Complex N-Glycosylation Stabilizes Surface Expression of Transient Receptor Potential Melastatin 4b Protein*

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Background: N-Glycosylation is important for the function and regulation of ion channels, but has not been examined for Trpm4.

Results: N-Glycosylation is not required for surface expression, but complex N-glycosylation stabilizes Trpm4b surface expression.

Conclusion: Complex N-glycosylation of Trpm4b has important functional implications.

Significance: By promoting surface expression, N-glycosylation contributes importantly to calcium regulation by Trpm4b.

N-Glycosylation is important for the function and regulation of ion channels. We examined the role of N-glycosylation of transient receptor potential melastatin (Trpm) 4b, a membrane glycoprotein that regulates calcium influx. Trpm4b was expressed in vivo in all rat tissues examined. In each tissue, Trpm4b had a different molecular mass, between ~129 and ~141 kDa, but all reverted to ~120 kDa following treatment with peptide:N-glycosidase F, consistent with N-glycosylation being the principal form of post-translational modification of Trpm4b in vivo. In six stable isogenic cell lines that express different levels of Trpm4b, two forms were found, high mannose, core-glycosylated and complex, highly glycosylated (HG), with HG-Trpm4b comprising 85% of the total Trpm4b expressed. For both forms, surface expression was directly proportional to the total Trpm4b expressed. Complex N-glycosylation doubled the percentage of Trpm4b at the surface, compared with high mannose N-glycosylation. Mutation of the single N-glycosylation consensus sequence at Asn-988 (Trpm4b-N988Q), located near the pore-forming loop between transmembrane helices 5 and 6, prevented glycosylation, but did not prevent surface expression, impair formation of functional membrane channels, or alter channel conductance. In transfection experiments, the time courses for appearance of HG-Trpm4b and Trpm4b-N988Q on the surface were similar. In experiments with cycloheximide inhibition of protein synthesis, the time course for disappearance of HG-Trpm4b from the surface was much slower than that for Trpm4b-N988Q. We conclude that N-glycosylation is not required for surface expression or channel function, but that complex N-glycosylation plays a crucial role in stabilizing surface expression of Trpm4b.

Transient receptor potential (Trp)2 melastatin 4b (Trpm4b) is a member of a large superfamily of mammalian proteins consisting of 28 cation channels. The principal function of Trpm4b has been identified as being the regulation of calcium influx (1–4). Trpm4b transports monovalent but not polyvalent cations and is activated by intracellular calcium. Channel activation induced by a rise in intracellular calcium results in cell depolarization, which reduces the electrochemical driving force for the passive influx of calcium into the cell. Excellent reviews on the biophysical properties and physiological regulation of this channel have been published (5–8). Given its important role in regulating calcium influx by membrane depolarization, it is evident that stable surface expression of Trpm4b is critical for its proper functioning.

N-Glycosylation serves numerous biological roles (9–11). For membrane-associated glycoproteins including some ion or water channels, N-linked glycosylation is implicated in the correct protein folding and assembly of functional channel complexes and may be required for surface expression (12–16). For some members of the Trp superfamily, N-glycosylation and its regulation have important effects on channel function (17–19), including channel activity, permeability, and sensitivity to activator/agonist and antagonist (20–23).

N-Glycosylation influences the surface expression of some Trp channels, but findings to date have been varied, and the specific effect depends on the channel involved. In the case of Trpc6, three different N-glycosylation mutants were found to have the same cellular distribution as the wild-type channel, suggesting little effect on surface expression (20). Similarly, with Trpv1, no significant difference was found in the total amounts of wild-type and Trpv1-N604T at the cell surface (22). With Trpm8, mutating the single N-glycosylation site at Asn-934 was reported to significantly reduce but not eliminate transport to the plasma membrane (24, 25), although studies from another laboratory reported that the same mutation failed

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‡ The abbreviations used are: Trp, transient receptor potential; CG, core-glycosylated; Endo H, endoglycosidase H; HG, highly glycosylated; PNGaseF, peptide:N-glycosidase F; TM, transmembrane; Trpm4b, Trp melastatin 4b.
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to produce any major defect in surface expression (21, 26). N-Glycosylation of Trpv4 at Asn-651 affects surface expression, but it is the nonglycosylated mutant that exhibits an increase in plasma membrane targeting (27).

Two “hot-spots” for N-glycosylation have been identified in Trp gene products, a site on the extracellular loop bridging transmembrane (TM) helices 1 and 2, and a site immediately adjacent to the pore-forming loop, between TM helices 5 and 6 (19). Sequence analysis revealed the presence of a consensus N-linked glycosylation site, NX/S/T, where X represents any amino acid except Pro (28), at Asn-988 of mouse Trpm4b. This site is situated near the pore-forming loop between TM helices 5 and 6 (29). We recently reported that Trpm4b can be found in core-glycosylated (CG; “high mannose”) and highly glycosylated (HG; “complex”) forms (30), but the specific site of N-glycosylation has not been identified, and the potential role of N-glycosylation in the function and the surface expression of Trpm4b has not been examined.

