A single conserved proline residue determines the membrane topology of stomatin

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Stomatin is an integral membrane protein which is widely expressed in many cell types. It is accepted that stomatin has a unique hairpin-loop topology: it is anchored to the membrane with an N-terminal hydrophobic domain and the N- and C-termini are cytoplasmically localized. Stomatin is a prototype for a family of related proteins, containing among others MEC-2 (mechanosensory protein 2) from Caenorhabditis elegans, SLP (stomatin-like protein)-3 and podocin, all of which interact with ion channels to regulate their activity. Members of the stomatin family partly localize in DRMs (detergent-resistant membrane domains) enriched in cholesterol and sphingolipids. It has been proposed that a highly conserved proline residue in the middle of the hydrophobic domain directly binds cholesterol and that cholesterol binding is necessary for the regulation of ion channels. In the present study we show that a small part of the stomatin pool exists as a single-pass transmembrane protein rather than a hairpin-loop protein. The highly conserved proline residue is crucial for adopting the hairpin-loop topology: substitution of this proline residue by serine transfers the whole stomatin pool to the single-pass transmembrane form, which no longer localizes to DRMs. These results suggest that formation of the hairpin loop is inefficient and that the conserved proline residue is indispensable for formation of the hairpin loop. The single-pass transmembrane form exists also for SLP-3 and it should be considered that it mediates part of the physiological functions of stomatin and related proteins.

Key words: mechanosensitive ion channel, mechanosensory protein-2 (MEC-2), membrane topology, podocin, stomatin, stomatin-like protein-3 (SLP-3).

INTRODUCTION

Stomatin was originally isolated as an integral membrane protein of erythrocytes [1–3]. It is absent in mature red cells of patients suffering from overhydrated hereditary stomatocytosis (OMIM #185000) [2,4], a form of haemolytic anaemia that is characterized by a membrane leak of monovalent cations. This ion misbalance has led early on to the hypothesis that stomatin may regulate the activity of ion channels. Gene sequencing has not revealed any mutation in the stomatin gene in patients suffering from stomatocytosis, however, indicating that the lack of stomatin is secondary to another disease-causing defect. Expression of stomatin is not restricted to erythrocytes and can be detected in many tissues [3], including sensory neurons of the trigeminal and spinal ganglia [5,6]. Its precise physiological functions in these tissues, as well as in erythrocytes, are unknown. Recently however, analysis of stomatin-knockout mice revealed a reduced sensitivity in a subtype of mechanoreceptor, the D-hair receptor, further implicating stomatin in the regulation of ion channels [7]. Moreover, it has recently been shown that stomatin enhances the transport of dehydroascorbic acid by GLUT1 (glucose transporter 1) in erythrocytes [8], which is important for mammals that are unable to synthesize vitamin C.

Stomatin is a 288-amino-acid protein. It contains a predicted hydrophobic domain towards its N-terminus (amino acids 26–54; Figure 1A) which targets the protein to the plasma membrane. Proteinase K treatment of intact erythrocytes does not degrade stomatin, however, suggesting that both the N- and C-terminus of stomatin face the cytoplasm [1], predicting a unique hairpin-loop topology for stomatin. Identification of Ser10 as a phosphorylation site confirmed the cytoplasmic location of the N-terminus [9]. Moreover, stomatin is palmitoylated [2], with Cys87 being the major and Cys97 a minor palmitoylation site [10]. Palmitoylation at these two residues that flank the hydrophobic domain supports the hairpin-loop topology. Palmitoylation targets membrane proteins to DRMs (detergent-resistant membrane domains) that are enriched in cholesterol and sphingolipids, and stomatin indeed localizes to DRMs [11,12]. DRMs are often equated with lipid rafts, an assumption that is not uncontested, however [13,14].

