The Cold and Menthol Receptor TRPM8 Contains a Functionally Important Double Cysteine Motif*§†

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We have investigated the glycosylation, disulfide bonding, and subunit structure of mouse TRPM8. To do this, amino-terminal c-myc or hemagglutinin epitope-tagged proteins were incorporated and expressed in Chinese hamster ovary cells. These modifications had no obvious effects on channel function in intracellular calcium imaging assays upon application of agonists, icilin or menthol, and cold temperatures. Unmodified TRPM8 migrates with an apparent mass of 129 kDa and can be glycosylated in Chinese hamster ovary cells to give glycoproteins with apparent masses of 136 and 147 kDa. We identified two potential N-linked glycosylation sites in TRPM8 (Asn-821 and Asn-934) and mutated them to show that only the site in the putative pore region at position 934 is modified and that glycosylation of this site is not absolutely necessary for cell surface expression or responsiveness to icilin, menthol, and cool temperatures. Enzymatic cleavage of the carbohydrate chains indicated that they are complex carbohydrates. The glycosylation site is flanked in the pore by two cysteine residues that we mutated, to prove that they are involved in a conserved double cysteine motif, which is essential for channel function. Mutation of either of these cysteines abolishes function and forces the formation of a non-functional complex of the size of a homodimer. The double cysteine mutant is also non-functional. Finally, we showed in Perfluoro-octanoic acid-polyacrylamide gels that TRPM8 can form a tetramer (in addition to dimer and trimer forms), consistent with current thinking that functional TRP ion channels are tetrameric.

In mammals, temperature is sensed through primary afferent sensory neurons whose cell bodies are located in the dorsal root and trigeminal ganglia. Signals from these cells are transmitted to the spinal cord and then to the brain where they are integrated and deciphered to evoke reflexive and cognitive responses. Growing evidence suggests that the principal molecular thermosensors in the sensory neurons belong to the family of transient receptor potential (TRP) channels. Six temperature-sensitive TRP channels have been identified so far, four belonging to the TRPV subfamily and sensing heat (TRPV1, TRPV2, TRPV3, and TRPV4) and TRPM8 and TRPA1, which are sensitive to cold temperatures. Some of these channels are also sensitive to compounds that mimic the sensation of heat or cold, for example, TRPV1 responds to capsaicin, the pungent ingredient of “hot” chili pepper, and TRPM8 is sensitive to the cooling compounds menthol and icilin.

The properties of TRPV1 have been extensively studied by electrophysiological, pharmacological, and biochemical methods (1). TRPM8 was cloned more recently (2, 3) and is less well studied.

TRPM8 is a non-selective cation channel predominantly expressed in a subpopulation of thermoceptive/nociceptive neurons found in dorsal root ganglia and in trigeminal ganglia. In dorsal root ganglia, TRPM8 is found in 5–10% of neurons that have a small diameter and do not stain with antibodies to neurofilament, CGRP or isolectin B4 or TRPV1 (2). In cultured dorsal root ganglia, the expression of TRPM8 and TRPV1 overlaps in about 50% of TRPM8 positive cells if they are grown in the presence of nerve growth factor (4).

TRPM8 is activated by cold temperature in the innocuous range with a threshold of ~22 °C, and when expressed in Chinese hamster ovary (CHO) cells, its response to icilin and cold, but not menthol, is modulated by changes in intracellular pH (5). TRPM8 activity is also influenced by transmembrane voltage (6) (7). Analysis of the biochemical properties of TRPM8 protein has been limited to in silico approaches. Hydrophobicity analysis of the protein reveals the presence of six hydrophobic, presumably transmembrane domains and a putative pore region between the last two transmembrane domains, which is presumed to be responsible for its ion permeation properties. In the present study, we address the question of whether TRPM8 undergoes post-translational modifications and investigate the importance of these in TRPM8 expression and function.

The role of post-translational modification in members of the TRP family has not been extensively studied. It is known that differences in N-linked glycosylation pattern in TRPC3 and TRPC6 are responsible for the distinct functional properties of the two genetically closely related cation channels (8). It is been reported also that TRPV1 (9) (10) and TRPV2 (11)

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§ The abbreviations used are: TRP, transient receptor potential; CHO, Chinese hamster ovary; HA, hemagglutinin; PBS, phosphate-buffered saline; Endo H, endoglycosidase H; PNGase F, peptide:N-glycosidase F; PFO, perfluoro-octanoic acid.