MATERIALS AND METHODS

Molecular Biology—Myc epitope-tagged expression plasmids of mouse Trpm4b (pMyc-Trpm4b) (NM_175130 (30)) and Trpm4a (pMyc-Trpm4a) were cloned into an expression vector, pCMV-Tag3C (Stratagene). To construct pMyc-Trpm4a, the cDNA sequence encoding the Trpm4a region was amplified by polymerase chain reaction (PCR) and cloned into the same expression vector. The amino acid sequence of mouse Trpm4a differs from that of Trpm4b by omission of the first N-terminal 186 amino acids (31). The single glycosylation consensus sequence at Asn-988 of Trpm4b was mutated to Gln in the pMyc-Trpm4b plasmid using PCR, and it is called pMyc-Trpm4b-N988Q. To construct pPuro-Myc-Trpm4b and pPuro-Myc-Trpm4b-N988Q for stable cell lines, the cDNA sequence of Myc-Trpm4b or Myc-Trpm4b-N988Q was cloned into an expression vector, pSELECT-puro-mcs (Invitrogen). All plasmids constructed by PCR amplification were verified by sequencing prior to transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen).

Cell Culture, Development of Stable Cell Lines—COS-7 cells were cultured in Dulbecco’s modified eagle medium (DMEM) with 4.5 g/liter glucose (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

To develop stable cell lines that express Myc-Trpm4b or Myc-Trpm4b-N988Q, COS-7 cells were transfected with pPuro-Myc-Trpm4b or pPuro-Myc-Trpm4b-N988Q. Transfections were performed using Lipofectamine 2000 (Invitrogen). Colonies that survived in a medium containing 1 μg/ml puromycin were selected. Expression of Myc-Trpm4b or Myc-Trpm4b-N988Q was examined from the colonies by immunoblotting. Six colonies, named Cmt4-1 through Cmt4-6, which showed different expression levels of Myc-Trpm4b, were chosen for further study. Two colonies, named N988Q-1 and N988Q-2, which showed different expression levels of Myc-Trpm4b-N988Q, were chosen for further study. Control stable cell lines, named CPuro, also were developed by transfecting an empty pSELECT-puro-mcs vector and selecting colonies that survived in the same condition.

Immunoblotting and Immunoprecipitation-coupled Immunoblotting—The chicken and rabbit anti-Trpm4 antibodies used in this study were described previously (30). Anti-Myc and anti-Hsc70 antibodies were purchased from Santa Cruz Biotechnology. Immunoblot and immunoprecipitation-coupled immunoblot analyses were performed as described (30). Total lysates from COS-7 cells transfected with various plasmids were prepared in lysis buffer (1.5 mM KH2PO4, 8 mM Na2HPO4 (pH 7.3), 3 mM KCl, 137 mM NaCl, and 1% Triton X-100) with freshly added protease inhibitor mixture (Roche Applied Science). The protein concentration was determined using a protein assay kit (Bio-Rad). To immunoprecipitate Trpm4, lysates were incubated with chicken anti-Trpm4 antisera and chicken IgY precipitating resin (GenScript, Piscataway, NJ) overnight at 4 °C. The resulting complex was isolated by centrifugation (800 × g) for 1 min. Trpm4 bound to the precipitating resin was eluted by boiling in 2× NuPAGE LDS sample buffer (Invitrogen) and was examined by immunoblotting. Immunoblot analysis of Hsc70 was performed to validate equal loading. Molecular masses were measured using GelAnalyzer 2010a software. Predicted molecular masses were determined from deduced amino acid sequences using Gene Runner 3.05 software.

Surface Biotinylation—To measure the abundance of Trpm4 in the plasma membrane, COS-7 cells transfected transiently or stably were washed with ice-cold PBS and incubated with 0.25 mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce) dissolved in Dulbecco’s PBS for 30 min at 4 °C. The biotinylation reaction was quenched by washing with and incubating in Tris-buffered saline (50 mM Tris (pH 7.4) and 150 mM NaCl) for 5 min. Total lysates were prepared from the cells in the lysis buffer (see above) for 30 min at 4 °C and cleared by centrifugation. Biotinylated proteins were isolated by NeutrAvidin-agarose beads (Pierce), washed with the cell lysis buffer, and eluted from the beads by boiling in 2× NuPAGE LDS sample buffer (Invitrogen). The resulting elutants were examined by immunoblotting for Trpm4. To validate separation of the biotinylated proteins, immunoblotting of Hsc70 also was performed.

