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A single conserved proline residue determines the membrane topology of stomatin

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Abbreviations used: HA, hemagglutinin; MES, 2-(*N*-morpholino)ethanesulfonic acid; PNGase F, *N*-glycosidase F;

Short title: Membrane topology of stomatin

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Membrane topology of stomatin

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 domain and N- and C-termini are cytoplasmically localized. Stomatin is a prototype for a family of related proteins, containing among others MEC-2 from *C. elegans*, stomatin-like protein (SLP) 3, and podocin, all of which interact with ion channels to regulate their activity. Members of the stomatin family partly localize in detergent resistant membrane domains (DRMs) 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. Here 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 is crucial for adopting the hairpin loop topology: substitution of this proline 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 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: stomatin, MEC-2, SLP-3, podocin, mechanosensitive ion channels, membrane topology

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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 hemolytic anemia 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 the Glut1-glucose transporter 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; Fig. 1A) that targets the protein to the plasma membrane. Proteinase K treatment of intact erythrocytes does not degrade stomatin, however, suggesting that both N- and C-terminus of stomatin face the cytoplasm [1], predicting a unique hairpin loop topology for stomatin. Identification of Ser-10 as a phosphorylation site confirmed the cytoplasmic location of the N-terminus [9]. Moreover, stomatin is palmitoylated [2], with Cys-30 being the major and Cys-87 a minor palmitoylation site [10]. Palmitoylation at these two residues that flank the hydrophobic domain support the hairpin loop topology. Palmitoylation targets membrane proteins to detergent resistant membrane domains (DRMs) 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 stomatin-like protein-3 (SLP-3, also called stomatin related olfactory protein or SRO), SLP-1 and podocin [15]. SLP-3 [16, 17], is essential for mechanosensitivity of a subset of mechanosensitive fibers 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 [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]. *C. elegans* contains several homologues of stomatin; the closest is MEC-2 [23]. MEC-2 is a component of a multiprotein complex, which also contains the 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]. Recently, 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 (P134) within the hydrophobic membrane-associated domain of MEC-2 disrupted cholesterol binding and it was concluded

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that Pro-134 binds cholesterol [21]. This proline residue is highly conserved in stomatin-like proteins.

Here 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 transfers the whole population of stomatins to the transmembrane form. Our results have important implications for the folding and membrane insertion of stomatin. Moreover, they suggest that mutation of the conserved proline 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 pRSSP6009 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), and SENP1 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 vesicular stomatitis virus glycoprotein (VSV-G) epitope (YTDIEMNRLGK) at their C-termini. For luminescence measurements, stomatin wild type and P47S mutant were tagged with the hemagglutinin (HA) epitope (YPYDVPDYA) at their C-termini. PCR-derived fragments were entirely 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 anesthesia from adult *Xenopus laevis* females and oocytes were isolated. Using mMessage mMachinE (Ambion, Austin, TX), capped cRNA was synthesized by SP6 RNA polymerase from cDNAs, which had been linearized by Mlu I. After injection of cRNA, oocytes were kept in oocyte ringer solution (OR-2; concentrations in mM: 82.5 NaCl, 2.5 KCl, 1.0 Na₂HPO₄, 5.0 HEPES, 1.0 MgCl₂, 1.0 CaCl₂, and 0.5 g/liter polyvinylpyrrolidone) at 19°C.

Immunoblot analysis and deglycosylation

0.1-10 ng of cRNA 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 thrice with NaCl solution (in mM: 125 NaCl, 5 KCl, 32 HEPES/NaOH, 5 glucose, 1 MgSO₄, 1 CaCl₂; pH 7.4), hemolysed by hypotonic shock in ice-cold 20 mM HEPES/NaOH (pH 7.4) solution (200 µl erythrocyte pellet in 50 ml). Ghost membranes were pelleted (15 000 x 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 (15 000 x g for 20 min at 4°C) and frozen at -80°C. When indicated, microsomal or erythrocyte membranes were treated with PNGase F as follows: they were dissolved in denaturing buffer (0.1% SDS / 50 mM β-mercaptoethanol), incubated at 56°C for 10 minutes, sodium phosphate (pH 8.0) was added to the final concentration of 80 mM and the samples were incubated for 3 hours to overnight at 37°C with PNGase F (Roche) in the presence of 1% Triton X100. Afterwards, they were supplemented with gel loading buffer (50 mM Tris, pH 6.8; 100 mM DTT; 2% SDS; 0.01 % Bromphenol blue; 10%

