**Ca\(^{2+}\)**-selective Transient Receptor Potential V Channel Architecture and Function Require a Specific Ankyrin Repeat*

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Transient receptor potential (TRP) proteins form cation-conducting ion channels with currently 28 known genes encoding TRP channel monomers in mammals. These monomers are thought to coassemble to form homo- or heterotetrameric channels, but the signals governing their assembly are unknown. Within the TRPV subgroup, TRPV5 and TRPV6 show exclusive calcium selectivity and play an important role in calcium uptake. To identify signals that mediate assembly of functional TRPV6, we screened domains for self-association using co-immunoprecipitation, sucrose gradient centrifugation, bacterial two-hybrid assays, and patch clamp analysis. Of the two identified interaction domains within the N-terminal region, we showed that the first domain encompassing the third ankyrin repeat is the stringing and structural element for physical assembly of subunits and when transferred to an unrelated protein enables its interaction with TRPV6. Deletion of this repeat or mutation of critical residues within this repeat rendered nonfunctional channels that do not co-immunoprecipitate or form tetramers. Suppression of dominant-negative inhibitors of TRPV6-specific currents was achieved by deletion of ankyrin (ANK) 3. We propose that the third ANK repeat initiates a molecular zipper-process that proceeds past the fifth ANK repeat and creates an intracellular anchor that is necessary for functional subunit assembly.

Experimental Procedures

Site-directed Mutagenesis—All experiments were conducted using hTRPV6 (GenBank accession number NM_018946). To obtain the three TRPV6 deletion mutants, a MluI restriction site (ACGCGT) was introduced by PCR at nucleotide positions 333–335 resulting in replacement of serine at position 112 by an arginine. The mutation was

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The abbreviations used are: TRP, transient receptor potential; ANK, ankyrin; aa, amino acid(s); GFP, green fluorescent protein; EGFP, enhanced GFP; HEP, human embryonic kidney; HA, hemagglutinin; F, farada(s); wt, wild-type; PM, pore mutant.
introduced using a reverse PCR primer containing the downstream SacI site and the indicated base pairs and a PCR-forward primer that included the SacI site of the pCAGGS-IRESEGFP vector. The SacI-cuttmed fragment was ligated into SacI-cut pCAGGS-TRPV6 vector. Deletion constructs were made by PCR with different forward primers containing an MluI recognition site and a reverse primer covering a unique TRPV6 EcoRV restriction site. The MluI/EcoRV fragments were ligated into the MluI and EcoRV sites of TRPV6-pCAGGS-IRESEGFP and for precipitation experiments were recloned into TRPV6-EGFP-pCDNA3. The D542A and 0.38AR and to 0.18m mon244 mutatons were obtained by using the QuickChange XL kit (Stratagene). The template for this reaction was the EGFP-tagged full-length TRPV6. TRPV6 antisense construct was obtained by cloning the entire coding sequence for TRPV6 antisense orientation into the MluI and EcoRV sites of pCAGGS-IRESEGFP for TRPV6-wt or TRPV6-the TRPV6-EGFP fusion protein (52p2) but not the EGFP that is co-expressed from the pCAGGS-IRES-EGFP for TRPV6-wt or TRPV6-the TRPV6-EGFP fusion protein (52p2) but not the EGFP that is co-expressed from the pCAGGS-IRES-EGFP and for precipitation experiments were recloned into TRPV6-EGFP-pCDNA3. The D542A and 0.38AR and to 0.18m mon244 mutatons were obtained by using the QuickChange XL kit (Stratagene). The template for this reaction was the EGFP-tagged full-length TRPV6. TRPV6 antisense construct was obtained by cloning the entire coding sequence for TRPV6 antisense orientation into the MluI and EcoRV sites of pCAGGS-IRESEGFP for TRPV6-wt or TRPV6-the TRPV6-EGFP fusion protein (52p2) but not the EGFP that is co-expressed from the pCAGGS-IRES-EGFP and for precipitation experiments were recloned into TRPV6-EGFP-pCDNA3. The D542A and 0.38AR and to 0.18m mon244 mutatons were obtained by using the QuickChange XL kit (Stratagene). The template for this reaction was the EGFP-tagged full-length TRPV6. TRPV6 antisense construct was obtained by cloning the entire coding sequence for TRPV6 antisense orientation into the MluI and EcoRV sites of pCAGGS-IRESEGFP for TRPV6-wt or TRPV6-the TRPV6-EGFP fusion protein (52p2) but not the EGFP that is co-expressed from the pCAGGS-IRES-EGFP and for precipitation experiments were recloned into TRPV6-EGFP-pCDNA3. The D542A and 0.38AR and to 0.18m mon244 mutatons were obtained by using the QuickChange XL kit (Stratagene). The template for this reaction was the EGFP-tagged full-length TRPV6. TRPV6 antisense construct was obtained by cloning the entire coding sequence for TRPV6 antisense orientation into the MluI and EcoRV sites of pCAGGS-IRESEGFP for TRPV6-wt or TRPV6-the TRPV6-EGFP fusion protein (52p2) but not the EGFP that is co-expressed from the pCAGGS-IRES-EGFP and for precipitation experiments were recloned into TRPV6-EGFP-pCDNA3.
antibody and either a monoclonal anti-GFP antibody (Fig. 1B) or monoclonal anti-TRPV6 (26B3) as detection antibody. The very C-terminal 32 amino acid residues of TRPV6 (aa 694–725) that are critical for binding the regulatory calmodulin (16) thus do not contribute to TRPV6-TRPV6 interaction. No signal was detected if the EGFP-tagged construct was expressed in HEK 293 cells and antibody 429 was used as the precipitating antibody. D, probing with an anti-HA antibody, immunoprecipitated TRPC3 with an anti-HA antibody that are critical for binding the regulatory calmodulin (16) thus do not contribute to TRPV6-TRPV6 interaction. No signal was detected if the EGFP-tagged construct was expressed in HEK 293 cells and antibody 429 was used as the precipitating antibody (Fig. 1B, right lane).