Close homologues of stomatin are SLP-3 [stomatin-like protein-3; also called SRO (stomatin-related olfactory protein)], SLP-1 and podocin [15]. SLP-3 [16,17] is essential for mechanosensitivity of a subset of mechanosensitive fibres of the mouse skin [18]. SLP-1 is a bipartite protein that contains a stomatin-like part at the N-terminus and a non-specific lipid-transfer domain at the C-terminus [19]. It is mainly expressed in the brain. Podocin is a podocyte-specific protein that interacts with nephrin [20] and the ion channel TRPC6 (canonical transient receptor protein 6) [21] at the slit diaphragm, a specialized intercellular junction that is part of the glomerular-filtration barrier. Mutations in podocin lead to autosomal recessive steroid-resistant nephrotic syndrome [22]. Caenorhabditis elegans contains several homologues of stomatin; the closest is MEC-2 (mechanosensory protein-2) [23]. MEC-2 is a component of a multiprotein complex, which also contains the

Abbreviations used: DRM, detergent-resistant membrane domain; DTT, dithiothreitol; ER, endoplasmic reticulum; HA, haemagglutinin; MEC, mechanosensory protein; OR-2, oocyte ringer solution; PNGase F, peptide N-glycosidase F; RLU, relative light unit; SENP1, SUMO1 (small ubiquitin-related modifier 1)-specific peptidase 1; SLP, stomatin-like protein; SUMO, small ubiquitin-related modifier; VSV-G, vesicular stomatitis virus glycoprotein.

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ion-channel subunits MEC-4 and MEC-10 [24]. MEC-2 regulates the activity of the channel subunits [25] and is essential for mechanosensitivity of the worm skin [23]. In summary, available evidence suggests that stomatin and related proteins have a role in the regulation of ion channels in general, and of mechanosensitive ion channels in particular.

SLP-3, SLP-1, podocin and MEC-2 all share the N-terminal hydrophobic domain with stomatin and are believed to adopt a similar hairpin-loop topology. SLP-3, podocin and MEC-2 also localize in DRMs [16,20,21]. Previously, it has been shown that podocin and MEC-2 bind cholesterol and that cholesterol binding is essential for the regulation of the associated ion channels [21]. Mutation of a proline residue (Pro134) within the hydrophobic membrane-associated domain of MEC-2 disrupted cholesterol binding and it was concluded that Pro134 binds cholesterol [21]. This proline residue is highly conserved in SLPs.

In the present study we show that a fraction of the stomatin and SLP-3 protein pool exists as a transmembrane form in addition to the hairpin-loop form that is associated to the inner leaflet of the membrane. Moreover, we show that the highly conserved proline residue within the hydrophobic domain determines the topology: substitution of the proline residue transfers the whole population of stomatins to the transmembrane form. The results of the present study have important implications for the folding and membrane insertion of stomatin. Moreover, they suggest that mutation of the conserved proline residue has no specific effect on cholesterol binding, but rather exerts its functional effects by unspecifically disrupting the hairpin-loop topology of stomatins.

**MATERIALS AND METHODS**

**Insertion of epitope tags and site-directed mutagenesis**

All cDNA constructs were cloned into the pRSP6009 oocyte expression vector [26]. Using PCR, stomatin was cloned from mouse heart cDNA, human SLP-3 from EST clone XM 090631 [provided by RZPD, Berlin, Germany (German Resource Centre for Genome Research)], and SENP1 [SUMO1 (small ubiquitin-related modifier 1)/sentrin/SMT3 (suppressor of mif two 3 homologue 1)-specific peptidase 1] from human brain cDNA respectively; clones were verified by sequencing.

Point mutations were introduced into the stomatin coding sequence by recombinant PCR using Pwo DNA polymerase (Roche Applied Science). For some experiments, stomatin and SLP-3 were tagged with the VSV-G (vesicular stomatitis virus glycoprotein) epitope (YTDIEMNRLGK) at their C-termini. For luminescence measurements, stomatin wild-type and the P47S mutant were tagged with the HA (haemagglutinin) epitope (YPYDVPDYA) at their C-termini. PCR-derived fragments were sequenced (MWG Biotech).

