Altered Biochemical Properties of Transient Receptor Potential Vanilloid 6 Calcium Channel by Peptide Tags

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Ion channels are commonly expressed in recombinant forms with peptide tags, which facilitates their molecular and electrophysiological studies. However, peptide tags may alter ion channel properties. Here we describe the differential effect of peptide tags on the biochemical properties of transient receptor potential vanilloid 6 (TRPV6) channels. Yellow fluorescent protein (YFP)-tagged wild-type TRPV6 (YFP-TRPV6WT) showed much lower levels of aggregate-like bands in Western blots than those of Myc-TRPV6WT. By contrast, the glycosylation level was higher with Myc-TRPV6WT than that with YFP-TRPV6WT. We additionally demonstrate that peptide tags affect the protein integrity of TRPV6 channels. Myc-TRPV6WT was expressed as an intact channel, whereas the pore mutants Myc-TRPV6D542A and Myc-TRPV6D542K were observed to be partially fragmented. By contrast, all YFP-tagged channels were intact, although the YFP-tagged pore mutants were less glycosylated than YFP-TRPV6WT. However, regardless of the peptide tag used, TRPV6D542K electrophysiologically inhibited TRPV6WT which indicates that all pore mutants are equivalent electrophysiologically, not biochemically. Thus, our findings suggest that peptide tags can produce unintended biochemical changes of ion channels which highlight the necessity of careful biochemical evaluation to clarify the roles of ion channels.

Key words peptide tag; transient receptor potential vanilloid 6; biochemical property; ion channel

Transient receptor potential vanilloid 6 (TRPV6) is a member of the TRP channel superfamily that consists of tetrameric nonselective cation channels.1) TRPV6, whose expression is transcriptionally regulated by 1α,25-dihydroxyvitamin D3, mediates Ca2+ absorption in the apical membrane of the duodenal epithelial cells.1) TRPV6 ablation decreases intestinal Ca2+ absorption, bone mineral density, and weight gain, all of which indicate that it plays an important role in Ca2+ homeostasis.2) In addition, immunosuppressive drugs, such as glucocorticoids, reduce TRPV6 expression in the duodenum, which has been implicated in the potential role of TRPV6 in glucocorticoid-induced osteoporosis.3) Thus, TRPV6 provides an important link between Ca2+ and human health. Furthermore, TRPV6 expression increases in prostate cancer cells which may couple intracellular Ca2+ homeostasis to tumor cell physiology.4) However, the molecular mechanisms of TRPV6 regulation are largely unknown.

The Asp542 residue in the pore region of TRPV6 is crucial for cation permeation, and therefore, TRPV6D542A and TRPV6D542K pore mutants are expressed as nonfunctional channels.5) In addition, these pore mutants may act as dominant-negative mutants by forming a heterotetrameric complex with wild-type TRPV6. Thus, reconstitution assays using these pore mutants as experimental controls or loss-of-function approaches as dominant negative mutants are invaluable in verifying the biological functionality of TRPV6, assessing its role in pathophysiological processes, or dissecting the related molecular mechanisms. Particularly, dominant negative mutant approaches are useful when the application of small interfering RNA is limited by long half-life of target proteins.

Despite several drawbacks, such as altered electrophysiological properties,6) peptide tags involving fluorescent proteins or epitope peptides have been widely used to confirm the correct expression of ion channels by providing a means to identify transfection-positive cells or to discriminate against the endogenously expressed channels. Biochemical approaches involving Western blot analysis, however, often fail to verify the expression of recombinant ion channels, mainly due to the difficulties in extracting ion channels from the lipid bilayer membranes, solubilizing ion channels, and preventing aggregate formation in the detergent-solubilized state.7,8) Nevertheless, biochemical assays of ion channels should be implemented to determine or verify their protein integrity or post-translational modifications involving glycosylations. In this study, we describe that peptide tags differentially alter the biochemical and electrophysiological properties of TRPV6 and its pore mutants as expressed in HEK 293 cells.