As a separate control for the specificity of the co-immunoprecipitation experiments, we used a cell line that stably expresses HA-tagged TRPC3 protein and transiently expresses EGFP-tagged full-length TRPV6. As expected, the anti-HA antibody could precipitate TRPC3, but it did not co-immunoprecipitate the EGFP-tagged full-length TRPV6 construct (Fig. 1, C and D). Conversely the polyclonal antibody 429 did not co-immunoprecipitate hTRPC3 (Fig. 1D).

Using the monoclonal anti-GFP antibody as precipitating antibody, we could also co-immunoprecipitate TRPV6 from the stable cell line transfected with interacting EGFP-tagged constructs (data not shown). However, because the monoclonal antibody was not as efficient in immunoprecipitating, we screened for interacting domains using antibody 429 as the precipitating antibody.

To narrow down the protein domain within TRPV6 that is responsible for TRPV6-TRPV6 interaction, we repeated the experiment described above after transient transfection and expression of a series of EGFP-tagged fusion proteins encompassing different protein domains of TRPV6. First we focused on the intracellular C-terminal (aa 584–694) and the N-terminal regions (aa 1–328) that contains six rather than three ANK repeats (17). Aligning the N-terminal TRPV6 sequence with the N-terminal region (aa 1–328) but neither with the C-terminal region (aa 584–694) nor with the intracellular loop between transmembrane domains 4 and 5 (aa 466–493) (Fig. 3A). We therefore subdivided the N-terminal domain into shorter fragments and narrowed down one interaction domain to the region around amino acids 116–163 that contains the third ANK repeat. The EGFP-tagged fragment covering ANK 1 and ANK 2 (aa 1–115) was not immunoprecipitated, but the fragment covering in addition ANK 3 and part of the adjacent sequence (aa 1–163) showed positive interaction (Fig. 3A). Controls for a comparable amount of expressed construct included monitoring the trichloroacetic acid-precipitated input (Fig. 3A, bottom) and the immunoprecipitant-supernatant of the expressed fusion

![Fig. 1](image1.png)

**Fig. 1.** Specificity of co-immunoprecipitation experiments. A, the anti-TRPV6 antibody 429 precipitates glycosylated and non-glycosylated wt-TRPV6 from a stable cell line (TRPV6s) but not from parental HEK cells. B, after transient expression of an EGFP-tagged TRPV6 lacking the C-terminal 429 epitope (TRPV6-Δ-EGFP) in both cell lines, the EGFP-tagged protein can be co-immunoprecipitated with wt-TRPV6 only in the stable cell line. C, after transient expression of TRPV6-EGFP in a stable HA-tagged TRPC3 cell line, anti-TRPV6 antibody (429) can immunoprecipitate full-length TRPV6-EGFP; however, immunoprecipitating TRPC3 with an anti-HA antibody does not co-immunoprecipitate TRPV6-EGFP as detected with a monoclonal anti-GFP antibody. D, probing with an anti-HA antibody, immunoprecipitated TRPC3 is detected (IPHA, arrows); however, no TRPC3 is co-immunoprecipitated with TRPV6. The asterisk (in B and D) indicates a cross-reacting band due to unspecific binding of the horseradish peroxidase-coupled goat-anti mouse antibodies (see also B, 293 cells and antibody 429 was used as the precipitating anti-detect antibody. The very C-terminal 32 amino acid residues of TRPV6 (aa 694–725) that are critical for binding the regulatory calmodulin (16) thus do not contribute to TRPV6-TRPV6 interaction. No signal was detected if the EGFP-tagged construct was expressed in HEK 293 cells and antibody 429 was used as the precipitating antibody (Fig. 1B, right lane).

