-fusion proteins the GFP tag is directly accessible to the proteinase. This means that the N- and C-termini are located on the cytosolic side of the membrane, in agreement with the bioinformatic prediction and the findings of immuno-EM analysis.

**DISCUSSION**

**Late endosomal/lysosomal targeting of TMEM192 is mediated by two DXLL-type dileucine motifs**

The performed experiments demonstrate that the two dileucine motifs localized between the N-terminus and the first transmembrane segment of TMEM192 represent critical determinants for intracellular trafficking of TMEM192. Disruption of these two motifs significantly impairs late endosomal/lysosomal sorting of TMEM192.

However, the systematic exploration of the cytosolic segments of TMEM192 for sorting signals using CD4 chimaeric constructs failed to demonstrate the involvement of the N-terminus. Under steady-state conditions both fusion proteins between the CD4 luminal and transmembrane domain and the N-terminal segment of TMEM192 (CD4–Nterm and CD4–Nterm\(_v\)) were localized at the plasma membrane. In contrast, similar approaches of examining potential late endosomal/lysosomal targeting signals with the chimaeric constructs between plasma membrane proteins and certain domains of interest have been successfully applied for the identification of targeting determinants in many lysosomal membrane proteins, such as CLN3 (ceroid-lipofuscinosis 3) [10], LAPT4m4α (lysosome-associated protein transmembrane 4α) [11], endolyn [22], mucolipin-1 [12] and sialin [23]. The results of the present study indicate that false negative results with such a strategy can be encountered, and that in the case of failed identification of sorting relevant domains by chimaeric constructs alternative approaches should be pursued.

Certain reasons may be considered why the two dileucine motifs did not act as late endosomal/lysosomal targeting signals in the context of a different protein, like in the CD4 chimaera. It should be kept in mind that the three-dimensional structure adopted by a segment of TMEM192 when fused to CD4 does not necessarily reflect its native structure. Therefore functional sorting motifs may not be in any case readily detectable by using CD4 chimaeric constructs. Another potential problem may be the topology and orientation with respect to the membrane, as a protein segment usually in type II orientation is made to be part of the type I transmembrane protein CD4. However, since the two motifs (residues 23/24 and 27/28) are localized approximately in the middle of the N-terminal tail consisting of 47 residues, the distance between the membrane and sorting motifs may not differ much between both orientations. Furthermore, in the case of sialin which exhibits an N-terminal segment of comparable length (41 residues) with a canonical dileucine motif in a similar position as in TMEM192,
a conventional CD4 chimaera containing the N-terminus of sialin was found to be targeted to lysosomes [23].

Although it may not be fully excluded that the topology and orientation are possible explanations why the N-terminus of TMEM192 fails to redirect CD4 to late endosomes/lysosomes, alternative explanations should be considered. The N-terminus and the two dileucine motifs, although obviously indispensable for late endosomal/lysosomal transport of TMEM192, may not be sufficient to mediate sorting of another protein on their own. Conceivably, additional determinants and/or sorting signals within TMEM192 may be required in order to render these motifs functional and finally initiate late endosomal/lysosomal targeting. It may only be speculated what these determinants are and where in the TMEM192 sequence they are localized. Studies with chimaeric proteins between the TGN integral membrane protein TGN38 and the late endosomal/lysosomal protein LAMP1 have indicated that even luminal domains and transmembrane segments can significantly contribute to the sorting of transmembrane proteins [24]. In addition, binding properties of sorting motifs can be modified by post-translational modifications which have been shown to play regulatory roles in intracellular trafficking. Well-known examples are phosphorylation [25] or lipidation, such as prenylation or palmitoylation [26,27].

Earlier reports indicated that TMEM192 may be present as a homodimer within the cell possibly linked by disulfide bridges. Although the structure of these dimers and the position of the N-terminus within the complex is not known, it is conceivable that dimerization may be a prerequisite for correct targeting of TMEM192. The mutant TMEM192-C266A which is devoid of disulfide bonds (Figure 6) was correctly targeted to late endosomes/lysosomes (results not shown). However, suppressed disulfide bond formation may not automatically prevent dimerization of TMEM192, and therefore this result should be interpreted with caution.

