Role of the Transient Receptor Potential Vanilloid 5 (TRPV5) Protein N Terminus in Channel Activity, Tetramerization, and Trafficking

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The epithelial Ca2+ channel transient receptor potential vanilloid 5 (TRPV5) constitutes the apical entry site for active Ca2+ reabsorption in the kidney. The TRPV5 channel is a member of the TRP family of cation channels, which are composed of four subunits together forming a central pore. Regulation of channel activity is tightly controlled by the intracellular N and C termini. The TRPV5 C terminus regulates channel activity by various mechanisms, but knowledge regarding the role of the N terminus remains scarce. To study the role of the N terminus in TRPV5 regulation, we generated different N-terminal deletion constructs. We found that deletion of the first 32 residues did not affect TRPV5-mediated 45Ca2+ uptake, whereas deletion up to residue 34 and 75 abolished channel function. Immunocytochemistry demonstrated that these mutant channels were retained in the endoplasmic reticulum and in contrast to wild-type TRPV5 did not reach the Golgi apparatus, explaining the lack of complex glycosylation of the mutants. A limited amount of mutant channels escaped the endoplasmic reticulum and reached the plasma membrane, as shown by cell surface biotinylation. These channels did not internalize, explaining the reduced but significant amount of these mutant channels at the plasma membrane. Wild-type TRPV5 channels, despite significant plasma membrane internalization, showed higher plasma membrane levels compared with the mutant channels. The assembly into tetramers was not affected by the N-terminal deletions. Thus, the N-terminal residues 34–75 are critical in the formation of a functional TRPV5 channel because the deletion mutants were present at the plasma membrane as tetramers, but lacked channel activity.

The epithelial Ca2+ channel TRPV5, the gatekeeper of active Ca2+ reabsorption in the kidney, is a member of the TRP superfamily. TRP channels are involved in many different biological processes ranging from sensory physiology, contributing to taste, olfaction, and vision to muscle proliferation and Ca2+ and Mg2+ homeostasis of the body (1, 2). Based on their sequence and structural homology, the mammalian TRP channel superfamily is divided into six subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPV (vanilloid). All TRP proteins have a common topology, including six transmembrane segments flanked by large cytoplasmic N- and C-terminal domains. They are suggested to assemble into tetramers, the hydrophobic loop between transmembrane 5 and 6 forming a putative core domain, ultimately leading to the formation of a cation-selective channel (1, 3).

Despite this common topology, the structure and function of the cytosolic N- and C-terminal domains between the different TRP subfamilies are quite diverse (4). The N terminus of the TRPM subfamily holds four TRPM homology regions, which are essential for proper functioning of these channels (5–8). Highly conserved ankyrin repeats are present in the N termini of TRPC, TRPV, and TRPA channels. Ankyrin repeats are composed of multiple protein/ligand interaction domains of ~33 residues each with a highly conserved helix-loop-helix structure (9, 10). ATP and calmodulin have been demonstrated to bind TRP ankyrin repeats; however, more binding factors remain to be identified (10, 11). Within the C terminus, a highly conserved short hydrophobic stretch, called the TRP box region, has been identified in TRPC, TRPM, and TRPV families (12). The TRP box region is located just after the sixth transmembrane segment and might serve as a coiled-coil zipper keeping the channel in a closed conformation (13). The C terminus of the TRPM subfamily harbors a coiled-coil region, which is involved in channel tetramerization and is critical for channel activation (7, 8, 14–16). The members TRPM6 and TRPM7 contain a unique α-kinase domain at the far end of the C terminus, which regulates channel activity by a variety of molecular mechanisms (17, 18).