MATERIALS AND METHODS

Plasmid Constructs and Cell Culture N-terminal Myc epitope- or yellow fluorescent protein (YFP)-tagged constructs were generated by cloning of TRPV6WT, TRPV6D542A, TRPV6D542K, and TRPV6WT into pCMV-Tag3A (Stratagene), pEYFP-C1, or pECFP-C1 vector (Clontech), respectively. Point mutations in TRPV6 were introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The constructs were validated by full-length cDNA sequencing. HEK 293 cells were used for electrophysiological, cytological, and biochemical studies. Each construct was transfected with FuGene-6 reagent according to the manufacturer’s instruction (Roche).

Western Blot Analysis After transfection for 24—48 h, the cells were harvested and resuspended with lysis buffers (50 mM Tris, 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), protease and phosphatase inhibitor cocktails) containing the detergents denoted in Results and Discussion. The cells were allowed to incubate on ice
for 30 min, and centrifuged at 12000 g for 10 min at 4 °C. The crude extracts were boiled for 10 min or incubated at 37 °C for 30 min with 2×Laemmli sample buffer and then resolved in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The expression of each TRPV6 construct was assessed by probing with anti-Myc epitope (Santa Cruz #sc-40 or Cell signaling #2272) or anti-GFP antibody. (Santa Cruz #sc-8334)

**Whole-Cell Patch Clamp Analysis**
Patch clamp experiments were performed in the tight seal whole-cell configuration at room temperature with three solutions: 1) standard internal solution (145 mM Cs glutamate, 10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 8 mM NaCl, 1 mM MgCl₂, 2 mM Mg-ATP, and 10 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N',N'-tetra acetic acid (EGTA); adjusted to pH 7.2 with CsOH), 2) extracellular solution (145 mM NaCl, 2 mM CaCl₂, 10 mM CsCl, 2.8 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES; adjusted to pH 7.4 with NaOH), and 3) divalent free solution (145 mM NaCl, 2.8 mM KCl, 10 mM CsCl, 10 mM glucose, 10 mM EGTA, 10 mM HEPES; adjusted to pH 7.4 with NaOH). Osmotic differences were corrected by adding mannitol to the extracellular solution. Membrane currents, filtered at 5 kHz, were recorded using an Axopatch 200B patch clamp amplifier (Axon Instruments). A gigaseal was achieved by glass pipettes with a resistance of ca. 2—4 MΩ. Before seal formation, the cells were kept in Ca²⁺-free medium and then exposed to the extracellular solution containing 2 mM Ca²⁺ for up to 5 min. The pClamp V8.0 and Digidata-1200 (Axon Instrument) were used for data acquisition and command pulse application. The sampling rate was 2 kHz.

**Statistics**
All data are expressed as mean±S.E. Student’s t-test for unpaired data was used for statistical analysis.

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**RESULTS AND DISCUSSION**

Because multi-pass transmembrane proteins involving ion channels are embedded in the lipid bilayer membranes and thereby generally insoluble in the aqueous environment, detergent solubilization is indispensable in an attempt at biochemical studies of ion channels.7,8) To determine an appropriate detergent that efficiently extracts and solubilizes TRPV6 from the membranes, the pellets of YFP-TRPV6 WT-transfected cells were treated with various detergents at the indicated concentrations (Fig. 1A). Western blot analysis revealed that SDS is an optimum detergent for YFP-TRPV6 detection; SDS shows a prominent monomeric band at 100 kDa and a very low level of aggregate-like spread bands with 250 kDa (Figs. 1A, B) which suggests that SDS solubilizes YFP-TRPV6 WT effectively. Aligned with previous results,9) we observed a band at 110 kDa that represents glycosylated TRPV6 (Figs. 1A, B).

We then constructed Myc-TRPV6 WT and examined the effect of detergents on its band patterns. Western blot analysis revealed that SDS provides an optimum result for Myc-TRPV6 WT detection; it produces a prominent monomeric band at 75 kDa and glycosylation bands near 85 kDa (Fig. 1D). However, aggregated spread bands with 250 kDa were still observed in Myc-TRPV6, suggesting that Myc-TRPV6 WT is less soluble than YFP-TRPV6 WT and prone to aggregate formation in the presence of SDS.