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Glycerol); control preparations of microsomal or erythrocyte membranes were treated in the same way but in the absence of PNGase F and supplemented with gel loading buffer. The membranes were then separated on a 12% Tris-glycine polyacrylamide-SDS gel and transferred to a polyvinylidene fluoride membrane (PolyScreen; PerkinElmer Life Sciences). For immunological detection, we used a polyclonal antibody raised in goat against an N-terminal epitope of mouse stomatin (N-14 antibody at a 1:250 dilution; sc-48309; Santa Cruz Biotechnology). For detection of the tagged constructs, we applied mouse peroxidase-coupled anti-HA (1:1000; Roche Applied Science) or mouse anti-VSV-G (0,5 µg/ml; Roche Applied Science). As secondary antibodies for western blotting we applied peroxidase-coupled anti-mouse (1:5000; Santa Cruz Biotechnology) and peroxidase-coupled anti-goat antibodies (1:2500; Santa Cruz Biotechnology). Bound antibodies were revealed by chemiluminescence (ECL or ECL-PLUS, GE Healthcare).

Sucrose density gradient centrifugation

About 30 oocytes, expressing wild-type stomatin or stomatin P47S, were homogenized on ice in 1 ml lysis buffer (1% Triton; 500 mM NaCl; 5 mM EDTA; 50 mM Tris, pH 7,4; 2mM PMSF, leupeptin, aprotinin and pepstatinA). The samples were incubated on ice for 30 min, large cell debris removed by centrifugation (1,000 X g at 4°C for 10 min), and the supernatants transferred in pre-chilled 12 ml centrifuge tubes (Beckmann) on ice. The probes were then mixed with 1,05 ml of an 85% sucrose solution in lysis buffer without Triton X100, carefully overlaid with 5 ml of a 45% and 2 ml of a 5% sucrose solution, each in lysis buffer without Triton X100. The tubes were placed in a SW 41 rotor (Beckman Ultracentrifuges) and centrifuged for 18 – 20 h at 144,000 X g (4°C). Afterwards, starting from the top fractions of 1 ml each 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 X g at 4 °C for 10 min). The pellets were washed with 500 µl 70 % ethanol (4°C) and centrifuged as before. The pellets were then dried for ~ 20 min in a vacuum concentrator, dissolved in gel loading buffer and analyzed by SDS – PAGE and immunoblotting as described above.

Precipitation of cholesterol with digitonin

Stomatin was co-precipitated with cholesterol from cell lysates by addition of digitonin as described [21, 28]. Microsomal membranes of oocytes, expressing epitope-tagged stomatins, were prepared as described above and resuspended in a buffer containing 20 mM Tris, pH 7.6, 100 mM NaCl, 2% BSA, 1 mM EDTA and 1% Triton. 10% digitonin dissolved in methanol was added to each sample in 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 sec at 12 000 x g. Pellets were washed carefully (the buffer was supplemented with digitonin or with pure methanol for the negative controls), resuspended in loading buffer and analyzed 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 two days at 19°C. The follicular membranes were removed manually and the oocytes were placed on ice, blocked with 1% BSA-ND96 and incubated with a monoclonal rat anti-HA antibody (clone 3F10; 0,5 µg/ml; Roche). The oocytes were washed several times and incubated with the horseradish peroxidase-coupled secondary antibody (goat anti-rat F(ab')₂; 0,8 µg/ml; Jackson ImmunoResearch Laboratories). Single oocyte chemiluminescence was detected