From the subfamily of TRPV channels, TRPV1–4 play a role in sensory signaling, whereas the Ca2+-selective and highly homologous TRPV5 and TRPV6 channels are crucial players in Ca2+ homeostasis. Like the other members of this TRPV subfamily the C terminus of TRPV5 is relatively short as compared with the N terminus. However, various molecular mechanisms regulate TRPV5 channel activity via the C terminus. In contrast, information regarding the role of the N terminus in...
TRPV5 function is more limited. One report revealed a critical role for a N-terminal protein kinase C (PKC) phosphorylation site in tissue kallikrein-mediated TRPV5 stimulation (19). This site was also necessary for PKC phosphorylation triggered by parathyroid hormone (20). Additionally, calmodulin has been demonstrated to bind the TRPV5 N terminus (21); however, the role in channel function has not been identified. Finally, residues 64–77 appeared essential for oligomerization of different N termini within the TRPV5 tetramer (22). Because TRPV5 is a rate-limiting factor in active Ca\(^{2+}\) reabsorption, understanding the molecular regulation of this Ca\(^{2+}\) channel is essential to elucidate the mechanisms of renal Ca\(^{2+}\) handling. The aim of our study was to gather more information regarding the role of the TRPV5 N terminus in channel regulation.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—Constructs containing N-terminally HA-tagged rabbit TRPV5 in pCB6 were obtained as described previously (23). This vector was used to develop HA- and FLAG-tagged N-terminal deletion constructs. First, using in vitro mutagenesis the restriction site BspEI was inserted directly after the HA tag. Second, a FLAG-tagged TRPV5 construct was generated by replacing the HA tag by the FLAG tag using restriction sites KpnI and BspEI. Third, using PCR the different N-terminal TRPV5 truncants were subcloned in the original vectors containing HA- and FLAG-tagged TRPV5 via restriction sites BspEI and XbaI. The tapasin-eGFP construct was a kind gift from the lab of Dr. K. Jalink (Amsterdam).

**Cell Culture and Transfection**—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Bio Whittaker Europe, Vervier, Belgium) containing 10% (v/v) fetal calf serum (PAA, Linz, Austria), 2 mM L-glutamine, 10 \(\mu\)M nonessential amino acids at 37 °C in a humidity-controlled incubator with 5% (v/v) CO\(_2\). For biochemical experiments cells were transiently transfected with the appropriate plasmids using polyethyleneimine (PEI; Brunswig/PolySciences) with a DNA:PEI ratio of 6:1. For patch clamp experiments, cells were transiently transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen). Transfected cells were used after 24 h.

**\(^{45}\)Ca\(^{2+}\) Uptake**—Radioactive Ca\(^{2+}\) uptake was determined using TRPV5 expressing HEK293 cells seeded on poly-L-lysine (Sigma)-coated 24-well plates. Cells were pretreated for 20 min with 25 \(\mu\)M BAPTA-AM, subsequently washed with PBS and incubated for 5 min in KHB buffer (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 0.1 mM CaCl\(_2\), 10 mM sodium acetate, 2 mM NaH\(_2\)PO\(_4\), 20 mM HEPES, pH 7.4, adjusted with NaOH), and finally incubated for 10 min with \(^{45}\)CaCl\(_2\) (1 \(\mu\)Ci/ml) in KHB buffer with voltage-gated Ca\(^{2+}\) channel inhibitors (10 \(\mu\)M felodipine, 10 \(\mu\)M verapamil). To block TRPV5-mediated \(^{45}\)Ca\(^{2+}\) uptake, cells were incubated with 10 \(\mu\)M ruthenium red during the 5- and 10-min incubation steps. After multiple washing steps with ice-cold stop buffer (110 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 10 mM sodium acetate, 0.5 mM CaCl\(_2\), 1.5 mM LaCl\(_3\), and 20 mM HEPES, pH 7.4, adjusted with NaOH) the uptake of \(^{45}\)Ca\(^{2+}\) was measured.

**Electrophysiology**—Patch clamp experiments were performed as described previously (24) in the tight seal, whole cell configuration at room temperature using an EPC-9 patch clamp amplifier computer controlled by the Pulse software (HEKA Elektronik, Lambrecht, Germany).