Interestingly, the level of glycosylation of Myc-TRPV6 WT appeared to be superior to that of YFP-TRPV6 WT. The ratio of unglycosylated to glycosylated TRPV6 seemed to be approximately >10 : 1 with YFP-TRPV6 WT and 1 : 1 with Myc-TRPV6 WT. These results suggest that certain structural changes in the cytosolic N-terminal domain by YFP conjugation can modulate luminal glycosylation processes of TRPV6. It is worthwhile to note that Myc-TRPV6 WT bands disappear at the expected position by sample boiling before

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Fig. 1. Western Blot Analysis Using TRPV6-Expressed HEK293 Cell Lysates

(A) YFP-TRPV6-transfected cells were treated with various detergents, including sodium dodecyl sulfate (SDS), sodium deoxycholate (SDOC), TritonX-100, n-dodecyl-β-D-maltoside (DDM), Tween 20, TritonX-114, and CHAPS. YFP-TRPV6 was assessed by probing with anti-GFP antibody. (B) Western blot analysis using the YFP-TRPV6-transfected cell lysates extracted with each detergent at 0.25%. (C) After Myc-TRPV6-transfected cells were treated with SDS at 0.25%, the extracts were mixed with 2×Laemmli sample buffer and boiled for 5 min or incubated at 37 °C for 30 min before gel loading. (D) Western blot analysis using the Myc-TRPV6-transfected cell lysates extracted with each detergent at 0.25%.
gel loading (Fig. 1C), which suggests that boiling in SDS induces high molecular aggregates of Myc-TRPV6 WT as described elsewhere. However, little difference in the band patterns of YFP-TRPV6 WT was observed between boiled and unboiled samples (data not shown), suggesting that YFP markedly enhances TRPV6 solubility in SDS.

As experimental controls or dominant negative mutants, the pore mutants of ion channels are useful to determine their role in pathophysiological processes or dissect the related molecular mechanisms. We thus generated TRPV6 pore mutants, Myc-TRPV6 D542A and Myc-TRPV6 D542K, as described previously. To ascertain there is no functionality of TRPV6 pore mutants, we performed a whole-cell patch clamp analysis. Current traces were recorded in response to a ramp (−100 to +100 mV) at a holding potential of −20 mV in extracellular solution without Ca2+ and Mg2+. The Na+ current density of Myc-TRPV6 WT was 485 ± 15 pA/pF (n = 7) at −100 mV (Figs. 2A, D). Subsequently, the addition of 30 mM Ca2+ to the extracellular solution caused a reduction of current amplitude and a notable positive shift of reversal potential (Fig. 2A). In contrast, Myc-TRPV6 D542A and Myc-TRPV6 D542K were nonfunctional channels (Figs. 2B—D).

In order to verify these results, we examined the correct expression of each construct by Western blot analysis. In the Myc-TRPV6 WT-transfected lysate, a prominent band was found at approximately 80 kDa (Fig. 2E). Unexpectedly, both Myc-TRPV6 D542A- and Myc-TRPV6 D542K-transfected lysates showed a markedly reduced band at 75 kDa, and concomitantly a new band appeared at approximately 50 kDa (Fig. 2E; arrowhead). Comparable results were obtained with another commercial antibody to Myc-epitope (Fig. 2F), and under this condition glycosylated bands with 85 kDa remained intact (Figs. 2E, F). However, glycosylation-defective mutant (Myc-TRPV6 N358Q) showed no fragmented bands at 50 kDa (Fig. 2G), indicating that glycosylation of Myc-TRPV6 does not correlate with the channel fragmentation. Our results suggest that the substitution of the Asp542 residue leads to loss of protein integrity particularly in unglycosylated Myc-tagged TRPV6.