As discussed above the assignment of the first of the two dileucine motifs to either the [DE]XXXL[LI] or the DXXLL pattern is not fully unequivocal. Owing to the presence of three consecutive acidic residues E[DE]DD [23] it may be seen as a DXXLL-type motif, which are also referred to as acidic cluster dileucine signals because the critical aspartate residue is regularly found within a cluster of acidic residues. An alignment of several characterized DXXLL motifs with the motif found in TMEM192 is provided in Figure 4(B). As can be seen, the number of acidic residues preceding the two leucine residues varies between different motifs. In the case of the CI-M6PR (cation-independent mannose 6-phosphate receptor), it was demonstrated that only the aspartate residue three positions N-terminal to the first of the two leucine residues (Figure 4B, shown in bold) is critical, whereas the others can be substituted without affecting the functionality of the motif [25]. Trafficking dependent on acidic cluster dileucine motifs has been reported for, e.g. the cation-dependent and cation-independent M6PR [28,29], sortilin [30], the LRP [LDL (low-density lipoprotein)-receptor-related protein] 9 [31,32] and the β-secretase BACE [33]. Similar motifs are present in LRP3 and the sortilin-related receptor SorLA [7].

Several features of the two identified DXXLL motifs in comparison with known motifs are remarkable. Most DXXLL motifs reported so far were found in the C-terminal section of single-pass type I transmembrane proteins, situated two to three residues N-terminally of the C-terminus [7]. Accordingly, the distance between the sorting signal and the membrane varies depending on the length of the C-terminal tail from 18 residues in BACE to more than 200 residues in LRP9 [7]. In contrast, TMEM192 is a multi-spanning membrane protein with four potential transmembrane segments. The two identified sorting motifs are localized within the N-terminal segment instead of the C-terminal tail and are separated from the N-terminus by more than 20 residues. Many examples of polytopic lysosomal

Figure 8 Proteinase K protection assay confirms cytosolic localization of the N- and C-termini of TMEM192

HeLa cells were transiently transfected with expression constructs encoding GFP-fusion proteins of TMEM192 (GFP-TMEM192, TMEM192-GFP) or Rab7 (Rab-7-GFP) or left untransfected (Ø). Cells were harvested 48 h after transfection, mechanically disrupted in isotonic buffer and a late endosome/lysosome-enriched fraction obtained by differential centrifugation. Organelles were incubated in the absence or presence of proteinase K and Triton X-100 respectively, for 30 min at 37 °C as indicated. Afterwards, the remaining proteinolytic activity was inactivated by addition of PMSF and SDS sample buffer followed by denaturation at 95 °C. Samples were subjected to Western blot analysis and the overexpressed fusion proteins (TMEM192, Rab7) were detected using an antibody against GFP. As a control, samples were analysed in parallel for the intralysosomally localized enzyme β-glucocerebrosidase (β-GC) and the cytoskeletal component β-tubulin. The molecular mass in kDa is indicated on the right-hand side.
membrane proteins utilizing several targeting motifs are known [8–12]. However, the positioning of the two dileucine motifs in direct vicinity to each other is a conspicuous feature. Proteins such as M6PRs that were identified previously to be dependent on DXXLL motifs are known to cycle between the TGN and endosomal compartments [7,8]. In contrast, TMEM192 is localized in late endosomes/lysosomes under steady-state conditions, as demonstrated for overexpressed and endogenous protein [15], and localization in the TGN or cycling between the TGN and endosomes has not so far been observed. As far as we are aware, TMEM192 is the first example of a lysosomal membrane protein whose targeting is dependent on dileucine motifs of the DXXLL type.

A common feature of several DXXLL motifs is the presence of one or more serine residues upstream of the acidic cluster (Figure 4B, underlined). In the case of the CD− (cation-dependent M6PR [34] as well as the CI-M6PR [25,35] it was demonstrated that these serine residues can be phosphorylated by CKII (casein kinase II). Sorting efficiency of the CI-M6PR was reported to be impaired when phosphorylation is prevented by mutation of the respective serine residue [25]. At a molecular level, phosphorylation of the serine enhances interaction of the targeting motif with the VHS domain of the GGA adapter proteins [36]. Together, in TMEM192 three phosphorylatable residues are located upstream of the first dileucine motif: Ser11, Thr15 and Ser17. Phosphorylation of these residues was detected in two proteomic studies [37,38] and Ser17 is part of a sequence context fitting to the [ST]XX[DE] consensus motif for phosphorylation by CKII [7]. In order to assess any potential influence of these putatively phosphorylated residues on intracellular trafficking of TMEM192, a triple mutant devoid of all phosphorylatable amino acids in these three positions (TMEM192-S11A,T15A,S17A) was created. The steady-state subcellular localization of this mutant was indistinguishable from that of wild-type TMEM192 (results not shown), indicating that phosphorylation of any of these three residues is dispensable for late endosomal/lysosomal targeting of TMEM192. Nevertheless other determinants within the sequence context surrounding the dileucine motifs may be relevant for functionality of the motifs. Therefore it may be of interest to systematically delete different parts of the N-terminal tail, especially of the section between the identified motifs and the membrane, and to analyse targeting of these mutants.