N-Glycosylation—To prevent N-glycosylation of Trpm4, COS-7 cells were treated with 0.5 μg/ml tunicamycin (Sigma) for 30 min prior to transfection. To remove N-linked glycans from high mannose (core-glycosylated) glycoproteins, cell lysates were incubated with endoglycosidase H (Endo H; New England BioLabs) for 1 h at 37 °C. To remove N-linked glycans from both complex and high mannose glycoproteins, cell lysates or immunoprecipitated samples from rat tissues were incubated with peptide:N-glycosidase F (PNGaseF; New England BioLabs) for 1 h at 37 °C. The resulting reaction mixtures were examined by immunoblotting.

Inhibition of Protein Synthesis—To reduce the expression of Trpm4, stable cell lines expressing Myc-Trpm4b (CmT4-6) and Myc-Trpm4b-N988Q (N988Q-2) were transfected with siRNA targeting Trpm4 (Santa Cruz Biotechnology) following the protocols provided by the manufacturer. As a negative control, the stable cell lines were transfected with a control siRNA (Santa Cruz Biotechnology). Expression of Trpm4 in total lysate and surface plasma membrane was examined 24 h after transfection, as described above.
To inhibit all protein synthesis, stable cell lines expressing Myc-Trpm4b (CmT4-6) and Myc-Trpm4b-N988Q (N988Q-2) were treated with 0.1 mg/ml cycloheximide. Expression of the Trpm4 in total lysate and surface plasma membrane was measured as described above.

**Patch Clamp Electrophysiology**—Patch clamp electrophysiology studies were performed as described (32, 33) using stable cell lines expressing Myc-Trpm4 (CmT4-6), and Myc-Trpm4-N988Q (N988Q-2). Membrane currents were recorded in intact cells using the nystatin-perforated whole cell configuration (34) and in cell-free isolated membrane patches using the inside-out configuration (35). For whole cell macroscopic recordings, we used a nystatin-perforated patch technique with a bath solution containing 145 mM CsCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 32.5 mM HEPES, 12.5 mM glucose (pH 7.4). The pipette solution contained 145 mM CsCl, 8 mM MgCl$_2$, and 10 mM HEPES (pH 7.2). For inside-out patch recording, we used a bath solution containing 145 mM CsCl, 4.96 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM EGTA, 32.5 mM HEPES, 12.5 mM glucose 12.5 (pH 7.4).

The pipette contained 145 mM CsCl, 1 mM MgCl$_2$, 0.2 mM CaCl$_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.3).

**RESULTS**

**Validation of the Anti-Trpm4 Antibodies**—Antibodies targeting the N-terminal 612-amino acid-long cytoplasmic region of rat Trpm4b recently were developed and used to detect Trpm4 in injured spinal cord tissues of mice and rats (30). With tissues from both species, only the Trpm4b isoform, with a molecular mass of ~135 kDa, was detected. To determine whether these antibodies also detect the Trpm4a isoform, lysates from COS-7 cells transfected with expression vectors encoding either Myc-epitope tagged Trpm4a (pMyc-Trpm4a) or Myc-epitope tagged Trpm4b (pMyc-Trpm4b) were examined by immunoblotting and immunoprecipitation-coupled immunoblotting. Immunoisolation was performed using chicken anti-Trpm4 antibody. Immunoblots developed using rabbit anti-Trpm4 antibody and anti-Myc antibody were essentially identical (Fig. 1), consistent with a high degree of specificity of the anti-Trpm4 antibodies toward Trpm4, regardless of the isoform.

In this experiment, the molecular mass of Myc-Trpm4a was estimated as ~105 kDa (Fig. 1 and Table 1). Myc-Trpm4b showed two distinct bands, with estimated molecular masses of ~135 and ~147 kDa (Fig. 1 and Table 1). Two forms of Trpm4b with similar molecular masses recently were shown to represent CG and HG Trpm4b (30).

**Organ-specific N-Glycosylation of Trpm4**—Glycosylation of Trpm4b in cultured cells has been demonstrated (30), but glycosylation in vivo has not been extensively studied. Lysates from various rat tissues were examined by immunoprecipitation-coupled immunoblotting. Expression of Trpm4b was detected in various tissues including brain, spinal cord, heart, kidney, liver, lung, skeletal muscle, and small intestine (Fig. 2A, *top panel*). None of these tissues, except possibly small intestine, showed Trpm4a expression. Expression of Trpm4b was high in the small intestine, lung, and spinal cord, intermediate in the kidney and skeletal muscle, and low in the brain, heart, and liver. The relative expression level of Trpm4 in the different rat tissues is summarized in Fig. 2B.

In contrast to observations in COS-7 cells, where two distinct forms of Trpm4b, CG and HG, were detected (Fig. 1), only a single form of Trpm4b was detected in rat tissues (Fig. 2A, *top panel*). The molecular mass of Trpm4b in the different rat tissues varied between ~129 and ~141 kDa.