**Immunocytochemistry**—TRPV5-expressing cells were grown on coverslips. Culture medium was removed, and cells were washed with cold PBS and subsequently fixed for 30 min at room temperature with 4% w/v formaldehyde in PBS. After washing twice with PBS, cells were treated with permeabilization buffer (0.2% v/v Triton X-100 in PBS with 0.1% w/v BSA) for 15 min. Then, cells were treated with 50 mM NH\(_2\)Cl for 15 min and washed with PBS and once with permeabilization buffer. Goat serum dilution buffer (16% v/v donkey or goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 0.2% v/v Triton X-100 in PBS) was applied before incubations with primary and secondary antibodies. Primary antibodies were diluted 1:200 in goat serum dilution buffer and incubated at 4 °C overnight. Secondary antibodies, also diluted 1:200 in goat serum dilution buffer, were conjugated to Alexa Fluor dyes (Invitrogen). Secondary antibody incubations were for 1 h at 37 °C. Cells were imaged by confocal microscopy (Olympus FV1000).

**Cell Surface Biotinylation and Internalization Assay**—HEK293 cells were transfected with different TRPV5 constructs in pCB6 using PEI. After 2 days, cell surface proteins were biotinylated for 30 min at 4 °C using sulfo-NHS-LC-LC-biotin or sulfo-NHS-SS-biotin (0.5 mg/ml; Pierce) as described previously (25). In short, cells were kept at 4 °C or incubated for 30 min at 37 °C to allow endocytosis from the plasma membrane. TRPV5 internalization was measured by treating cells with fresh 100 mM 2-mercaptoethanesulfonic acid sodium salt (mesna) for 3 times 20 min at 4 °C. After treatment with 120 mM iodoacetic acid to quench mesna, cells were lysed in 150 mM NaCl, 5 mM EGTA, 50 mM Tris, pH 7.5 adjusted with HCl, Triton 1% (v/v), and protease inhibitors at 4 °C and centrifuged at 14,000 \(\times\) g for 10 min. Finally, biotinylated proteins in the supernatant were precipitated using neutravidin-coupled beads (Pierce) and analyzed by immunoblotting using anti-HA antibodies (2367; Cell Signaling Technology). Anti-β-actin (A5441; Sigma) was used to detect nonspecific labeling of intracellular proteins.

**Co-immunoprecipitation**—HEK293 cells expressing both HA-TRPV5 and FLAG-TRPV5 were lysed in 150 mM NaCl, 5 mM EGTA, 50 mM Tris, pH 7.5 adjusted with HCl, Nonidet P-40 0.5% (v/v), and protease inhibitors: 1 mM PMSF, 10 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin, 5 \(\mu\)g/ml aprotanin at 4 °C. After centrifugation at 16,000 \(\times\) g for 15 min at 4 °C, the supernatant containing 3 \(\mu\)g of total protein was added to 30-\(\mu\)l equivalents of protein A/G plus-agarose beads (Santa Cruz Biotechnology), which were preincubated for 3 h at room temperature with 2 \(\mu\)l of polyclonal anti-FLAG antibody raised in rabbit (F7425; Sigma) in 0.7 ml of IPP500 (500 mM NaCl, 10 mM Tris pH 8.0 adjusted with HCl, 0.5% (v/v) Nonidet P-40, 1 mM PMSF, 10 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin, 5 \(\mu\)g/ml aprotanin). As a control for nonspecific antibody binding 2 \(\mu\)l of rabbit anti-GFP antibody (home-made) was bound to protein A/G plus-agarose beads. The beads were washed three times with the lysis buffer described above. Cell lysate and antibody-bound beads were incubated overnight at 4 °C. Subsequently, the beads were washed with lysis buffer, and proteins were eluted in Laemmli as described previously (26).
Oligomerization Assay—HA-tagged TRPV5, TRPV5-Δ1–38 and TRPV5-Δ1–75 were expressed in HEK293 cells. Cell lysates were mixed with 24 perfluorooctanoic acid (PFO) sample buffer (100 mM Tris, 20% v/v glycerol, 8% w/v PFO (Sigma-Aldrich), 0.005% w/v bromphenol blue, pH 8.0, adjusted with NaOH) and run on a 4–12% Tris-glycine acrylamide gel (Invitrogen) in 25 mM Tris, 192 mM glycine, 0.5% w/v PFO (pH 8.5, adjusted with NaOH) at 4 °C. Subsequently TRPV5 proteins were detected by immunoblotting using anti-HA antibodies.