**Maintenance of oocytes and injection of cRNA**

Animal care and experiments followed approved institutional guidelines at the Universities of Würzburg and Tübingen. Ovaries were surgically removed under anaesthesia from adult *Xenopus laevis* females and oocytes were isolated. Using mMessage mMachene (Ambion), capped cRNA was synthesized by SP6 RNA polymerase from cDNAs, which had been linearized by MluI. After injection of cRNA, oocytes were kept in OR-2 (oocyte ringer solution: 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM Na2HPO4, 5.0 mM Hepes, 1.0 mM MgCl2, 1.0 mM CaCl2 and 0.5 g/l polyvinlypyrrolidone, pH 7.3) at 19°C.

**Immunoblot analysis and deglycosylation**

cRNA (0.1–10 ng) coding for the tagged proteins was injected into oocytes. Microsomal membranes were prepared 2 days after injection as previously described [27]. In addition, white membranes were prepared from mouse erythrocytes. Erythrocytes were washed three times with NaCl solution [125 mM NaCl, 5 mM KCl, 32 mM Hepes/NaOH, 5 mM glucose, 1 mM MgSO4 and 1 mM CaCl2 (pH 7.4)] and haemolysed by hypotonic shock in ice-cold 20 mM Hepes/NaOH (pH 7.4) solution (200 μl of erythrocyte pellet in 50 ml of buffer). Ghost membranes were pelleted by centrifugation (15 000 g for 20 min at 4°C) and re-opened with ice-cold 10 mM Hepes/NaOH (pH 7.4) solution. Finally, white membranes were pelleted by centrifugation (15 000 g for 20 min at 4°C) and frozen at −80°C. When indicated, microsomal or erythrocyte membranes were treated with PNGase F (peptide N-glycosidase F; Roche) or a mouse anti-(VSV-G) antibody (YaCm1 antibody at a 1:250 dilution; sc-48309, Santa Cruz Biotechnology). For detection of the tagged constructs, we applied a mouse peroxidase-coupled anti-HA antibody (1:1000; Roche Applied Science) or a mouse anti-(VSV-G) antibody.
Sucrose-density-gradient centrifugation

Approx. 30 oocytes expressing wild-type stomatin or stomatin P47S mutant were homogenized on ice in 1 ml of lysis buffer [1 % (v/v) Triton X-100, 500 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl (pH 7.4), 2 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml aprotinin and 1 μg/ml pepstatin A]. The samples were incubated on ice for 30 min, large cell debris was removed by centrifugation (1000 g at 4 °C for 10 min), and the supernatants were transferred in pre-chilled 12 ml centrifuge tubes (Beckman) on ice. The probes were then mixed with 1.05 ml of an 85 % (w/v) sucrose solution in lysis buffer without Triton X-100, carefully overlaid with 5 ml of a 45 % (w/v) sucrose solution, each in lysis buffer without Triton X-100. The tubes were placed in a SW41 rotor (Beckman) and centrifuged for 18–20 h at 34 150 rev./min (4 °C). Afterwards, starting from the top, fractions each of 1 ml were carefully collected, transferred to 2 ml tubes and thoroughly mixed with an equal volume of methanol to precipitate the proteins. The samples were incubated overnight at −20 °C and centrifuged (18 000 g at 4 °C for 10 min). The pellets were washed with 500 μl of 70 % (v/v) ethanol (4 °C) and centrifuged as above. The pellets were then dried for approx. 20 min in a vacuum concentrator, dissolved in gel-loading buffer and analysed by SDS/PAGE and immunoblotting as described above.