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with an Orion II Microplate Luminometer (Berthold detection systems; Pforzheim, Germany) in relative light units (RLUs) per sec. 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 mean \pm S.E.M. Statistical analysis was performed with the unpaired 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 weight expected for stomatin (~31 kD) (Fig. 1B). In addition to this expected antigen, we consistently observed another antigen of higher molecular weight of ~42 kD. This antigen of 42 kD was not present in lysates from uninjected oocytes, suggesting it was related to stomatin (Fig. 1, lanes 1 and 2). The relative amount of the 42 kD antigen was variable but it was always less abundant than the 31 kD antigen (Fig. 1B). The same pattern was consistently detected, also with a polyclonal anti-stomatin antibody (for example, see Fig. 2A). The 42 kD antigen could not be explained by dimerization of stomatin because the expected size of a dimer would be ~65 kD. We considered that the higher molecular weight antigen represented a form of stomatin that was post-translationally modified. It is known that stomatin is palmitoylated on two cysteine residues: C30 and C87; Cys-30 is the major palmitoylation site [10]. Each palmitate group should increase the apparent molecular weight by much less than 1 kD, however, suggesting that palmitoylation cannot account for the 42 kD form of stomatin. Similarly, it was shown that phosphorylation of Ser-10, the sole phosphorylation site on stomatin in erythrocytes [9], does not substantially increase the apparent molecular weight of stomatin [9], also excluding phosphorylation as the basis for the 42 kD antigen.

According to the proposed hairpin loop topology of stomatin, we considered sumoylation as another possible intracellular modifications. The small ubiquitin-like molecule 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 SENP-1 and co-expressed it in oocytes with stomatin. This did not reduce the abundance of the large antigen (result 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 *N*-glycosidase F (PNGase F) made the 42 kD form completely disappear (Fig. 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 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 in the primary sequence of mouse stomatin (NXS/T, where X is any amino acid, except proline); they all localize to the C-terminus (Fig 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 Val of Thr130 in the most proximal consensus site (N¹²⁸A¹²⁹T¹³⁰, Δ N1) reduced the molecular weight of the large stomatin form by ~2-3 kD (Fig. 2B, lane 2), which is the expected size for a single *N*-glycan. Combined disruption of the first and second sites, Δ N1/N2, by simultaneous substitutions T130V and T137N reduced the molecular weight further (Fig. 2B; lane 3). And combined disruption of the first three

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proximal sites, $\Delta N1/2/3$, by simultaneous substitutions T130V, T137N, and N159Q led to a small pool of stomatin that was only a few kD larger than the major pool (Fig 2B; lane 4). This small pool of stomatin was still sensitive to PNGase F (not shown). Combined substitution of all five consensus sequences completely eliminated the large stomatin form (Fig. 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 Fig. 2B (lanes 2 and 3), removal of individual *N*-glycans reduced the molecular weight, 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 case stomatin passes across the membrane but not in case 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 to oocytes expressing a non-tagged stomatin (Fig. 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 artifact due to overexpression in the heterologous oocyte system and searched for evidence of the *N*-glycosylated form *in vivo*. We analyzed protein extracts from mouse erythrocytes with a polyclonal anti-stomatin antibody. Like in *Xenopus* oocytes we observed a second antigen of ~42 kD in addition to the expected 31 kD antigen (Fig. 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 (Fig. 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 stomatin-like proteins (SLPs)

We asked whether *N*-glycosylation is specific for stomatin or is shared by other stomatin-like proteins (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 kD and, in addition, another form of higher molecular weight of ~35 kD (Fig. 3B, lane 1). Similar to the large molecular weight form of stomatin, the large form of SLP-3 was sensitive to PNGase F: treatment with PNGase F made this form completely disappear (Fig. 3B, lane 2), showing that it arose by *N*-glycosylation of SLP-3. In contrast to stomatin, the *N*-glycosylated form of SLP-3 was equally abundant as the unmodified form suggesting that about 50% of the SLP-3 pool in oocytes was a “single-pass” transmembrane protein. SLP-3 is 65% identical to 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 could readily account for the smaller size difference (4 kD) between small (un-glycosylated) and large (glycosylated) form of SLP-3.

Proline 47 in the middle of the membrane-associated domain establishes the 'hairpin' topology of stomatin

Stomatin has a proline residue (P47) that localizes to the middle of the hydrophobic, membrane-associated domain (Fig. 4A). Since proline is a strong alpha-helix breaker that induces turns in the protein conformation, we hypothesized that P47 may be crucial for adopting the membrane-associated hairpin loop topology of stomatin. We substituted P47 by a serine. For this P47S variant the 31 kD form that corresponds to the unmodified form of stomatin was completely absent, whereas the large 42 kD form that corresponds to the glycosylated form was the sole antigen (Fig. 4B, lane 1). Treatment of stomatin P47S with PNGase F reduced the molecular weight of the entire stomatin pool to 31 kD, corresponding to the completely deglycosylated form of the protein (Fig. 4B, lane 2). These results show that the P47S substitution completely transferred stomatin into an N-glycosylated "single pass" transmembrane form.