Molecular Modeling—The crystal structure of delta endotoxin Cry8Ea1 (Protein Data Base ID code 3EB7) was used as a template to generate a model of the beginning of the TRPV5 N terminus (residues 10–46). Sequence homology for this region was 35%. YASARA was employed for modeling.

Statistical Analysis—In all experiments, data are expressed as means ± S.E. Statistical comparison was determined using a one-way ANOVA followed by the Newman-Keuls multiple comparison test. p values < 0.05 were considered significant.

RESULTS

TRPV5 Lacking the first 38 N-terminal Residues Is Not Functional—To analyze the role of the N terminus in the regulation of the TRPV5 channel, several channel deletion constructs were generated, each missing parts of the N terminus. To study the role of the ankyrin domains in TRPV5 function, deletion constructs TRPV5-Δ1–75 and TRPV5-Δ1–161 were developed missing ankyrin domains 1 and 1–3, respectively (Fig. 1A). Additionally, three constructs lacking the first 25, 32, and 38 N-terminal residues, but with intact ankyrin domains, were generated (Fig. 1A). HEK293 cells were transfected with these constructs to study protein expression and function using 45Ca2+ uptake assays. Immunoblotting of wild-type TRPV5 (TRPV5-WT) demonstrated a double band (Fig. 1B), representing a core and complex glycosylated form (23). Removal of the first 38 or 75 amino acids completely prevented complex glycosylation, whereas this was only slightly reduced for TRPV5-Δ1–32 (Fig. 1B). Unfortunately, deletion mutants TRPV5-Δ1–25 and TRPV5-Δ1–161 showed severely reduced protein expression compared with TRPV5-WT, so these constructs were not included in the functional analysis (data not shown).

The activity of the deletion mutants was measured by the influx of radioactive 45Ca2+ into TRPV5-expressing HEK293 cells. Removal of the first 32 amino acids of the N terminus did not affect TRPV5-mediated 45Ca2+ influx (Fig. 1C). In contrast, removal of the first 38 or 75 amino acids abolished TRPV5 activity because the 45Ca2+ influx levels were not different from...
the TRPV5 blocker ruthenium red (Fig. 1C). To investigate further the mechanism by which removal of the N terminus abolishes TRPV5 function, we used TRPV5-Δ1–38 because this was the minimal deletion eliminating channel activity. Patch clamp analysis was implemented to study whether TRPV5-Δ1–38 was permeable to Na⁺. In contrast to TRPV5-WT, the TRPV5-Δ1–38 mutant did not exhibit Na⁺ currents different from mock-transfected cells (Fig. 1D and E).

**TRPV5-Δ1–38 Is Retained in the Endoplasmic Reticulum**—Because a lack of complex glycosylation can be a direct result of retention in the endoplasmic reticulum, the localization of TRPV5-Δ1–38 was visualized using immunocytochemistry. In short, HEK293 cells coated on coverslips were transfected with TRPV5-WT or TRPV5-Δ1–38. To investigate the presence in the Golgi apparatus cells were stained with the Golgi marker α-giantin. TRPV5 localization was determined using an antibody against the HA tag. TRPV5-WT appeared mainly in vesicles and specifically co-localized with α-giantin (Fig. 2A). In contrast, TRPV5-Δ1–38 was not localized to vesicles and did not specifically overlap with the Golgi marker (Fig. 2A). To study whether TRPV5-Δ1–38 was retained in the endoplasmic reticulum, GFP-fused tapasin, an endoplasmic reticulum resident protein, was co-expressed. In contrast to TRPV5-WT, TRPV5-Δ1–38 co-localized with the endoplasmic reticulum protein tapasin-GFP (Fig. 2B).