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are N-glycosylated at a single residue when expressed in heterologous systems, but the role of this modification is still unknown. We also examined the possible role of cysteine residues in intermolecular and intramolecular disulfide bridges in TRPM8, which have been shown to be implicated in the function of members of this family of channels (12) (13). To study the effect of post-translational modifications and tertiary and quaternary structure on TRPM8 function, we used a mutagenesis approach where potential motifs for N-glycosylation and cysteine residues were mutated. We also investigated if, by analogy with TRPV1, TRPM8 assembles in homo-tetrameric complexes. We hope that these studies will form the basis of an increased understanding of the important structural features of TRPM8 and the similarities and differences to other members of this intriguing family of proteins.

MATERIALS AND METHODS

Construction of c-myc-TRPM8 and Hemagglutinin (HA)-TRPM8 Expression Vectors—To enable immunoprecipitation and identification in Western blot experiments, c-myc and HA tags were inserted into the NH2-terminal coding region of TRPM8 expression vectors. The mouse TRPM8 cell line was generated as described previously (2). New constructs were generated using the QuikChange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA), and sequences were confirmed by automated DNA sequencing.

Surface Biotinylation Assay—Surface expression of TRPM8 was demonstrated by biotinylation of surface proteins on intact CHO cells, followed by solubilization, streptavidin precipitation, and Western blotting. Cells were rinsed in PBS/Ca2+/Mg2+ (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9.6 mM Na2HPO4, 1 mM MgCl2, 0.1 mM CaCl2 (pH 7.4)) three times at room temperature then incubated in 2 ml of a solution containing 1 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Perbio Science) in PBS/Ca2+/Mg2+ for 30 min at room temperature. Cells were rinsed and the reaction quenched by incubation in PBS/Ca2+/Mg2+ containing 100 mM glycine. After two further washes in PBS/Ca2+/Mg2+, cells were lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% SDS containing protease inhibitor mixture (Sigma).

Cells and Transfections—The mouse TRPM8 cell line was generated as described previously (2). New constructs were transfected into CHO FRT cells as described previously (2). Stable transfections were performed using FuGENE 6 reagent (Roche Diagnostics). Combinations of alternatively HA and c-myc-tagged C929A, C940A wild type TRPM8 were co-expressed for pulldown experiments. 48 h after transfection cells were harvested in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture (Sigma). Co-immunoprecipitation was performed on 0.5 mg of total protein lysate overnight at 4 °C using monoclonal anti-HA-agarose conjugate (Sigma) following the manufacturer’s instructions. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-c-myc antibody.

Site-directed Mutagenesis of TRPM8—Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA), and sequences were confirmed by automated DNA sequencing.

Endoglycosidase H (Endo H) and PNGase Digestion—10 μg of total proteins from CHO expressing unmodified mouse TRPM8 or mutant N934Q were boiled for 10 min at 100 °C in presence of 1% β-mercaptoethanol and 0.5% SDS. Digestion was performed in a total volume of 35 μl with 0.2 units of endoglycosidase H (New England Biolabs, Hitchin, UK) in the presence of 50 mM sodium citrate or 0.03 unit of peptide-N-glycosidase F (New England Biolabs) in the presence of 50 mM sodium phosphate and 1% Nonidet P-40 for 2 h at 37 °C. Reactions were stopped by addition of SDS-PAGE sample buffer and analyzed by Western blotting with anti-c-myc antibody as described above.

Perfluoro-octanoic Acid (PFO)-PAGE—PFO-PAGE was performed using the method described by Ramjeesingh et al. (15). Total cell lysate from CHO cells expressing c-myc-TRPM8
construct was prepared in PBS using an homogenizer (Omni International Inc., Marietta, GA) for 10 s at maximum speed and clarified by centrifugation for 10 min at maximum speed. 15 μg of lysate was mixed with doubly concentrated sample buffer (100 mM Tris base, 20% (v/v) glycerol, 0.005% bromphenol blue (pH 8.0)) and NaPFO (Sigma) was added to 0.8% (w/v). After 30 min at room temperature samples were electrophoresed on 5% Tris-HCl gels (Bio-Rad) using PFO running buffer (25 mM Tris base, 192 mM glycine, 0.5% (w/v) PFO (pH 8.5)). The transfer and immunostaining were performed as described above.