We determined cell surface expression of the P47S variant using the luminescence assay. Luminescence was about 300-fold higher for stomatin P47S carrying the HA-tag at its C-terminus than for untagged controls (Fig. 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 that was targeted to the cell surface. Luminescence was approximately 5-fold higher for the P47S mutant than for wild type stomatin ($p < 0.05$), reflecting the larger fraction of the transmembrane form. Western blots performed on the same batch of oocytes used for luminescent measurements, demonstrated that the total amount of tagged P47S protein was comparable to the total amount of wild type stomatin (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 detergent resistant membrane domains and directly binds cholesterol. The P47S mutation abolishes detergent resistance and the association with cholesterol

Our results 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 analyzed by SDS-PAGE and Western blotting. Stomatin was enriched in the fractions towards the top of the gradient, corresponding to the detergent resistant fractions, and in some fractions at the bottom of the gradient, corresponding to the "heavy" detergent sensitive fractions (Fig. 5). Stomatin distributes similarly between detergent resistant and detergent sensitive fractions also in erythrocytes [12]. Unfortunately, we were not able to detect the large form of stomatin in these gradients, neither in the detergent sensitive nor in the detergent resistant fractions. We attribute this lack of detection to the low abundance of the large stomatin form. The P47S variant, however, which consists only of the 42 kD fully glycosylated form, was clearly detected in the detergent sensitive fractions but never in the detergent resistant fractions towards the top of the gradient, 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 whether digitonin can co-precipitate stomatin. This was indeed the case (Fig. 6, lane 3). We never found co-precipitation of the large transmembrane form, however ($n = 3$). Moreover, digitonin also did not co-precipitate the P47S mutant. These results suggest that the small form of stomatin but not the large transmembrane form

directly bound cholesterol, supporting the hypothesis that only the small hairpin form but not the large transmembrane form is associated with DRMs.

DISCUSSION

Stomatin has a unique hairpin loop topology [1, 9, 10]. Our results show that Pro-47 within the hydrophobic domain is indispensable for formation of this unique topology (Fig. 4). Even in the presence of this proline residue, however, a small part of the stomatin population exists as a single-pass transmembrane form with the N-terminus intracellular and the C-terminus extracellular (Fig. 2), showing that the folding of stomatin into a hairpin loop is intrinsically inefficient. Fig. 7 provides a scheme that illustrates this basic finding of our study.

A proline residue in a peptide chain cannot form hydrogen bonds that stabilize an α -helix; moreover, it restricts the rotation of the backbone of the chain. As a consequence, proline has a tendency to break α -helices. It is therefore likely that Pro-47 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, Fig. 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 (Figs. 1-3) is certainly the reason why this form has so far not been described. A high-molecular weight 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 (P134) strongly increased the relative amount of the high-molecular weight form [31]. Since it was suggested that P134 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 (N128 and N264, Fig. 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 weight than the hairpin loop form. Therefore, our results suggest that the high molecular weight form of MEC-2 is a single-pass *N*-glycosylated transmembrane protein. We showed that stomatin carrying the P47S mutation does not co-fractionate with DRMs, in contrast to the hairpin loop form (Fig. 5). This result suggests that substitution of P134 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 weight form of MEC-2 is indeed an *N*-glycosylated form and considering that stomatin and SLP-3 both exist as an *N*-glycosylated form (Figs. 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 these proteins exists as a transmembrane form. Because of its low abundance compared to 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 (Figs. 2 and 4) and in native cells (Fig. 3A). In addition, we showed that ~50% of the total SLP-3 pool is *N*-glycosylated suggesting that it also adopts the transmembrane topology (Fig. 3B). Further studies will reveal whether the transmembrane form is just a misfolded byproduct of stomatin and related proteins or whether it mediates part of their physiological functions.

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FIGURE LEGENDS

Fig. 1 A minor fraction of stomatin carries a modification increasing its molecular weight by ~10 kD.