**Plasma Membrane Regulation of TRPV5-Δ1–38 Is Affected**—The presence of TRPV5-WT channels at the plasma membrane cannot be visualized by immunocytochemistry. Therefore, other biochemical experiments like cell surface biotinylation were used to investigate the expression of TRPV5 at the plasma membrane (27). Because TRPV5-Δ1–38 was retained in the endoplasmic reticulum we investigated whether this mutant still reached the plasma membrane. Cell surface proteins of cells expressing TRPV5-WT or TRPV5-Δ1–38 were biotinylated and subsequently precipitated with neutravidin-agarose beads. TRPV5 protein expression in whole cell lysate and biotinylated fraction was determined using immunoblotting. As a negative control, β-actin was used to ensure cell surface specificity of our analysis (supplemental Fig. 1). Strikingly, both TRPV5-WT and TRPV5-Δ1–38 were biotinylated and subsequently precipitated with neutravidin-agarose beads. 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Next, PFO-PAGE was implemented to investigate whether TRPV5-Δ1–38 forms a tetrameric complex at the plasma membrane. PFO is a mild detergent, retaining oligomeric structures, and was successfully applied on other TRP channels (29). Biotinylated fractions and whole cell lysates of TRPV5-WT, TRPV5-Δ1–38, and TRPV5-Δ1–75 were analyzed by PFO-PAGE and subsequent immunoblotting. All subjected proteins were present in four different oligomeric states, demonstrating tetramerization of the truncated channels at the plasma membrane.

**Residues Gln<sup>31</sup>** and Asp<sup>34</sup> Are Essential for Proper TRPV5 Function—To identify the precise part of TRPV5 critical for proper channel functioning, the residues surrounding amino acid 32 were investigated (Fig. 5A). First, two additional deletion mutants were generated, TRPV5-Δ1–34 and TRPV5-Δ1–36. TRPV5 function was determined by introducing TRPV5-WT and the different deletion mutants into HEK293 cells, and subsequent the amount of $^{45}$Ca<sup>2+</sup> influx was measured. Significant $^{45}$Ca<sup>2+</sup> influx was observed for TRPV5-WT and TRPV5-Δ1–32 (Fig. 5B). In contrast, deletion mutants TRPV5-Δ1–34, -Δ1–36, and -Δ1–38 were not permeable for Ca<sup>2+</sup> because they did not exhibit any TRPV5-mediated $^{45}$Ca<sup>2+</sup> influx (Fig. 5B). Immunoblotting demonstrated the absence of complex glycosylation of TRPV5-Δ1–34, -Δ1–36, and -Δ1–38 (Fig. 5B).

Next, single point mutations were introduced in the TRPV5 protein to identify the residues critical for channel function. Based on alignments of the rabbit TRPV5 sequence with other species, the conserved residues Gln<sup>31</sup> and Asp<sup>34</sup>, located around the N-terminal region essential for TRPV5 function, were selected (Fig. 5A). To determine the effect of these residues in TRPV5 function the amino acids were mutated into an alanine and subsequently studied by assessing the $^{45}$Ca<sup>2+</sup> influx. $^{45}$Ca<sup>2+</sup> influx was significantly reduced in the double point mutant Q31A/D34A (Fig. 5C). Furthermore, this mutant lacked complex glycosylation. TRPV5 modeling using the YASARA bioinformatics program suggested the presence of these critical residues within a helix (Fig. 5D). Alanine substitution of three similar residues outside the helix (Gln<sup>30</sup>, Gln<sup>41</sup>, and Glu<sup>42</sup>), rendering a triple point mutant (QA/QA/EA), did not affect TRPV5 function (Fig. 5C).