Intracellular Calcium Measurements—Intracellular calcium levels ([Ca^{2+}]_i) in cell populations was measured by fluorescence using a Flexstation (Molecular Devices, Sunnyvale, CA). Cells were plated in 96-well plates (Corning, NY) at an approximate density of 25,000 cells per well and grown overnight. Cells were loaded with 2 μM Fura-2 AM (Molecular Probes, Leiden, The Netherlands) for ~1 h in the presence of 2.5 mM probenecid and 0.01% pluronic F-127 at 37 °C. Loading and experiments were performed in HEPES buffer (138 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2, 2 mM CaCl_2, 10 mM glucose, 10 mM HEPES (pH 7.4)). Emission intensity at 520 nm was measured at intervals of 4 or 5 s, using excitation wavelengths of 380 and 340 nm. The [Ca^{2+}]_i in groups of individual cells was measured using a fluorescent microscope-based system. Cells were grown on glass cover slips covered with poly-d-lysine, loaded with Fura-2 AM as described above, and then placed in a laminar flow perfusion chamber and continuously perfused with HEPES buffer. Cold stimuli were applied locally (flow rate approximately 3 ml min^{-1}) via a fine pipette positioned very close to the cells being studied. Cold stimulation consisted of a linear decrease in perfusate temperature from 36 to 12 °C at a constant rate of 1 °C per second, controlled by a regulated Peltier device (Marlow Industries, Dallas, TX). The temperature of the perfusate was monitored by a thermocouple positioned in the tip of the pipette and temperature traces recorded on a chart recorder.

Images of a group of cells were captured every 2 s with excitation at 340 and 380 nm and emission measured at 520 nm using a PTI imaging system (South Brunswick, NJ). Analysis of ratios of emission intensity at 340 nm/380 nm excitation in individual cells was determined using the PTI ImageMaster software.

Agonists—(−)-Menthol was from Sigma, and icilin was from Tocris Cookson (Bristol, UK).

Calculation and Statistics—Data are presented as mean ± S.E. for the number of experiments indicated (n). Curve fitting was performed using GraphPad Prism software (GraphPad Ltd.). Statistical significance was calculated using Student's t test for differences or using equivalence test where no difference was expected between groups (16).

RESULTS

TRPM8 Tagged with c-myc or HA Retains Activity—To facilitate identification of mouse TRPM8 protein, we inserted either a c-myc or a HA epitope tag in the NH_{2} terminus of mouse TRPM8, 14 amino acids after the initiating methionine. The resulting constructs were expressed in CHO cells and could be detected readily in a Western blot (Fig. 1A). Intracellular calcium measurements showed that the insertion of either epitope tag does not modify the responsiveness of the receptor to agonists, icilin and menthol, and cold (Fig. 1, B and C).

TRPM8 Is a Glycoprotein—Western blotting of recombinant mouse TRPM8 in denaturing polyacrylamide gels showed a strong signal at 147 kDa and a weaker one at 136 kDa (Fig. 1A), which were larger than the size predicted from cDNA sequence of the tagged protein of 129 kDa. The higher molecular mass bands are likely to be due to glycosylation. To verify the presence of potential glycosylation motifs, we compared the predicted sequence of the mouse, rat, and human TRPM8 proteins and searched for conserved NXS/T
motifs (where X is any amino acid except proline) and found asparagine within conserved motifs at positions Asn-821 and Asn-934 (Fig. 2). To test whether these motifs were glycosylated, we mutated the asparagine to glutamine at each site, expressed the resulting recombinant protein in CHO cells, and performed Western analysis on extracted proteins. Mutation of site Asn-821 made no difference to the observed pattern of bands upon Western blotting however mutation of site Asn-934 resulted in a single band of 129 kDa molecular mass that corresponds to the predicted size of unmodified TRPM8 (Fig. 3).