(A) Linear scheme of stomatin, illustrating the hydrophobic domain (black) and the positions of the phosphorylation site S10, the palmitoylation sites C30 and C87, the conserved P47, and the VSV-G epitope. (B) Membrane preparations from uninjected oocytes (lane 1) and from stomatin-expressing oocytes (lane 2) were run side-by-side on a polyacrylamide gel; stomatin was tagged with the VSV-G epitope. The two antigens of different molecular weight reacting with a monoclonal anti-VSV antibody are marked by arrowheads. These results were reproduced with several different batches of oocytes. Note that the X-ray films were overexposed to better reveal the 42 kD antigen.

Fig. 2 The large form of stomatin arises by N-glycosylation.

(A) Microsomal fractions from stomatin-expressing oocytes, which were either untreated (lane 1) or pre-treated with PNGase F (lane 2), were analyzed in parallel by SDS-PAGE and stomatin detected by immunoblotting with a polyclonal anti-stomatin antibody (N-14 antibody, Santa Cruz biotechnology). Similar results were observed with three different batches of oocytes. (B) Top, linear scheme of stomatin, illustrating the hydrophobic domain (black) and the approximate positions of the five putative N-glycosylation sites located at the C-terminus. Bottom, immunoblot analysis of stomatin variants containing mutations of one or several of the consensus sites for N-glycosylation. Removal of the putative N-glycosylation sites resulted in a cumulative reduction of the molecular weight in steps of ~2-3 kD (lanes 2 – 5). (C) Surface expression of stomatin in intact oocytes. Stomatin was tagged with the HA epitope at its C-terminus; untagged stomatin served as a negative control. Luminescence (mean \pm S.E.M.), normalized to the values of untagged stomatin is shown. $n = 18$. Relative light units for untagged stomatin were 3054 ± 1111 (first frog, 8 oocytes) and 693 ± 259 (second frog, 10 oocytes), respectively. **, $p < 0.01$, t -test.

Fig. 3 Effects of PNGase F on stomatin from mouse erythrocytes and on stomatin-like-protein (SLP) 3.

(A) Effects of PNGase F on endogenous stomatin of mouse erythrocytes. The membrane preparation was split in two halves, one was incubated for 3 hours with PNGase F (lane 2), the other was treated similarly except that PNGase F was lacking (lane 1). Stomatin was visualized using the N-14 anti-stomatin polyclonal antibody. The identity of the antigen of ~70 kD is unclear. (B) Effects of PNGase F on stomatin like protein (SLP) 3; SLP-3 was tagged with the VSV-G epitope at its C-terminus. Microsomal protein fractions extracted from SLP-3-expressing oocytes, which were either untreated (lane 1) or pre-treated with PNGase F (lane 2), were analyzed in parallel by SDS-PAGE and SLP-3 detected by immunoblotting with a monoclonal anti-VSV antibody.

Fig. 4 A single proline residue in the membrane domain determines the membrane topology of stomatin

(A) Schematic presentation of stomatin. The hydrophobic intramembrane (IM) domain is shown as a black bar. The sequences of this domain of mouse stomatin, human SLP-3, SLP-1, and podocin and *C. elegans* MEC-2 are shown; proline 47 (*) of stomatin is conserved in all these proteins. Numbering refers to stomatin. (B) Stomatin P47S mutant was expressed in oocytes and analyzed by PNGase F treatment and immunoblotting as

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before. The P47S mutation leads to glycosylation of the whole stomatin pool. The results were reproduced with preparations from several independent batches of oocytes. **(C)** Surface expression of stomatin P47S in intact oocytes. Stomatin wildtype and P47S tagged at their C-termini with the HA-tag were expressed in oocytes; oocytes injected with untagged stomatin served as a negative control. Luminescence (mean \pm S.E.M.), normalized to the values of untagged stomatin is shown. The results for tagged and untagged stomatin are from Fig. 2. $n = 18$. **, $p < 0.01$.

Fig. 5 The P47S mutation abolishes the association of stomatin with DRMs.

Oocytes expressing stomatin wild type **(A)** or stomatin P47S **(B)** were homogenized and subjected to sucrose density gradient centrifugation. Starting from the top of the gradient, nine fractions were collected, separated by PAGE, and stomatin detected by the N-14 anti-stomatin antibody. Stomatin was enriched in buoyant fractions 2 and 3 (detergent resistant) and less abundantly in fractions 6 – 8 (detergent sensitive); the P47S variant was detected almost exclusively in detergent sensitive fractions 6 – 8. The results shown were reproduced with two different batches of oocytes. Note that the large (42 kD) form of wild type stomatin could not be detected in these experiments.