Further studies were performed using an alternative peptide tag in order to determine the universality of the observed alteration on protein integrity. We thus generated YFP-TRPV6 WT, YFP-TRPV6 D542A, and YFP-TRPV6 D542K constructs. No difference in electrophysiological properties was found between Myc-tagged and YFP-tagged constructs (Figs. 3A—C). The Na+ current density of YFP-TRPV6 WT was 742 ± 21 pA/pF (n = 8) at −100 mV (Figs. 3A, D). YFP-TRPV6 D542A and YFP-TRPV6 D542K were found to be nonfunctional channels (Figs. 3B—D).

Nonetheless, Western blot analysis showed a striking difference concerning protein integrity. No cleaved band was detected in YFP-TRPV6 D542A or YFP-TRPV6 D542K or in YFP-TRPV6 WT. Interestingly, in our experiments with YFP constructs, a band at 110 kDa was observed only with YFP-TRPV6 WT. This new band is similar to that of YFP-TRPV6 N358Q, indicating that YFP-tagged pore mutants impair glycosylation and suggesting that certain structural changes by the substitution of the Asp542 residue interfere with glycosylation of TRPV6.

TRPV6 are known to form a tetrameric complex. Because glycosylated forms of Myc-tagged pore mutants of TRPV6 are intact (Fig. 2), they can act as dominant negative mutants by interacting with TRPV6 WT. To get experimental evidence concerning dominant negative effects of the pore mutants, we first generated cyan fluorescent protein (CFP)-TRPV6 WT. The Na+ current density of CFP-TRPV6 WT was 417 ± 13 pA/pF (n = 5) at −100 mV (Fig. 4A). When Myc-TRPV6 D542A or Myc-TRPV6 D542K was co-expressed with CFP-TRPV6 WT (Figs. 4B, C, F), minimal Na+ current density was observed which indicates that the fragmented channels act as dominant negative mutants by inhibiting the channel.
nel activity of TRPV6\textsuperscript{WT}. As expected, YFP-TRPV6\textsuperscript{D542A} and YFP-TRPV6\textsuperscript{D542K} also inhibited the activity of CFP-TRPV6\textsuperscript{WT} (Figs. 4D—F). These results demonstrated that the pore mutants of TRPV6 examined are electrophysiologically equivalent, but not biochemically.

This study demonstrates that peptide tags differentially affect the biochemical properties of TRPV6 in terms of its solubility according to various detergents or heat treatment and its glycosylation level. Our data also show that the substitution of the Asp\textsuperscript{542} residue results in loss of protein integrity or a defect in glycosylation depending on the peptide tag used. Our results suggest that the Asp\textsuperscript{542} residue of TRPV6 is crucial for forming an appropriate structure which is needed for glycosylation and protein stability, in addition to the crucial role of its cation permeation. In addition, we provide the basis for future investigation aiming at delineating the significance of the cytoplasmic N-terminal domain on luminal glycosylation processes.

In whole-cell patch clamp experiments, all TRPV6 pore mutants used were found to be electrophysiologically non-functional and to be capable of inhibiting the activity of TRPV6\textsuperscript{WT}. Therefore, those mutants may be used to remove the activity of endogenously expressed TRPV6, which is useful for the electrophysiological validation of TRPV6 expres-
sion. However, YFP-tagged pore mutants of TRPV6 were defective in glycosylation as well as cation permeation. Moreover, Myc-tagged pore mutants of TRPV6 were partially fragmented. Thus, our results indicate that the pore mutants are unsuitable for negative controls evaluating the roles of calcium permeation.

Because the availability of high quality antibodies to ion channels, particularly in TRP channels, is commercially limited, peptide tags are readily used to quantitate TRP channel research. In addition, peptide tag strategies have been commonly used to understand the biological roles and the molecular mechanisms underlying trafficking, subcellular localization, and subunit assembly of ion channels. However, our study indicates that the careful characterization of tagged channels is required to avoid potential pitfalls in addition to electrophysiological evaluation. Thus, our findings emphasize the need of experiments to verify biochemical properties of ion channels.

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