**30°C Incubation Recovers Function of TRPV5-Δ1–38 and TRPV5-Q31A/D34A**—Retention of proteins at the endoplasmic reticulum, as was observed for TRPV5-Δ1–38, is often a result of protein misfolding (30). Incubation at lower temperatures is known to rescue proteins that are retained in the endoplasmic reticulum, allowing them to pass the endoplasmic quality control system (31, 32). To investigate whether this procedure can recover the function of TRPV5-Δ1–38 and TRPV5-Q31A/D34A, HEK293 cells were transfected with these constructs and subsequently cultured at 30°C for 18 h. TRPV5 channel activity was measured by the influx of radioactive $^{45}$Ca<sup>2+</sup>. Activity of the TRPV5-Δ1–38 mutant channel was nearly recovered (Fig. 6A). Remarkably, $^{45}$Ca<sup>2+</sup> influx was restored in the Q31A/D34A mutant, and Western blotting demonstrated a double band (Fig. 6, A and B), indicating recovery of complex glycosylation.

Next, the cell surface were probed with sulfo-NHS-SS-biotin, and cells were incubated for 30 min at 37°C to allow internalization of biotinylated proteins. Next, cells were treated with the membrane-impermeant mesna to remove all biotin bound to remaining cell surface proteins. Treatment with mesna removed ~80% of all biotin for both TRPV5-WT and TRPV5-Δ1–38 (Fig. 3, C and D). A 30-min chase at 37°C resulted in a significant elevation of TRPV5-WT protein amounts (Fig. 3, C and D). These channels were internalized and, therefore, resistant to mesna treatment. In contrast, a 30-min chase for cells treated expressing TRPV5-Q31A/D34A—Tetrameric assembly of TRPV5-Δ1–38 N-terminal deletion mutant. A, FLAG-tagged and HA-tagged TRPV5-WT and N-terminal deletion mutants were immunoprecipitated (IP) using the anti-FLAG antibody, and subsequently co-immunoprecipitated TRPV5 channels were determined using the anti-HA antibody. Of note, antibodies used for precipitation are visible on immunoblot as depicted by the arrows. n.a., no antibody; n.s.a., control antibody for nonspecific binding. B, cell surface expression of TRPV5 tetramers was determined by submitting biotinylated fractions to PFO-PAGE analysis.

![Figure 4. Tetramerization of the TRPV5 Δ1–38 N-terminal deletion mutant.](image-url)

- **A**: FLAG-tagged and HA-tagged TRPV5-WT and N-terminal deletion mutants were immunoprecipitated (IP) using the anti-FLAG antibody, and subsequently co-immunoprecipitated TRPV5 channels were determined using the anti-HA antibody. Of note, antibodies used for precipitation are visible on immunoblot as depicted by the arrows. n.a., no antibody; n.s.a., control antibody for nonspecific binding.
- **B**: Cell surface expression of TRPV5 tetramers was determined by submitting biotinylated fractions to PFO-PAGE analysis.
**DISCUSSION**

This study demonstrated the instrumental role of the TRPV5 N terminus in channel activity based on the following findings. Deletion of the first 38 residues of the N terminus abolished TRPV5 activity. The TRPV5-H90041–38 protein exhibited no complex glycosylation and appeared to be retained in the endoplasmic reticulum. Hence, plasma membrane expression of TRPV5-H90041–38 was strongly reduced. However, the TRPV5-H90041–38 mutant is still able to form tetramers at the plasma membrane. Thus, although TRPV5-H90041–38 channels reach the plasma membrane, they are not active, suggesting that the first 38 residues of the N terminus are critical for the formation of a functional channel.