Fig. 6 Wild type stomatin but not the P47S mutant co-precipitates with cholesterol.

Membrane preparations of oocytes expressing stomatin wild type (left) or stomatin P47S (right) were used for this assay. The 31 kD form of stomatin co-precipitated in the presence (lane 3) but not in the absence (lane 2) of digitonin. The 42 kD form, corresponding to the glycosylated, transmembrane form, did not co-precipitate under any of the conditions (lanes 2, 3, 5, and 6). The lysates demonstrating expression of the proteins are shown in lanes 1 and 4. Note that X-ray films were over-exposed for long time in order to reveal also faint signals.

Fig. 7 Scheme illustrating the biosynthetic pathways leading to the hairpin loop and the transmembrane topology of stomatin.

Details in the text. Whether Cys-30 becomes palmitoylated in the transmembrane form is unknown. ER, endoplasmic reticulum; IC, intracytoplasmic; EC, extracytoplasmic; LR, "lipid raft" (assuming that DRMs are identical to lipid rafts).

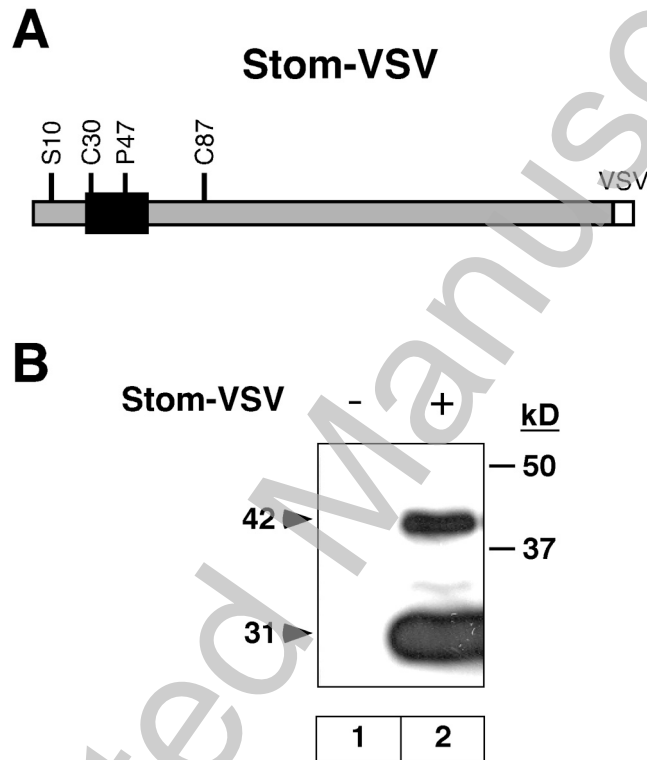


FIGURE 1

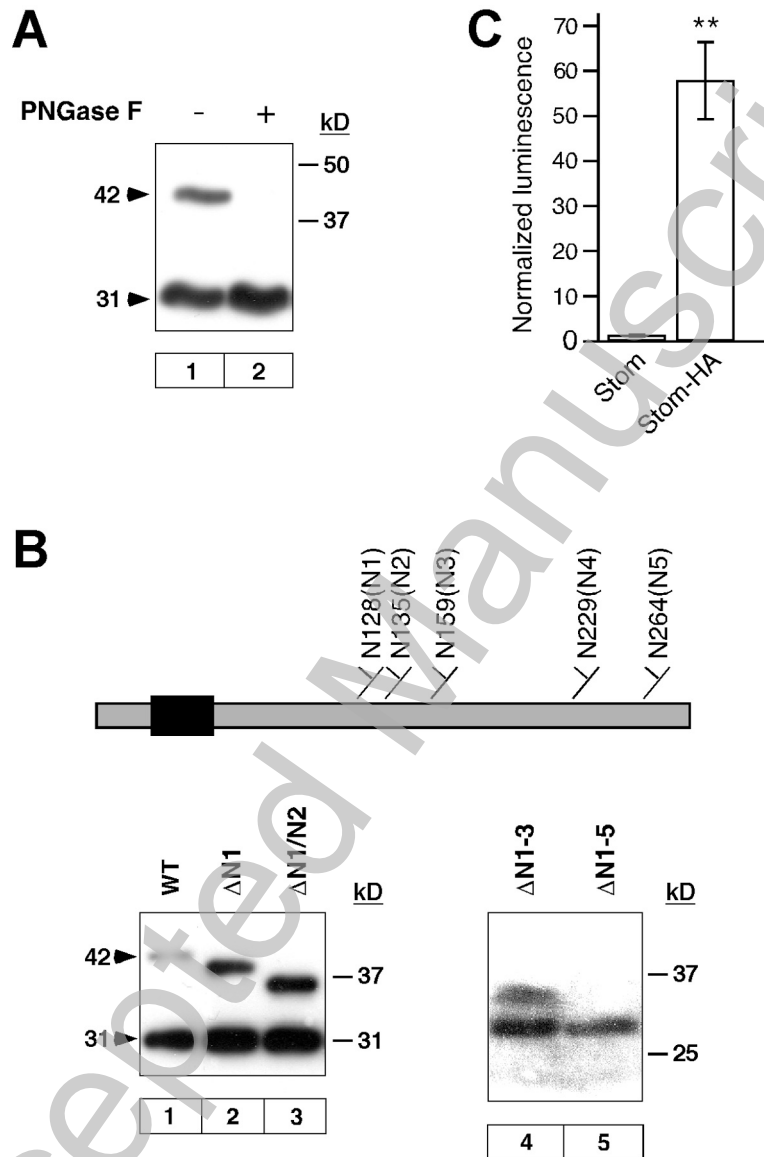
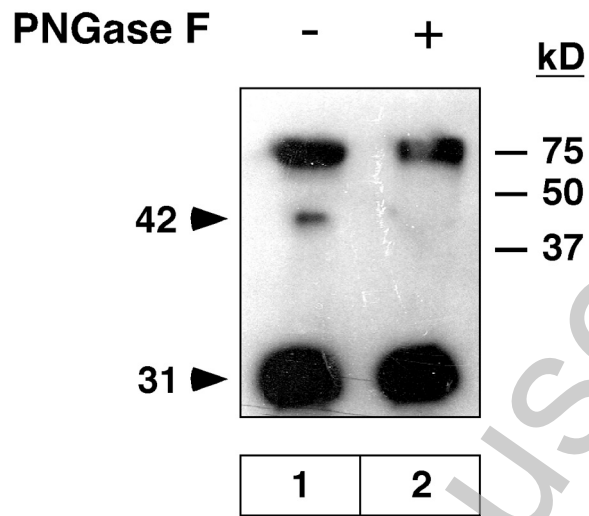