We tested whether organ-specific differences in the molecular mass of Trpm4b could be due to N-glycosylation. Immunoprecipitated fractions were incubated with PNGaseF, which releases the intact oligosaccharide from both core and highly N-glycosylated proteins and hydrolyzes the original Asn residue to Asp (36). Immunoblots revealed that with all tissues, PNGaseF treatment yielded a single protein band with a molecular mass of ~120 kDa (Fig. 2A, *middle panel*). These findings were confirmed by running samples without and with PNGaseF treatment in adjacent lanes (Fig. 2C). These data suggested that N-glycosylation is the principal type of post-translational modification that Trpm4b undergoes in vivo and that different tissues from the same experimental animal show unique glycosylation patterns, representing tissue-specific differences in N-glycan terminal processing (37, 38). The degree of N-glycosylation of Trpm4b was relatively high in the lung and kidney, and low in the brain, spinal cord, and skeletal muscle. In the different tissues, the N-glycans attached to Trpm4b were estimated by gel migration to contribute 8–18% to the mass of the protein. After deglycosylation with PNGaseF, the faster migrating band from small intestine had a molecular mass ~105 kDa, possibly representing Trpm4a.

**N-Glycosylation and Surface Expression of Trpm4a and Trpm4b**—N-Glycosylation is known to have important effects on proper folding and plasma surface targeting of many proteins. We therefore asked what the role of N-glycosylation might be for Trpm4, focusing specifically on surface expression, which is necessary for its function.

We examined the expression of Trpm4a and Trpm4b in COS-7 cells pretreated with 0.5 μg/ml tunicamycin, an antibiotic that blocks the addition of carbohydrate molecules to Asn residues of glycoproteins (39). Tunicamycin treatment led to the expression of the fast migrating forms of Myc-Trpm4b and Myc-Trpm4a, with molecular masses of 128 and 104 kDa (Fig. 3A, *left half*).
We examined the surface expression of Trpm4a and Trpm4b in tunicamycin-treated COS-7 cells. Both Trpm4a and Trpm4b were detected in biotin-labeled surface proteins (Fig. 3A, right half), indicating that both proteins reached the plasma membrane despite the absence of glycosylation (3). Quantification showed that inhibiting N-glycosylation of CG-Trpm4a and of CG-Trpm4b affected surface expression minimally, whereas inhibiting N-glycosylation of HG-Trpm4b reduced surface expression by half, from 17% to 8% (Fig. 3B).

High Mannose N-Glycosylation of Trpm4a and Trpm4b—To further examine the effect of N-glycosylation on surface expression, we studied a single cell line (CmT4-6) in which we partially inhibited Trpm4b expression using different amounts of siRNA (Fig. 4). As observed previously, with both glycosylation forms, Trpm4b expression at the surface was linearly related to the total Trpm4b expressed (Fig. 5B). In this experiment, 14% of HG-Trpm4b was present at the surface, whereas only half this amount, 7% of CG-Trpm4b was found on the surface (Fig. 5B), reinforcing the importance of complex glycosylation for surface expression.

Glycosylation-deficient Trpm4b Mutant—Three independent experiments (Figs. 3B, 4C, and 5B) showed that complex N-glycosylation at least doubled the percentage of Trpm4b at the surface, compared with high mannose N-glycosylation, whereas the tunicamycin experiment suggested that N-glycosylation was not an absolute requirement for surface expression. To examine further the requirement for N-glycosylation, we developed a glycosylation-deficient Trpm4b mutant.

Sequence analysis revealed the presence of only a single consensus N-linked glycosylation site, NX(S/T), where X represents any amino acid except Pro (28), at Asn-988 of mouse Trpm4b (Fig. 6A). The amino acid sequence in this region is highly conserved across species (Fig. 6B). We exchanged Asn for the structurally very similar Gln, which differs by only one methylene group in the amino acid side chain, thus minimizing the possibility of conformational disruption and preserving the local charge distribution. Mutation of Asn at position 988 to Gln resulted in the mutant plasmid, pMyc-Trpm4b-N988Q. We developed two independent stable cell lines that express different levels of Trpm4b-N988Q.

Mutation of Asn-988 led to the disappearance of the N-glycosylated forms of the protein and resulted in a single band of ~128 kDa (Fig. 6C), the same as that observed in tunicamycin-treated cells (see Fig. 3A). PNGaseF treatment of wild-type Trpm4b collapsed the HG and CG forms into a single protein band, as expected, but had no effect on Trpm4b-N988Q (Fig. 6C), consistent with Asn-988 being the only N-glycosylation site on Trpm4b.

The expression of Trpm4b-N988Q was examined in total lysate and in biotin-labeled surface protein. Biotinylation experiments showed that nonglycosylated Trpm4b-N988Q reached the plasma membrane (Fig. 6D), consistent with our earlier finding with tunicamycin-treated cells. The ratio of surface to total expressed was 6.1 ± 1.7% (n = 3).