Precipitation of cholesterol with digitonin

Stomatin was co-precipitated with cholesterol from cell lysates by the addition of digitonin as described previously [21,28]. Microsomal membranes of oocytes, expressing epitope-tagged stomatins, were prepared as described above and resuspended in a buffer containing 20 mM Tris/HCl (pH 7.6), 100 mM NaCl, 2 % (w/v) BSA, 1 mM EDTA and 1 % (v/v) Triton X-100. Digitonin (10 %) dissolved in methanol was added to each sample at a ratio of 1:10 of the whole volume. The same amount of pure methanol was added as a negative control to the rest of the samples. The tubes were rotated for 30 min at 4 °C and centrifuged for 10 s at 12 000 g. Pellets were washed carefully (the buffer was supplemented with digitonin or with pure methanol for the negative controls), resuspended in loading buffer and analysed by SDS/PAGE and immunoblotting as described above.

Quantification of surface expression

Surface expression was determined for HA-tagged constructs as previously described [29]. Briefly, oocytes were injected with 10 ng of cRNA and incubated in OR-2 solution for 2 days at 19 °C. The follicular membranes were removed manually and the oocytes were placed on ice, blocked with 1 % (w/v) BSA-ND96 and incubated with a monoclonal rat anti-HA antibody (0.5 μg/ml; clone 3F10, Roche). The oocytes were washed several times and incubated with the horseradish peroxidase-coupled secondary antibody [0.8 μg/ml; goat anti-rat F(ab′)2, Jackson ImmunoResearch Laboratories]. Single oocyte chemiluminescence was detected with an Orion II Microplate Luminometer (Berthold Detection Systems) in RLUs (relative light units)/s. Surface expression was quantified for oocytes from two different frogs (for each n = 8–10 oocytes). For each batch, RLUs were normalized to the mean of untagged stomatin. Results are reported as means ± S.E.M. Statistical analysis was performed using the unpaired Student’s t test.

RESULTS

A small fraction of stomatin is N-glycosylated and passes the cell membrane

Western blot analysis of the membrane fraction of Xenopus oocytes expressing VSV-tagged mouse stomatin revealed strong immunoreactivity of an antigen of the molecular mass expected for stomatin (∼31 kDa) (Figure 1B). In addition to this expected antigen, we consistently observed another antigen of a higher molecular mass of ∼42 kDa. This antigen of 42 kDa was not present in lysates from uninjected oocytes, suggesting it was related to stomatin (Figure 1B, lanes 1 and 2). The relative amount of the 42 kDa antigen was variable, but it was always less abundant than the 31 kDa antigen (Figure 1B). The same pattern was consistently detected, also with a polyclonal anti-stomatin antibody (for example, see Figure 2A). The 42 kDa antigen could not be explained by dimerization of stomatin because the expected size of a dimer would be ∼65 kDa. We considered that the higher molecular mass antigen represented a form of stomatin that was post-translationally modified. It is known that stomatin is palmitoylated on two cysteine residues, Cys38 and Cys35; Cys35 is the major palmitoylation site [10]. Each palmitate group should increase the apparent molecular mass by much less than 1 kDa, however, suggesting that palmitoylation cannot account for the 42 kDa form of stomatin. Similarly, it was shown that phosphorylation of Ser10, the sole phosphorylation site on stomatin in erythrocytes [9], does not substantially increase the apparent molecular mass of stomatin [9], also excluding phosphorylation as the basis for the 42 kDa antigen.

According to the proposed hairpin-loop topology of stomatin, we considered SUMOylation as another possible intracellular modification. SUMO is covalently attached to lysine residues of the target protein. SUMO can be cleaved from its target proteins by specific proteases, SENPs [30]. We cloned SENP1 and co-expressed it in oocytes with stomatin. This did not reduce the abundance of the large antigen (results not shown), however, arguing against SUMOylation of stomatin.