FIGURE 2

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A
Erythrocytes



B
SLP-3

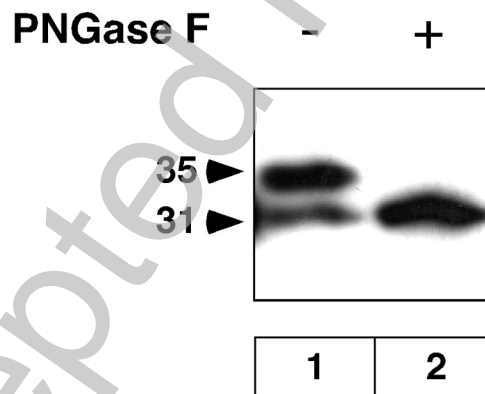


FIGURE 3

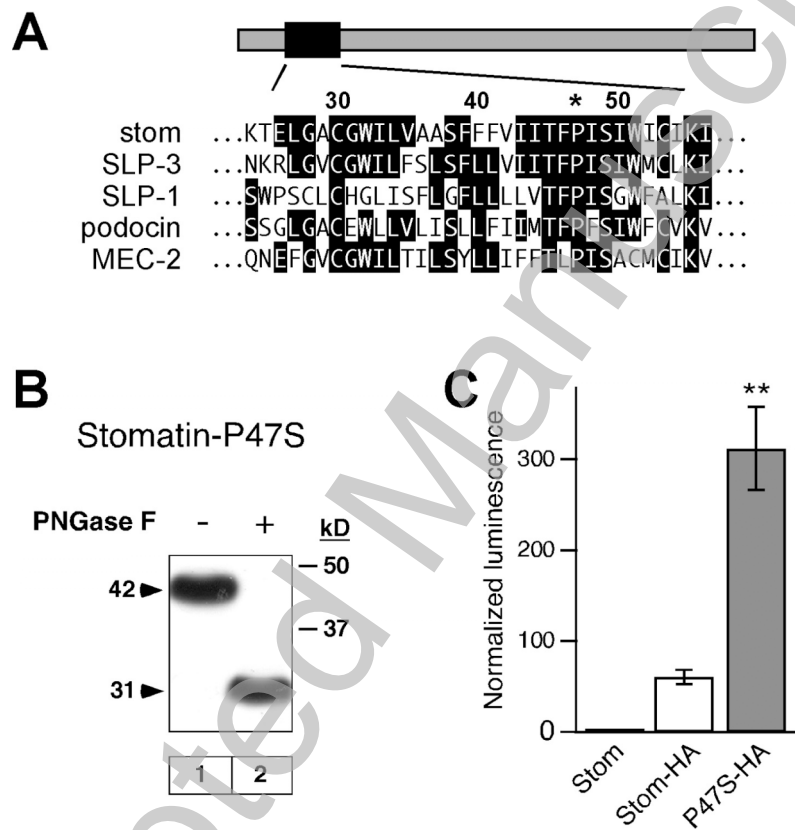


FIGURE 4

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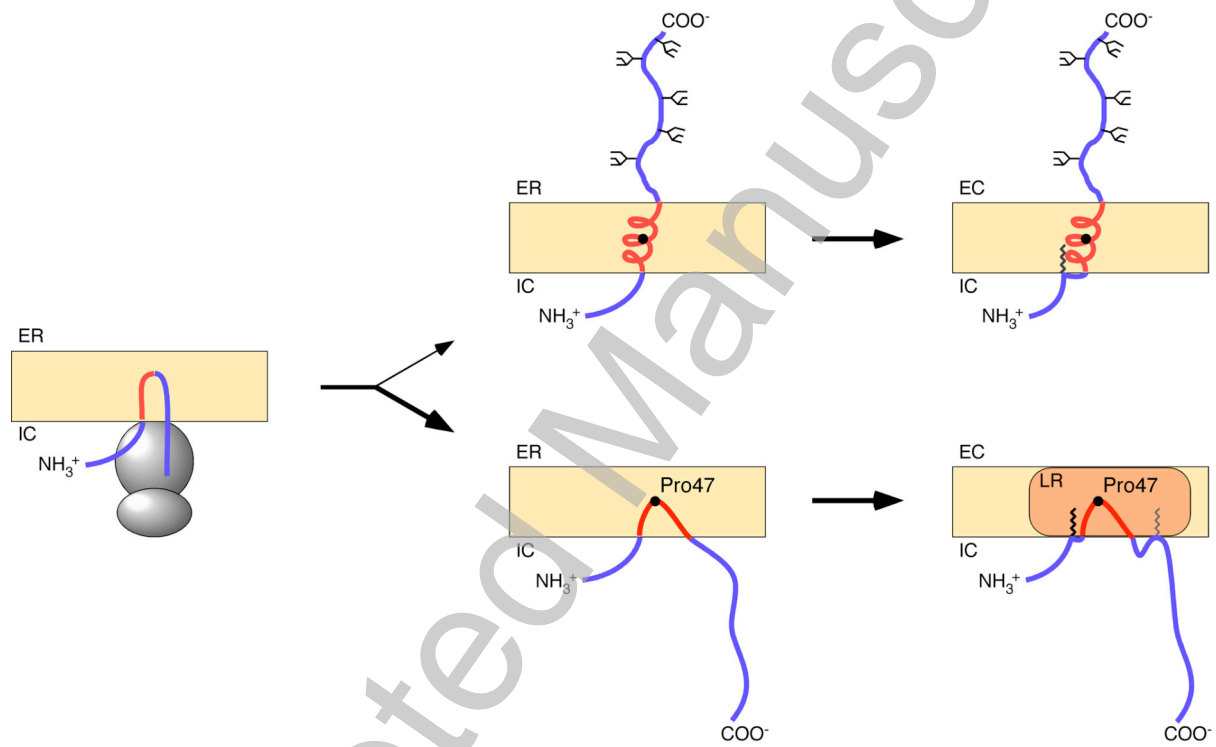


FIGURE 7

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