Mutation or removal of N-terminal regions abrogates function of many TRP channels studied (8, 33–36); however, the underlying mechanism remains poorly understood. Within the TRPV subfamily, N-terminal deletion mutants or splice variants of TRPV1, TRPV4, and TRPV6 were investigated. Deletion of ankyrin repeats 1 and 3 in TRPV4 and ankyrin repeat 3 in TRPV6 disabled channel function. These channels could not tetramerize anymore (34, 35). This suggests that certain ankyrin domains within the N terminus are critical for proper channel tetramerization. Assembly dysfunction of TRPV4 resulted in retention in the endoplasmic reticulum, whereas the intracellular localization of the N-terminal TRPV6 mutant was not different from the wild-type channel (34, 35). In TRPV1, deletion of the first 114 and more residues abrogated channel function (33). Interestingly, this is exactly the start of the first ankyrin repeat (9). Plasma membrane expression was not different from the wild-type channel (33). Our study demon-

![Figure 5. Identification of key residues in N-terminal region of TRPV5.](image)

**FIGURE 5. Identification of key residues in N-terminal region of TRPV5.** A, multiple alignment of rabbit, human, rat, and mouse TRPV5 protein sequences, respectively. Conserved residues Gln31 and Asp34 are depicted in bold. B, 45Ca²⁺ influx in HEK293 cells transiently expressing TRPV5-WT (n = 11), TRPV5-D1–32 (n = 11), TRPV5-D1–34 (n = 11), TRPV5-D1–36 (n = 11), and TRPV5-D1–38 (n = 11). TRPV5-mediated 45Ca²⁺ uptake is blocked by the addition of 10 μM ruthenium red (RR). *, p < 0.05 versus TRPV5-WT (CTR). Error bars, S.E. Protein expression was determined by immunoblotting (inset). C, 45Ca²⁺ influx by TRPV5-WT (n = 12), the double point mutant TRPV5-Q31A/D34A (n = 11), and triple point mutant TRPV5-QA/QA/EA (n = 12). *, p < 0.05 versus TRPV5-WT CTR. D, model of TRPV5 N terminus showing residues 10–46 using YASARA. Mutated residues are indicated.
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FIGURE 6. Activity of TRPV5-WT, TRPV5-Δ1–38, and TRPV5-Q31A/D34A in cells cultured at reduced temperature. A, 45Ca²⁺ influx in HEK293 cells transiently expressing mock construct (n = 9), TRPV5-WT (n = 9), TRPV5-Δ1–38 (n = 9), and TRPV5-Q31A/D34A (n = 9) cultured for 18 h at 37 °C and 30 °C, respectively. *, p < 0.05 versus TRPV5-WT. Error bars, S.E. B, protein expression of TRPV5-WT, TRPV5-Δ1–38, and TRPV5-Q31A/D34A as determined by immunoblotting.

Stratified that N-terminal deletion mutants were retained in the endoplasmic reticulum. Removal of the first ankyrin repeat (TRPV5-Δ1–75) did not affect channel tetramerization. Thus, elimination of essential ankyrin repeats disables the formation of tetramers, whereas deletion of other parts of the N terminus abrogates channel function due to a different molecular mechanism.

Functional and biochemical experiments revealed that removal of the first 38 residues of the TRPV5 N terminus resulted in loss of channel function and lack of complex glycosylation. Furthermore, most of the TRPV5-Δ1–38 mutant was retained in the endoplasmic reticulum and did not reach the Golgi apparatus, which is in line with the lack of complex glycosylation. Quality control within the endoplasmic reticulum recognizes and retains misfolded proteins or protein complexes (30). Strikingly, some TRPV5-Δ1–38 complexes were not retained by the endoplasmic reticulum quality control and reached the plasma membrane. PFO-PAGE demonstrated that these mutants formed tetramers at the plasma membrane; however, functional analysis did not show any current for these mutants. The absence of activity of TRPV5-Δ1–38 tetramers at the plasma membrane together with the retention of the majority of protein in the endoplasmic reticulum indicates that the TRPV5-Δ1–38 subunits were probably not folded correctly. Thus, an intact TRPV5 N terminus is not required for channel tetramerization, but the N-terminal residues 34–75 are probably essential for correct protein folding and thus normal channel function. The assembly of misfolded proteins into oligomers has been observed by others (37, 38).