The presence of a nonglycosylated glycoprotein at the cell surface does not imply that it is functional. We used patch clamp electrophysiology to assess for functional channels. Cells expressing Trpm4-N988Q were studied using a nystatin patch whole cell configuration with Cs⁺ as the charge carrier. Mac-

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**TABLE 1**

**Observed vs. predicted mass of Trpm4**

| Trpm4        | Predicted mass (kDa) | 4–12% Gel observed mass* (kDa) | 3–8% Gel observed mass (kDa) | Where observed         |
|--------------|----------------------|--------------------------------|-----------------------------|------------------------|
| Nonglycosylated |                      |                                 |                             |                        |
| rTrpm4b      | 135                  | 127                            | 120                         | Rat tissues + PNGaseF  |
| Myc-mTrpm4b  | 138                  | 134                            | 128                         | COS-7 + tunicamycin    |
| rTrpm4a      | 115                  | 134                            | 128                         | N988Q                  |
| Myc-mTrpm4a  | 118                  | 108                            | 105                         | Rat small intestine + PNGaseF |
| Glycosylated |                      |                                 |                             |                        |
| HG-Myc-mTrpm4b | 150                 | 147                            | 104                         | COS-7 + tunicamycin    |
| CG-Myc-mTrpm4b | 137                 | 135                            | 104                         | COS-7                 |

* The values shown are averages of two or three replicates.
roscopic currents elicited by depleting ATP reversed near 0 mV and were typical of Trpm4b currents (Fig. 7, A–C). These macroscopic currents were blocked by the hydroxytricyclic compound, 9-hydroxyphenanthrene (9-phenanthrol), which blocks Trpm4b preferentially (40) (Fig. 7, A–C).

With Trpv1, N-glycosylation at Asn-604, which is near the pore-forming loop between TM helices 5 and 6, is known to affect permeability, with glycosylation required for pore dilation and loss of selectivity (22). Because Asn-988 of mTrpm4b is situated near the pore-forming loop between TM helices 5 and 6 (29), we asked whether the N988Q mutation would affect conductance. Single channel recordings of inside-out patches from cells expressing Trpm4-N988Q were performed. A single channel conductance of 28 picosiemens was observed with Cs⁺/H¹¹⁰⁰ⁱ as the charge carrier, which was not significantly different from 26 picosiemens (p = 0.1) observed with wild-type Trpm4b (Fig. 7, D–F).

**Trpm4b Stability**—The foregoing experiments indicated that surface expression of Trpm4b was greatly favored by complex N-glycosylation, but that glycosylation was not required for surface expression. These findings could be due to more efficient surface trafficking with complex N-glyco-
sylation. However, examining surface expression as a function of time after transfection showed that the nonglycosylated mutant, Trpm4-N988Q, appeared at the surface as rapidly as HG-Trpm4b (Fig. 8, A and C). This was not due to any appreciable difference in the rates of synthesis of HG-Trpm4b compared with Trpm4b-N988Q, as indicated by measurements of total Trpm4b as a function of time (Fig. 8, B and D). These findings suggested that surface trafficking was unlikely to be the main factor responsible for greater surface expression of HG-Trpm4b.

An alternative explanation for greater surface expression of HG-Trpm4b might be that complex N-glycosylation stabilizes Trpm4b, impairing its turnover. Support for this hypothesis was obtained in experiments in which protein synthesis was halted using cycloheximide. Examining surface expression of HG-Trpm4b as a function of time showed that it declined only very slowly after cessation of protein synthesis (Fig. 9, A and C). By contrast, surface expression of Trpm4b-N988Q declined significantly more rapidly after cessation of protein synthesis (Fig. 9, A and C). Notably, total HG-Trpm4b remained nearly constant for 48 h, in contrast to the rapid loss of total Trpm4b-N988Q (Fig. 9, B and D). These findings are consistent with complex N-glycosylation stabilizing Trpm4b and impairing its turnover.

DISCUSSION

The principal findings of the present study are as follows: (i) Trpm4b is found in vivo with variable forms of complex N-glycosylation in all rat tissues sampled, with our data showing that N-glycosylation is the principal type of post-translational modification that this protein undergoes in vivo and that different tissues from the same experimental animal show unique glycosylation patterns, representing tissue-specific differences in N-glycan terminal processing. (ii) The presence of the 186
amino acids at the N terminus of Trpm4a prevents complex cell-surface expression but not core N-glycosylation at Asn-802 of Trpm4a. (iii) Despite its location near the pore-forming loop, N-glycosylation of Asn-988 of Trpm4b does not influence single channel conductance of Trpm4b. (iv) Although an important determinant of surface expression of Trpm4b is the total amount expressed, complex N-glycosylation plays a crucial role in stabilizing Trpm4b, thereby promoting its surface expression compared with the core-glycosylated and nonglycosylated forms.