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protein and the amount of precipitated TRPV6 wild-type protein (by stripping and reprobing blots with antibody 26B3). Due to weak expression of construct 192–328 (ANK 5 + 6), see Fig. 3A, **, we could not exclude a further binding site downstream of amino acid 163. Construct 158–328 (ANK 4–6) also showed a positive interaction albeit weaker than construct 1–191, indicating that a second interaction site exists downstream of the ANK 3 domain (Fig. 3A). A short construct containing ANK domains 3 and 4 (aa 116–191) that ought to contain the first N-terminal interaction site co-immunoprecipitated only very weakly (Fig. 3B). ANK 3 alone showed an even weaker interaction, and no interaction was seen for ANK 4 alone (Fig. 3B).

As all constructs contain a C-terminal EGFP tag, we used fluorescence microscopy to monitor their expression and localization. Interestingly all three constructs shown in Fig. 3B were strongly expressed; however, ANK 3 + 4 (aa 116–191) and ANK 3 alone (aa 116–163) were targeted to mitochondria. Fig. 3C shows the fusion protein EGFP fluorescence of living transfected cells in the left panel and Texas Red fluorescently labeled mitochondria of the same cells in the right panel. The finding that most of the recombinant fusion proteins covering ANK 3 and ANK 3 + 4 reside primarily within the mitochondria might explain why only minor amounts are available close to TRPV6-containing membranes for interaction with full-length TRPV6. In addition, this finding may indicate that the interactions observed after immunoprecipitation are not due to an artificial association of overexpressed proteins or protein fragments during solubilization of cells.

Analysis of 116–191—Is the TRPV6 region 116–191 including ANK 3 and 4 alone sufficient for subunit interaction, and can the interaction with full-length TRPV6 be restored by forcing the interaction domain construct out of the mitochondria? To answer these questions, a foreign protein, the four-transmembrane γ1 subunit of voltage-gated calcium channels was inserted between the ANK 3 + 4 (aa 116–191) fragment of TRPV6 and the EGFP tag (Fig. 4). γ1-EGFP alone, when expressed in TRPV6-expressing cells, localized to the plasma membrane (data not shown) but could not be co-immunoprecipitated with TRPV6 (Fig. 4A). However, fusing the ANK 3 + 4 domain (aa 116–191) to the N terminus of γ1 indeed was sufficient to both restore plasma membrane localization of 116–191 and, more importantly, to enable an interaction between the γ subunit and TRPV6 channels as seen by co-immunoprecipitation of TRPV6 and ANK 3 + 4-γ1-EGFP (Fig. 4B). The ANK 3 + 4 region thus can act as a strong association signal when localized to TRPV6-expressing membranes.

We also investigated multimerization of EGFP alone and ANK 3 + 4-EGFP. Solubilized transfected cells with an EGFP expression vector both under weakly denaturing conditions (−: 4% SDS, 37 °C) and under denaturing conditions (+: 4% SDS, 10 mM dithiothreitol, 1.43 mM β-mercaptoethanol, 95 °C) yielded in either case a monomeric EGFP protein in the standard SDS-PAGE Western analysis (Fig. 4C). However, adding the ANK 3 + 4 region resulted in formation of higher molecular weight ANK 3 + 4-EGFP variants with at least two bands running at the apparent molecular weights of the dimer and trimer (Fig. 4C). Those variants were visible under − solubilization conditions but were much weaker under + conditions. We also investigated whether ANK 1 + 2-EGFP and ANK 5 + 6-EGFP are able to form multimers under these conditions. In contrast to ANK 3 + 4-EGFP, we could not readily detect bands at molecular weights of the dimer and trimer (data not shown).