Therefore we also considered N-glycosylation. Unexpectedly, treatment of membrane proteins prepared from stomatin-expressing oocytes with PNGase F made the 42 kDa form completely disappear (Figure 2A), demonstrating that the large form arose by N-glycosylation of stomatin. This was a surprising finding because the enzymes mediating N-glycosylation are strictly restricted to the lumen of the ER (endoplasmic reticulum) and Golgi, indicating that part of the stomatin polypeptide chain was exposed to the lumen of these compartments. There are five potential consensus sequences for N-glycosylation (NXS/T, where X is any amino acid, except proline) in the primary sequence of mouse stomatin; they all localize to the C-terminus (Figure 2B; for simplicity, the sites are numbered from 1 to 5). N-glycosylation of stomatin would thus predict that the stomatin C-terminus was exposed to the ER lumen. In order to corroborate this finding, we mutated the five consensus sequences individually or in combination.

Substitution by asparagine of Thr37 in the second most proximal consensus site (Asn135→Thr136→Thr137, ΔN2) reduced the molecular mass of the large stomatin form by approx. 2–3 kDa (Figure 2B, lane 2), which is the expected size for a single N-glycan. Combined disruption of the first and second sites, ΔN1/N2, by simultaneous substitutions T130V and T137N...
reduced the molecular mass further (Figure 2B, lane 3). Combined disruption of the first three proximal sites, ΔN1/2/3, by simultaneous substitutions T130V, T137N and N159Q led to a minor pool of stomatin that was only a few kDa larger than the major pool (Figure 2B, lane 4). This minor pool of stomatin was still sensitive to PNGase F (results not shown). Combined substitution of all five consensus sequences completely eliminated the large stomatin form (Figure 2B, lane 5). These results clearly establish that a small subpopulation of stomatin heterologously expressed in *Xenopus* oocytes carried glycans on at least four asparagine residues on the C-terminal part of the protein. As can be seen in Figure 2B (lanes 2 and 3), removal of individual N-glycans reduced the molecular mass, but increased the abundance of the residual N-glycosylated stomatin, an effect which was consistently observed.

N-glycosylation predicted that the C-terminus was exposed to the lumen of the ER, suggesting a 'single-pass' transmembrane topology for this variant. If this form of stomatin also reaches the cell surface, this topology would predict an extracellular location for the C-terminus of this stomatin variant. To verify this prediction, we performed a surface expression assay with oocytes expressing stomatin bearing an HA tag at its C-terminus. In this assay the HA tag is specifically detected by a monoclonal antibody and bound antibodies are quantified via a secondary antibody and a luminescence reaction [29]. In intact cells, the C-terminal tag should be accessible from the extracellular side only in the case where stomatin passes across the membrane, but not in the case where both termini are on the cytoplasmic face of the plasma membrane. This assay indeed revealed more than 50-fold higher luminescence of oocytes expressing stomatin–HA compared with oocytes expressing a non-tagged stomatin (Figure 2C; *P* < 0.01), providing additional evidence that the glycosylated form of stomatin is a transmembrane protein. In addition, this result suggested that this form is targeted to the cell surface.

We asked whether the large stomatin form is an artefact due to overexpression in the heterologous oocyte system and searched for evidence of the N-glycosylated form in vivo. We analysed protein extracts from mouse erythrocytes with a polyclonal anti-stomatin antibody. As in *Xenopus* oocytes we observed a second antigen of ~42 kDa in addition to the expected 31 kDa antigen (Figure 3A, lane 1). This form was only a minor fraction of the whole stomatin pool of mouse erythrocytes, but it was completely absent in samples treated with PNGase F (Figure 3A, lane 2), suggesting that a fraction of stomatin is N-glycosylated also in native cells. Since erythrocytes lack most intracellular organelles,
the transmembrane N-glycosylated form of stomatin is probably present on the plasma membrane of erythrocytes.