Functional analysis of TRPM8 and the N934Q mutant showed no significant difference in responses to menthol (p < 0.05) or icilin (p < 0.05) (Fig. 3A). The calculated pEC_{50} for menthol is 4.02 ± 0.35 and 3.81 ± 0.27 (n = 8) and for icilin is 6.37 ± 0.17 and 6.33 ± 0.25 (n = 6) for the c-myc-TRPM8 and N934Q mutant constructs, respectively.

Analysis of [Ca^{2+}]_i increase due to cold activation showed that this mutant responds to cold with a threshold of activation similar to wild type (~22 °C), but the amplitude of the response is generally smaller (Fig. 3).

We further investigated the pattern of N-glycosylation at position Asn-934 to determine whether it was susceptible to hydrolysis using Endo H or peptide:N-glycosidase F (PNGase F). Endo H cleaves between the two N-acetylglucosamine residues in the chitobiose core of N-linked high mannose and hybrid oligosaccharides, whereas PNGase F cleaves the amide bond between the asparagine and the first N-acetylglucosamine residue of all types of glycoproteins (17). The N-glycosyl carbohydrate of mouse TRPM8 was not sensitive to Endo H hydrolysis whereas enzymatic digestion with PNGase F reduced the molecular mass to 130 kDa, close to the size predicted from the cDNA sequence. Neither enzyme affected the migration of the N934Q mutant (Fig. 3).

To test whether the reduced responsiveness of mutant N934Q to cold could be the result of a less efficient trafficking of the receptor to the cell surface, we performed a surface biotinylation assay. Mutant N934Q appears to have lower levels of cell-surface expression compared with wild type TRPM8 and N821Q mutant, and this could be related to the glycosylation state of the protein (Fig. 3C).

We noticed that only the most heavily glycosylated form of TRPM8 (147 kDa) is expressed at the cell surface (Fig. 3C, pull-down fraction), whereas the less heavily glycosylated form of
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that of the wild type TRPM8, demonstrating that cysteines in the pore are not essential for transport of TRPM8 to the cell surface (Fig. 4C).

Investigation of the glycosylation patterns of normal and mutant TRPM8 proteins by Western blotting reveals that TRPM8 and the C929A/C940A double mutant have similar migration patterns, whereas the C929A and the C940A mutants show weaker expression of the higher molecular mass glycosylated form of 147 kDa compared with the smaller glycosylated form of 136 kDa (Fig. 4A). Thus, the unpaired cysteines affect the degree and type of N-linked glycosylation in the endoplasmic reticulum.

Under non-reducing conditions, TRPM8 migrates as a monomer so we conclude that these cysteine residues are not normally responsible for intermolecular disulfide bridging. However, when either cysteine is mutated, a band corresponding to the size of a dimer is detectable, suggesting that a proportion of TRPM8 may be forced into a disulfide-bridged homo-dimer form. As expected, simultaneous mutation of both cysteines in the pore prevents putative dimers from forming (Fig. 4B). This suggests that under normal circumstances, these cysteines are likely to be either reduced or involved in an intramolecular disulfide bridge within the putative pore-forming loop of TRPM8. In an attempt to determine whether the high molecular mass band appearing under non-reducing condition in the single cysteine mutants is a homodimer, we co-transfected CHO cells with TRPM8 mutants tagged with either HA or c-myc. Thus, C929A tagged with either HA or c-myc, C940A tagged with either c-myc or HA, and unmutated TRPM8 with either tags were expressed and co-immunoprecipitated from CHO lysates to see if TRPM8 C929A and C940A could form a dimer that is not present in unmutated TRPM8. Upon pull-down of the tagged TRPM8 a band migrating with the mass of TRPM8 was visible for all constructs (Fig. 4D). Thus, there is strong self-association of TRPM8 subunits that is not cysteine-dependent, and this prevents observation of any disulfide bonding homodimer in this experiment.

Since the essential conserved cysteine motif flanks the glycosylation site in the putative pore region, our data suggest that the cysteine residues may be able to form an intramolecular disulfide bond to create a loop within the putative pore-forming region of TRPM8 with a complex carbohydrate N-linked to its apex (Fig. 5).