With Trpm4b, we identified Asn-988 as the only acceptor site for N-linked glycans. Trpm4b subunits with a single point mutation (N988Q) showed no glycosylation. Although its surface turnover was more rapid, the N988Q mutant was able to assemble into functional channels with a single channel conductance similar to wild-type Trpm4b. Although N-glycosylation was not found to be required for channel function of Trpm4b, it is possible that more subtle functional effects of protein glycosylation may be detected by other electrophysiological experiments.

N-Glycosylation influences the surface expression of several Trp channels. With Trpv5, cleavage of a single N-linked oligosaccharide by Klotho, a glucuronidase enzyme, activates and stabilizes the protein at the surface (41). In the case of Trpv5, the glycosylation site is at Asn-358, which is located within the extracellular loop between the first and second transmembrane segments (41). As noted by Cohen (19), Trp channels with a glycosylation site near the pore-forming loop between helices 5 and 6 tend to share the common feature that glycosylation of this site affects surface expression. With Trpv4, mutation of a single N-glycosylation site near the pore-forming loop increases the expression of membrane-bound channels compared with wild type (27). With Trpm8, mutating the single N-glycosylation site at Asn-934, which is near the pore-forming loop, is reported to significantly impair but not eliminate transport to the plasma membrane (24, 25), although studies by another group did not confirm this (21, 26). In the study on Trpv4 cited above (27), the authors did not characterize the type of glycosylation involved. In the studies on Trpm8, the effects observed on two protein bands following treatment with PNGaseF and Endo H suggested the presence of a nonglycosylated and a complex glycosylated form (Endo H was without effect) (24, 25). As shown here, it is specifically complex N-glycosylation of Asn-988 that significantly affects surface expression of Trpm4b, compared with both core-glycosylated (Endo H-sensitive) and nonglycosylated forms.

Most studies on Trp glycosylation have been performed on heterologous systems, with artificially high levels of expressed channels. Native and recombinant channels may act differently, due to differences in glycosylation. Malkia et al. (42) found...
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![Diagram](image)

**FIGURE 8.** N-Glycosylation does not influence trafficking to the surface membrane. A and B, immunoblotting for Trpm4b was performed from biotin-labeled surface proteins (A) and from total lysate (B) at various times after transfection of COS-7 cells with pMyc-Trpm4b or pMyc-Trpm4b-N988Q, as indicated. The numbers in individual lanes indicate the number of hours after transfection. C and D, densitometric analyses of surface expressed (C) and total expressed (D) Trpm4b (empty circles) and Trpm4b-N988Q (filled circles) at the various times after transfection; mean ± S.E. (error bars) of four replicates; data normalized to values at 0 h.

**FIGURE 9.** Complex N-glycosylation stabilizes surface expression of Trpm4b. A and B, immunoblotting for Trpm4b was performed from biotin-labeled surface proteins (A) and from total lysate (B) at various times after stopping protein synthesis using cycloheximide in cell lines stably expressing Trpm4b or Trpm4b-N988Q, as indicated. The numbers in individual lanes indicate the number of hours after adding cycloheximide. C and D, densitometric analyses of surface expressed (C) and total expressed (D) Trpm4b (open circles) and Trpm4b-N988Q (filled circles) at the various times after stopping protein synthesis; mean ± S.E. (error bars) of four replicates; data normalized to values at 0 h.

important differences in the gating properties of native and recombinant Trpm8 channels, leading to large changes in voltage sensitivity and thermal threshold. In heterologous systems, Trpv1 appears as an N-glycosylated protein whereas in the native system this modification is not detected (18). Here, we found that Trpm4b is present ubiquitously in normal rat tissues, where it exhibits variable forms of complex N-glycosylation. Normal rat tissues did not express core glycosylated Trpm4b, which is commonly observed in cultured cells. To date, CG-TRPM4b has been identified in vivo only under conditions of CNS injury, when it co-associates with Sur1 to form Sur1-Trpm4 channels (30). In this case, the presence of co-associated Sur1 appears to mimic the effect of the "missing" 186-amino acid N terminus of Trpm4 in preventing complex N-glycosylation. Further work will be required to clarify the role of N-glycosylation in vivo, including the different forms of N-glycosylation identified in the various rat tissues, both normal and after injury.

Stable surface expression of Trpm4b is critical for its proper functioning in regulating calcium influx. It is evident that the number of channels present at the cell surface is the principal factor that determines the magnitude of the depolarization induced by Trpm4b in response to a given concentration of intracellular calcium; if too few channels are present, an insufficient depolarization will lead to calcium overload and its deleterious consequences. The data presented here show that complex N-glycosylation serves a crucial role in stabilizing the surface expression of Trpm4b.