**N-glycosylation is a common feature of SLPs**

We asked whether N-glycosylation is specific for stomatin or is shared by other SLPs. To investigate this question, we chose SLP-3 [16,17], which was recently implicated in touch sensation in mice [18]. Western blot analysis of the membrane fraction of *Xenopus* oocytes expressing human SLP-3 revealed an antigen of the expected size of ∼31 kDa and, in addition, another form of a higher molecular mass of ∼35 kDa (Figure 3B, lane 1). Similar to the large molecular mass form of stomatin, the large form of SLP-3 was sensitive to PNGase F: treatment with PNGase F made this form completely disappear (Figure 3B, lane 2), showing that it arose by N-glycosylation of SLP-3. In contrast with stomatin, the N-glycosylated form of SLP-3 was equally as abundant as the unmodified form suggesting that approx. 50% of the SLP-3 pool in oocytes was a single-pass transmembrane protein. SLP-3 is 65% identical with stomatin at the amino acid level, but has only two of the five consensus sequences for N-glycosylation of stomatin (the two distal ones), which readily accounts for the smaller size difference (4 kDa) between the small (un-glycosylated) and large (glycosylated) form of SLP-3.

**Pro47 in the middle of the membrane-associated domain establishes the ‘hairpin’ topology of stomatin**

Stomatin has a proline residue (Pro47) that localizes to the middle of the hydrophobic, membrane-associated domain (Figure 4A). Since proline is a strong α-helix breaker that induces turns in the protein conformation, we hypothesized that Pro47 may be crucial for adopting the membrane-associated hairpin-loop topology of stomatin. We substituted Pro47 by a serine residue. For this P47S variant, the 31 kDa form that corresponds to the unmodified form of stomatin was completely absent, whereas the large 42 kDa form that corresponds to the glycosylated form was the sole antigen (Figure 4B, lane 1). Treatment of stomatin P47S with PNGase F reduced the molecular mass of the entire stomatin pool to 31 kDa, corresponding to the completely deglycosylated form of the protein (Figure 4B, lane 2). These results show that the P47S substitution completely transferred stomatin into an N-glycosylated single-pass transmembrane form.

Next we determined cell-surface expression of the P47S variant using the luminescence assay. Luminescence was approx. 300-fold higher for stomatin P47S carrying the HA tag at its C-terminus than for untagged controls (Figure 4C; P < 0.01), confirming that the C-terminus of the glycosylated form of stomatin was extracellular. This result shows that this variant was a transmembrane protein which was targeted to the cell surface. Luminescence was approx. 5-fold higher for the P47S mutant than for wild-type stomatin (P < 0.05), reflecting the larger fraction of the transmembrane form. Western blot analysis performed on the same batch of oocytes used for luminescence measurements demonstrated that the total amount of tagged P47S protein was comparable with the total amount of wild-type stomatin (results not shown), confirming that the 5-fold increase of the luminescence with the P47S variant was specifically associated with an increased amount of the transmembrane form of stomatin.

**Stomatin localizes to DRMs and directly binds cholesterol. The P47S mutation abolishes detergent resistance and the association with cholesterol**

The results of the present study described above strongly suggested that the P47S mutation ‘switches’ the protein topology from a membrane-associated hairpin form to an N-glycosylated transmembrane form. It is established that stomatin localizes to DRMs [11,12]. We next studied whether the transmembrane form of stomatin also localizes to DRMs. We performed sucrose-density-gradient centrifugation of lysates from *Xenopus* oocytes and isolated nine fractions, which we analysed by SDS/PAGE and Western blotting. Stomatin was enriched in the detergent-resistant and detergent-sensitive fractions in these gradients, neither in the detergent-resistant fractions, nor in the detergent-sensitive fractions. We attribute this lack of detection to the low abundance of the large stomatin form. The P47S variant, however, which consists of only the 42 kDa fully glycosylated form, was clearly detected in the detergent-sensitive fraction of the gradient (Figure 5), suggesting that the transmembrane form does not localize to DRMs.

DRMs are enriched in cholesterol and cholesterol can be precipitated by the detergent digitonin [28]. Therefore we tested...
A proline residue in a peptide chain cannot form hydrogen bonds that stabilize a $\alpha$-helix; moreover, it restricts the rotation of the backbone of the chain. As a consequence, proline has a tendency to break $\alpha$-helices. It is therefore likely that Pro47 has a specific role in the formation of the hairpin-loop topology of stomatin. There are other highly conserved residues adjacent to Pro$^{47}$, however (e.g. Thr$^{45}$ and Ser$^{49}$, Figure 4A), and we cannot rule out that these residues also have a role in formation of the hairpin-loop topology.