TRPM8 Forms a Homotetrameric Complex—To investigate the quaternary structure of TRPM8, we used a gel analysis technique that gently disrupts the plasma membrane with a weak detergent (PFO) while maintaining weak interactions between proteins before and during electrophoresis. PFO gel analysis of TRPM8 protein expressed in CHO cells reveals the presence of multimeric forms of the ion channel (Fig. 6). Careful size determination indicated proteins of 128 and 153, 303, 434, 520 kDa, which is consistent with the size expected for TRPM8 protein in the monomeric (both glycosylated forms) dimeric, trimeric, and tetrameric forms. We analyzed wild type and all the mutants forms of TRPM8 by PFO-PAGE and found that all are able to form dimers, trimers, and tetramers despite the fact that some of them are non-functional (data not shown). These data show that neither glycosylation nor perturbation of the double cysteine motif flanking the glycosylation site in the putative pore region are essential for transport of TRPM8 to the cell surface (Fig. 4C).
cysteine motif modulate multimerization of TRPM8 in the PFO gel system.

**DISCUSSION**

We have characterized the multimeric structure and the impact of post-translational modifications, such as N-glycosylation and disulfide bond formation, on the structure and function of TRPM8. This ion channel is gated by cooling compounds, such as icilin and menthol, and temperatures below 23 °C and may act as a coincidence detector of multiple sensory stimuli like the polymodal heat receptor, TRPV1 (2, 18). The cooling compound icilin has recently been shown to bind to near the base of transmembrane domains 2 and 3 (19), in a region implicated in binding capsaicin and capsazepine in TRPV1 (14, 20). Menthol also binds to a determinant in transmembrane domain 2 as well as to a site in the carboxyl terminus (21), suggesting that this transmembrane region may be a conserved general binding site for TRP channel ligands.

**Tagging and Glycosylation**—Insertion of an epitope tag into the NH$_2$-terminal region of TRPM8 did not affect responses to the agonists icilin or menthol or to cool temperatures. Although TRPM8 contains two potential N-glycosylation sites (Asn-821 and Asn-934) that are conserved in rat, mouse, and human sequences, only the asparagine at position 934, in the putative pore forming loop is significantly glycosylated. TRPV1 can also be glycosylated in the pore loop region (10), at a site that occurs near the beginning of the predicted pore loop, whereas the TRPM8 site occurs in the second half of the loop. In CHO cells, mouse TRPM8 is expressed as two different forms of 136 and 147 kDa. Analysis of the sensitivity of these forms to endoglycosidase F demonstrated that they are both N-glycosylated and hence are modifications of asparagine 934. Endoglycosidase H is unable to cleave the carbohydrate chains of these proteins suggesting that they are either of the complex or the low mannose type, and they are not of either the hybrid or high mannose type (17). The observation that the single glycosylation site Asn-934 occurs in two different glycosylation states (136 and 147 kDa) may be explained in two ways: either there is a mixed population of complex glycosylated mature receptors, or the 136 kDa band represents immature receptor molecules. The lower band of TRPM8 protein (136 kDa) is not transported to the cell surface and may represent an immature, low mannose form of the receptor not yet delivered to the trans-Golgi compartments where complex glycoconjugates are formed.

N-Linked glycosylation is implicated in the correct protein folding and assembly of functional ion channel complexes for nicotinic acetylcholine receptors (22) and for P2X$_2$ receptor (23). Conversely, glycosylation is not necessary for TRPV1 function (10) nor is it essential for correct expression and function of the Shaker or HERG potassium channels (5, 24), which are structurally related to the TRP proteins. Although mutation of N934 in TRPM8 results in a loss of detectable glycosylation and in a lower level
of surface expression, it has no obvious effect on responses to icilin, menthol, or cold in intracellular calcium imaging assays. Thus glycosylation is not necessary for TRPM8 function, although it is possible that there may be more subtle effects of protein glycosylation on the functional characteristics of TRPM8, which may be detected by more sensitive electrophysiological analysis.

Critical Cysteine Residues—The glycosylation site at Asn-934 is located between two cysteine residues in the putative pore forming region. The presence of intact disulfide bonds between extracellular cysteine residues seems to be a key structural feature that is necessary for proper folding and expression of many ion channels. The inwardly rectifying potassium channel, Kir2.1, contains two cysteine residues which form an intramolecular disulfide bond bridge that holds the molecule in a permissive conformation and destruction of the disulfide bridge prevents the protein from forming a functional ion channel (25). In addition, some ligand-gated channels, including the nicotinic acetylcholine receptor (26) and P2X (27), contain disulfide bridges in their extracellular loop, which are essential for receptor trafficking to the cell surface.