Disulfide bond formation and membrane topology of TMEM192

The cytosolic localization of both the N- and C-termini of TMEM192 and thereby the overall topology were experimentally corroborated by two different approaches. Consequently, cytosolic localization of Cys266 has to be assumed, although this cysteine residue, according to the mutagenesis studies, mediates disulfide bond formation between two TMEM192 monomers. In general, disulfide bond formation is considered to be restricted to the oxidative environment of the endoplasmic reticulum leading to the presence of disulfide bridges in extracellular proteins, and proteins or protein domains present in the lumen of the secretory pathway and endo-/lysosomal compartment [39]. In contrast, the redox state in the cytosol does not favour the formation of disulfide bonds [39] owing to the presence of two major thiol-reducing systems, the cysteine-containing tripeptide glutathione and the protein thioredoxin [40]. Nevertheless disulfide bonds in cytosolic enzymes have been observed under conditions of oxidative stress when the capacity of these protection mechanisms is exhausted [41]. Furthermore, the reversible oxidation and reduction of thiols and disulfides in cytosolic enzymes was suggested as a means of regulation for enzymatic activity and metabolic pathways [42]. Additional examples show that exceptions from the general rule may exist: two intramolecular disulfide bridges were identified in the cutaneous fatty-acid-binding protein from rats which is localized in the cytosol [43]. Furthermore, a single-chain Fv antibody ectopically expressed in stably transformed plant cells was reported to form intramolecular disulfide bonds in the cytosol [44]. In this context it was speculated whether the redox state of the cytoplasm is homogeneous or whether zones with a less reductive environment may exist due to some form of compartmentalization [44].

In the case of TMEM192, the observed disulfide could also represent an artefact caused by oxidation of the free thiols after disruption and extraction of the cells [42]. Nevertheless, this finding may provide important structural information about TMEM192. For such an artificial disulfide formation to occur, the C-termini of two TMEM192 molecules would need to be in close proximity to each other and should remain associated upon cell extraction and solubilization of membrane proteins in order to allow oxidation of these two cysteine residues and covalent stabilization of the dimer. Presumably, the C-terminal part of TMEM192 is critically involved in mediating the interaction between two TMEM192 monomers. As the cysteine residue identified (Cys266) is not conserved among most species it seems likely that this residue (and therefore also the disulfide) is not critical for dimerization and possibly also the function of TMEM192.

The molecular function of TMEM192 remains currently unknown. The results of the present study regarding late endosomal/lysosomal targeting, complex formation and topology of TMEM192 will certainly facilitate functional studies. For instance, the identification of two critical targeting motifs makes it possible to redirect overexpressed TMEM192 to the cell surface. This allows uptake or electrophysiological studies in order to test for a possible transport or channel function of TMEM192, which has been successfully performed for a variety of late endosomal/lysosomal transporters [6]. From a more general point of view, the findings of the present study have revealed extended or possibly even novel functions of a known type of sorting motif that had not been implicated in the sorting of lysosomal proteins previously. Therefore it may be anticipated that the analysis of the sorting determinants of further emerging novel lysosomal membrane proteins may provide further novel insights into the mechanisms and pathways involved in late endosomal/lysosomal targeting.

AUTHOR CONTRIBUTION

Jörg Behnke performed most of the experiments in the present study and analysed the experimental data. Eeva-Liisa Eskelinen analysed the membrane topology of TMEM192 by immuno electron microscopy. Paul Saftig obtained grant support and contributed to the design of the study. Bernd Schröder designed and supervised the study, and wrote the manuscript. All authors contributed to the editing of the manuscript.

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REFERENCES