Besides the role of the TRPV5 N terminus in channel function, we made some other interesting observations during this study. First, the co-occurrence of TRPV5-Δ1–38 tetramerization and retention in the endoplasmic reticulum indicates that tetramerization of TRPV5 channels takes place in the endoplasmic reticulum. Oligomerization of proteins is indeed known to take place in the endoplasmic reticulum and is a prerequisite to pass quality control of the endoplasmic reticulum (30, 39). Second, several TRPV5-Δ1–38 mutants escaped the endoplasmic reticulum quality control eventually appearing at the plasma membrane. These channels did not display any complex glycosylation, indicating that this small subset of channels reached the plasma membrane without being processed by the Golgi apparatus. Protein targeting to the plasma membrane independent from the Golgi apparatus has been observed by others (40). Finally, we observed that the plasma membrane retrieval of TRPV5-Δ1–38 mutant was delayed. This could be related to the absence of complex glycosylation, which has been implicated in the retrieval of TRPV5 channels from the plasma membrane (28). This delayed retrieval overestimates the amount of TRPV5-Δ1–38 proteins that reaches the plasma membrane.

In 2003, it was demonstrated that TRPV5 has a tetrameric stoichiometry, which results in a functional channel with an assumed ring-like structure around a central pore (23). A few years later, Chang et al. demonstrated interactions between GST-fused TRPV5 N termini and between GST-fused TRPV5 N- and C termini (22). The N-terminal residues 64–77 were necessary for these interactions and were therefore described as a multimerization domain. We demonstrated that deletion of residues 1–75 abolished channel function and that these channels were still able to form tetramers. Thus, although the TRPV5 N termini interact, they are not critical for tetramerization of the channel. The TRPV5-Δ1–75 mutant lacks the multimerization domain 64–77, possibly hindering proper folding into a functional channel. The role of the multimerization domain 64–77 in a TRPV5 tetramer remains to be investigated.

In addition, we pinpointed the N-terminal residues essential for proper channel function. Therefore, TRPV5 protein sequences from several species were aligned, and within the first 38 N-terminal residues only several residues appeared to be conserved. Residues Gln³¹ and Asp³⁴ were close or within the N-terminal region essential for TRPV5 function. Furthermore, modeling with delta endotoxin Cry8Ea1, displaying low sequence homology with the TRPV5 N terminus, suggested that these residues were located in the center of a helix. Alanine substitution of Gln³¹ and Asp³⁴ rendered a nonfunctional TRPV5 mutant, lacking complex glycosylation. Mutation of three similar residues, Gln⁴⁰, Gln⁴¹, and Glu⁴², just outside the putative helix did not affect channel function. This indicates that the region around residues 31–34 might be essential for correct folding of the channel or binding of other factors.

Finally, TRPV5-Δ1–38 and TRPV5-Q31A/D34A mutants were rescued at reduced temperature, pointing to misfolding as the reason for retention at the endoplasmic reticulum and activity at the membrane. For the TRPV5-Q31A/D34A mutant complex glycosylation was restored as well, suggesting recovery of trafficking through the Golgi apparatus. Thus, the putative helix around residues Q31A/D34A is of major structural importance, affecting both channel processing and function.

This report demonstrated the essential role of the TRPV5 N terminus in channel function. Deletion of the N terminus from residue 34 further abrogated channel function and resulted in endoplasmic retention. Interestingly, we found that the first 75 residues of TRPV5, containing the first ankyrin repeat, are not necessary for channel tetramerization. The role of other ankyrin repeats in TRPV5 tetramerization remains to be established.
Role of N Terminus in TRPV5 Function

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