Mutation of either Cys-929 or Cys-940 in the pore loop of TRPM8 resulted in a non-functional ion channel. Non-reducing Western blots demonstrated that each of these TRPM8 mutants on their own resulted in an additional band with the apparent size of a dimer. The double cysteine mutant and non-mutated versions both migrated as monomers. This result suggests that the cysteines are reactive. The cysteines at positions 929 and 940 of TRPM8 are essential for function, since mutating either of them ablated the ability of TRPM8 to respond to icilin, menthol, or to cool temperatures. Loss of function was not due to a failure of the mutant TRPM8 to traffic to the cell surface, since all three mutants could be cell-surface biotinylated.

Our data are consistent with the two conserved pore cysteines being reactive and normally forming an intramolecular disulfide-bonded loop with N-linked complex carbohydrate at the apex (Fig. 6). It is likely that the N-linked carbohydrate will be hydrated and will draw the attached protein structure toward the aqueous phase of the extracellular side of the ion channel pore. In the TRP family of channels, TRPM2, TRPM3, TRPM7, TRPV4 and TRPC1, share the motif of conserved cysteine residues flanking a putative N-glycosylation site in the pore region. While this paper was under review, the double cysteine motif was shown to be essential for agonist activation of TRPM2 in a dominant negative manner (28), confirming the importance of the motif for function in two different members of the TRP family. Thus, these channels share similar structural and functional features, which may represent a more general post-translational modification mechanism for the modulation of ion channel function. Such a modification could enable redox modulation of channel function, however, the biological significance of the modification remains to be elucidated.

Our experiments showed that there is strong protein-protein interaction between TRPM8 subunits, hindering the analysis of the importance of disulfide bonding in homodimer formation involving either normal or cysteine mutant TRPM8. The protein-protein interaction was not sufficient to enable formation of a functional channel.

Disulfide bonds are formed in the endoplasmic reticulum before N-linked oligosaccharides are processed in the Golgi apparatus. The double cysteine mutant is glycosylated to a similar extent as unmodified TRPM8, with higher levels of the heavily glycosylated protein (147 kDa) form compared with the smaller (136 kDa) form in whole cell extracts. The double cysteine motif in the pore is not essential for correct N-glycosylation. In addition, both single cysteine mutants show more of the 136-kDa band than the 147-kDa band in Western blotting, suggesting the double cysteine motif influences glycosylation of TRPM8, and perturbing it reduces the efficiency of synthesis of mature, glycosylated TRPM8.

Quaternary Structure—Several TRP family proteins have been shown to be incorporated into heteromeric or homomeric complexes. For example, in the canonical TRP family, Clapham and co-workers (23) have shown that TRPC1, TRPC4, or TRPC5 can be expressed as heteromers with either of the diacylglycerol-activated TRPC3 or TRPC6 subunits. It is likely that all functional TRP channel are hetero- or homotetramers by analogy to the structurally related voltage-gated potassium ion channel family (29). TRPV1 has been shown to be able to form a homotetrameric complexes under pseudo-native conditions (30), and here we have used the same approach to show that TRPM8 can also assemble in a tetrameric complex and that mutation of the potential N-linked glycosylation sites and the cysteines in the pore has no effect on the formation of tetrameric TRPM8.

Further motifs in TRPM8 remain to be studied. In silico analysis reveals the presence of other conserved motifs in mouse, rat, and human TRPM8 protein, which could be investigated to gain a better understanding of the structural features of this receptor. Two examples of these features in the COOH-terminal tail are the “TRP domain” region, which is found in other members of this family (31) and is of unknown function and a putative coiled-coil domain at the extreme carboxyl terminus. Coiled-coil domains are involved in protein-protein interaction and have been shown to be implicated in subunit oligomerization of GABA<sub>B</sub> channels (32) and some TRP channels (33). Future studies will aim to investigate the roles that these motifs play in determining the protein structure of TRPM8 and related ion channels.

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