REFERENCES

1. Launay, P., Fleig, A., Perraud, A. L., Scharenberg, A. M., Penner, R., and Kinet, J. P. (2002) TRPM4 is a Ca2+-activated nonselective cation channel mediating cell membrane depolarization. *Cell* **109**, 397–407

2. Murakami, M., Xu, F., Miyoshi, I., Sato, E., Ono, K., and Iijima, T. (2003) Identification and characterization of the murine TRPM4 channel. *Biochem. Biophys. Res. Commun.* **307**, 522–528

3. Launay, P., Cheng, H., Srivatsan, S., Penner, R., Fleig, A., and Kinet, J. P. (2004) TRPM4 regulates calcium oscillations after T cell activation. *Science* **306**, 1374–1377

4. Nilius, B., Prenen, J., Yang, J., Wang, C., Owsianik, G., Janssens, A., Voets, T., and Zhu, M. X. (2005) Regulation of the Ca2+-activated nonselective cation channel TRPM4. *J. Biol. Chem.* **280**, 6423–6433

5. Guinamard, R., Sallé, L., and Simard, C. (2011) The non-selective monovalent cationic channels TRPM4 and TRPM5. *Adv. Exp. Med. Biol.* **704**, 147–171

6. Liman, E. R. (2007) in *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades* (Liedtke, W. B., and Heller, S., eds) pp. 203–212, CRC Press, Boca Raton, FL

7. Vennekens, R., and Nilius, B. (2007) Insights into TRPM4 function, regulation and physiological role. *Handb. Exp. Pharmacol.* **179**, 269–285

8. Ullrich, N. D., Voets, T., Prenen, J., Vennekens, R., Talavera, K., Droogmans, G., and Nilius, B. (2005) Comparison of functional properties of the Ca2+-activated cation channels TRPM4 and TRPM5 from mice. *Cell Calcium* **37**, 267–278

9. Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**, 631–664

10. Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126**, 855–867

11. Banerjee, D. K. (2012) N-Glycans in cell survival and death: cross-talk between glycosyltransferases. *Biochim. Biophys. Acta* **1820**, 1338–1346