The low relative abundance of the transmembrane form of stomatin (Figures 1–3) is certainly the reason why this form has so far not been described. A high-molecular-mass form of MEC-2, however, has recently been observed [31]. In agreement with our own results with stomatin, substitution of the conserved proline in the hydrophobic domain of MEC-2 (Pro$^{134}$) strongly increased the relative amount of the high-molecular-mass form [31]. Since it was suggested that Pro$^{134}$ in MEC-2 binds cholesterol [21], it was speculated that MEC-2 with cholesterol bound migrated more quickly than MEC-2 lacking cholesterol [31]. We propose an alternative explanation. The proximal and the distal consensus sequences for N-glycosylation of stomatin (Asn$^{128}$ and Asn$^{264}$, Figure 2B) are conserved in MEC-2; in addition, MEC-2 contains a third consensus sequence at the distal C-terminus that is not present in stomatin. Thus a transmembrane form of MEC-2 would likely be N-glycosylated and have a larger apparent molecular mass than the hairpin-loop form. Therefore the results of the present study suggest that the high molecular mass form of MEC-2 is a single-pass N-glycosylated transmembrane protein. We showed that stomatin carrying the P47S mutation does not co-precipitate with DRMs, in contrast with the hairpin-loop form (Figure 5). This result suggests that substitution of Pro$^{134}$ in MEC-2 binds cholesterol [21], it was speculated that MEC-2 with cholesterol bound migrated more quickly than MEC-2 lacking cholesterol [31]. We propose an alternative explanation. The proximal and the distal consensus sequences for N-glycosylation of stomatin (Asn$^{128}$ and Asn$^{264}$, Figure 2B) are conserved in MEC-2; in addition, MEC-2 contains a third consensus sequence at the distal C-terminus that is not present in stomatin. Thus a transmembrane form of MEC-2 would likely be N-glycosylated and have a larger apparent molecular mass than the hairpin-loop form. Therefore the results of the present study suggest the that high molecular mass form of MEC-2 is a single-pass N-glycosylated transmembrane protein. We showed that stomatin carrying the P47S mutation does not co-precipitate with DRMs, in contrast with the hairpin-loop form (Figure 5). This result suggests that substitution of Pro$^{134}$ in MEC-2 might not have a direct effect on cholesterol binding, but rather abolishes cholesterol binding of MEC-2 through unspecific inhibition of localization in DRMs.

Assuming that the high-molecular-mass form of MEC-2 is indeed an N-glycosylated form, and considering that stomatin and SLP-3 both exist as an N-glycosylated form (Figures 2 and 3), it is likely that folding into a hairpin-loop structure is inefficient for all stomatin-related proteins and that a fraction of all of these proteins exist as a transmembrane form. Because of its low abundance compared with the hairpin-loop form, it is unlikely that the transmembrane form of stomatin is of physiological relevance. Formally, however, this possibility cannot be ruled out. Moreover, we showed that the transmembrane form of stomatin is present at the plasma membrane (Figures 2 and 4) and in native cells (Figure 3A). In addition, we showed that $\sim$50% of the total SLP-3 pool is N-glycosylated suggesting that it also adopts the
transmembrane topology (Figure 3B). Further studies will reveal whether the transmembrane form is just a misfolded by-product of stomatin and related proteins or whether it mediates part of their physiological functions.

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Figure 7  Scheme illustrating the biosynthetic pathways leading to the hairpin-loop and the transmembrane form of stomatin

For details please see the main text. Whether Cys30 becomes palmitoylated in the transmembrane form is unknown. IC, intracytoplasmic; EC, extracytoplasmic; LR, ‘lipid raft’ (assuming that DRMs are identical with lipid rafts).

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