12. Petrecca, K., Atanasiu, R., Akhavan, A., and Shrier, A. (1999) N-Linked glycosylation sites determine HERG channel surface membrane expression. *J. Physiol.* **515**, 41–48
13. Fozzard, H. A., and Kyle, J. W. (2002) Do defects in ion channel glycosylation set the stage for lethal cardiac arrhythmias? Sci. STKE 2002, pe19
14. Much, B., Wahl-Schott, C., Zong, X., Schneider, A., Baumann, L., Moosmang, S., Ludwig, A., and Biel, M. (2003) Role of subunit heteromerization and N-linked glycosylation in the formation of functional hyperpolarization-activated cyclic nucleotide-gated channels. J. Biol. Chem. 278, 43781–43786
15. Hendriks, G., Koudijs, M., van Balkom, B. W., Oorschot, V., Klumperman, J., Deen, P. M., and van der Sluijs, P. (2004) Glycosylation is important for cell surface expression of the water channel aquaporin-2 but is not essential for tetramerization in the endoplasmic reticulum. J. Biol. Chem. 279, 2975–2983
16. Napp, J., Monje, F., Stühmer, W., and Pardo, L. A. (2005) Glycosylation of Eag1 (Kv10.1) potassium channels: intracellular trafficking and functional consequences. J. Biol. Chem. 280, 29506–29512
17. Vannier, B., Zhu, X., Brown, D., and Birnbaumer, L. (1998) The membrane topology of human transient receptor potential 3 as inferred from glycosylation-scanning mutagenesis and epitope immunocytochemistry. J. Biol. Chem. 273, 8675–8679
18. Kedei, N., Szabo, T., Lile, J. D., Treanor, J. J., Olah, Z., Iadarola, M. J., and Blumberg, P. M. (2001) Analysis of the native quaternary structure of vanilloid receptor 1. J. Biol. Chem. 276, 28613–28619
19. Cohen, D. M. (2006) Regulation of TRP channels by N-linked glycosylation. Semin. Cell Dev. Biol. 17, 630–637
20. Dietrich, A., Mederos y Schnitzler, M., Emmel, J., Kalwa, H., Hofmann, T., and Gudermann, T. (2003) N-Linked protein glycosylation is a major determinant for basal TRPC3 and TRPC6 channel activity. J. Biol. Chem. 278, 47842–47852
21. Pertusa, M., Madrid, R., Morenilla-Palao, C., Belmonte, C., and Viana, F. (2012) N-Glycosylation of TRPM8 ion channels modulates temperature sensitivity of cold thermoreceptor neurons. J. Biol. Chem. 287, 18218–18229
22. Veldhuis, N. A., Lew, M. J., Abogadie, F. C., Poole, D. P., Jennings, E. A., Ivanusic, J. J., Eilers, H., Bunnell, N. W., and McIntyre, P. (2012) N-Glycosylation determinesionic permeability and desensitization of the TRPV1 capsain receptor. J. Biol. Chem. 287, 21765–21772
23. Wirkner, K., Hognestad, H., Jahnel, R., Huchó, F., and Illes, P. (2005) Characterization of rat transient receptor potential vanilloid 1 receptors lacking the N-glycosylation site N604. Neuroreport 16, 997–1001
24. Dragoni, I., Guida, E., and McIntyre, P. (2006) The cold and menthol receptor TRPM8 contains a functionally important double cysteine motif. J. Biol. Chem. 281, 37353–37360
25. Erler, I., Al-Ansary, D. M., Wissenbach, U., Wagner, T. F., Flockerzi, V., and Niemeyer, B. A. (2006) Trafficking and assembly of the cold-sensitive TRPM8 channel. J. Biol. Chem. 281, 38396–38404
26. Morenilla-Palao, C., Pertusa, M., Meseguer, V., Cabedo, H., and Viana, F. (2009) Lipid raft segregation modulates TRPM8 channel activity. J. Biol. Chem. 284, 9215–9224
27. Xu, H., Fu, Y., Tian, W., and Cohen, D. M. (2006) Glycosylation of the osmoreponsive transient receptor potential channel TRPV4 on Asn-651 influences membrane trafficking. Am. J. Physiol. Renal Physiol. 290, F1103–1109
28. Pless, D. D., and Lennarz, W. J. (1977) Enzymatic conversion of proteins to glycoproteins. Proc. Natl. Acad. Sci. U.S.A. 74, 134–138
29. Nilius, B., Prener, J., Janssens, A., Owsianik, G., Wang, C., Zhu, M. X., and Voets, T. (2005) The selectivity filter of the cation channel TRPM4. J. Biol. Chem. 280, 22899–22906
30. Woo, S. K., Kwon, M. S., Ivanov, A., Gerzanich, V., and Simard, J. M. (2013) The sulfonylurea receptor 1 (sur1)-transient receptor potential melastatin 4 (trpm4) channel. J. Biol. Chem. 288, 3655–3667
31. Nilius, B., Prener, J., Droogmans, G., Voets, T., Vennekens, R., Freichel, M., Wissenbach, U., and Flockerzi, V. (2003) Voltage dependence of the Ca2+-activated cation channel TRPM4. J. Biol. Chem. 278, 30813–30820
32. Chen, M., and Simard, J. M. (2001) Cell swelling and a nonselective cation channel regulated by internal Ca2+ and ATP in native reactive astrocytes from adult rat brain. J. Neurosci. 21, 6512–6521
33. Chen, M., Dong, Y., and Simard, J. M. (2003) Functional coupling between sulfonylurea receptor type 1 and a nonselective cation channel in reactive astrocytes from adult rat brain. J. Neurosci. 23, 8568–8577
34. Horn, R., and Marty, A. (1988) Muscarinic activation of ionic currents measured by a new whole-cell recording method. J. Gen. Physiol. 92, 145–159
35. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflagers Arch. 391, 85–100
36. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer, T. H., Jr. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. Anal. Biochem. 180, 195–204
37. Benallal, M., and Anner, B. M. (1995) Major organ-specific glycoproteins in isolated brain and kidney membranes identified as Na,K-ATPase sub-units by combined glycan-, lectin-, and immunoblotting. Biosci. Rep. 15, 21–36
38. Dennis, J. W., Granovskiy, M., and Warren, C. E. (1999) Protein glycosylation in development and disease. Bioessays 21, 412–421
39. Mahoney, W. C., and Duksin, D. (1979) Biological activities of the two major components of tunicamycin. J. Biol. Chem. 254, 6572–6576
40. Grand, T., Demion, M., Norez, C., Mettey, Y., Launay, P., Becq, F., Bois, P., and Guinamard, R. (2008) 9-Phenanthrol inhibits human TRPM4 but not TRPM5 cationic channels. Br. J. Pharmacol. 153, 1697–1705
41. Chang, Q., Hoefs, S., van der Kemp, A. W., Topala, C. N., Bindels, R. J., and Hoenderop, J. G. (2005) The sulfonylurea receptor 1 ( SUR1 ) -transient receptor potential melastatin 4 ( TRPM4 ) channel. J. Biol. Chem. 280, 22899–22906
42. Mälkiä, A., Madrid, R., Meseguer, V., de la Peña, E., Valero, M., Belmonte, C., and Viana, F. (2007) Bidirectional shifts of TRPM8 channel gating by temperature and chemical agents modulate the cold sensitivity of mammalian thermoreceptors. J. Physiol. 581, 155–174