If 116–191 contains the dominant interaction domain, deletion of this domain from full-length TRPV6 channels should abolish its biochemical interaction. To remove this domain we
created a novel restriction site just upstream of the sequence encoding ANK 3 by replacing a serine residue at position 112 of the TRPV6 protein with an arginine residue (see “Experimental Procedures”). This S112R mutant behaved like wild-type TRPV6 in biochemical and functional assays (see below). We used the following three TRPV6 deletion constructs in the co-immunoprecipitation experiments as described above. We compared with the TRPV6-Δ-EGFP construct (compare Fig. 1), the ΔANK 3 lacks amino acid residues 116–140 of TRPV6. The ΔANK 3 + and the ΔANK 3 + 4 constructs lack amino acid residues 116–165 and 113–191, respectively. With all three deletion constructs, we found that with an equivalent or even larger input of transfected EGFP-tagged protein compared with wild type interaction was virtually abolished (Fig. 5A). The signals that could be detected with long exposure of the films (faint bands seen in Fig. 5A) are due to nonspecific binding of minor amounts of protein to the protein A-Sepharose beads; similar signals could be detected after preincubation of cell lysates with protein A-Sepharose beads in the absence of precipitating antibody (Fig. 5B, lane 2). This nonspecific binding was not detected with the constructs comprising cytosolic portions of TRPV6 in the experiments described previously and is apparently due to the increase in hydrophobicity by including the six transmembrane segments.

The findings that deletion of ANK 3 is sufficient to abolish physical interaction were confirmed by functional studies. Whereas the EGFP-tagged wild-type TRPV6 (TRPV6-Δ-EGFP) showed currents with characteristics similar to untagged TRPV6 (11), proving that the tag itself does not disturb subunit assembly, neither deletion construct was able to form functional channels (ΔANK 3: 0 of 4 cells showed TRPV6-specific currents; data not shown). Fluorescence microscopy of these EGFP-tagged constructs did not reveal differences in localization between wild-type and deletion constructs (see also Fig. 7E). Deletion of the entire N-terminal region (Δ1–330) or all ANK repeats (Δ1–254) also yielded non-functional channels (data not shown).

Summarizing these results, two important conclusions can be drawn. 1) If ANK 3 is missing, TRPV6 cannot associate with itself; ANK 3 therefore represents the minimal structural requirement for TRPV6-TRPV6 association. 2) Any other association site necessary for assembly is by itself either not strong enough or masked by a change in tertiary structure due to deletion of ANK 3.

Are the core amino acids of ANK 3 thus the critical and specific amino acids necessary for subunit interaction, or are other amino acids involved that may not interact due to the steric reasons mentioned above? We attempted to address this concern by reinserting amino acids into the deletion construct that is derived from S112R-TRPV6. Due to our cloning strategy, the sequence 111TRELY140 is repeated after the reinserted amino acids. Therefore, we first reinserted 20 amino acids into the ANK 3 region (M5, Fig. 6A). M5 was unable to form tetramers as detected by immunoblot analyses of fractions after sucrose gradient centrifugation. The fractions with peak intensities of marker protein (ferritin, 440 kDa; catalase, 240 kDa; β-galactosidase, 116 kDa) are indicated above. IP, immunoprecipitation; WB, Western blot.

Fig. 5. Deletion of the ANK 3 + 4 region abolishes interaction. A, co-immunoprecipitation of TRPV6-Δ-EGFP (here denoted as Wt but lacking the 429 epitope) and TRPV6-Δ-EGFP with the indicated (Δ) additional deletions, respectively, with TRPV6 from the stable cell line as detected by the anti-GFP antibody. B, TRPV6 proteins containing the hydrophobic transmembrane regions can show a small amount of nonspecific binding to protein A-Sepharose beads (lane 2) when compared with the co-immunoprecipitation signal (lane 1). The asterisk indicates the cross-reacting band (see Fig. 1). Ab, antibody; WB, Western blot; IP, immunoprecipitation.

Fig. 6. Functional assembly of TRPV6 requires specific amino acid residues within ANK 3. A, schematic representation of sequences and modifications within ANK 3. The S112R mutation introduces a MuI site used in deletion constructs. AR/EA, point mutant within tagged wild type; M5, reinsertion of ANK sequence into 25-amino acid deletion resulting in replacement of LLARR with TRELr; +5p2, reinsertion of 52 amino acids into the ANK 3 deletion resulting in an additional five amino acids downstream of ANK 4; rb, rabbit; m, mouse; h, human; ND, not determined. B, a five-amino acid point mutant within ANK 3 (M5) does not interact with TRPV6, and insertion of additional amino acids alters interaction depending on their relative position. C, immunoprecipitation signals were detected after transfection into TRPV6 cells, immunoprecipitation with antibody 429, and detection with anti-GFP with similar blot exposure time. C, M5 abolishes TRPV6-specific calcium currents that can be recorded from TRPV6 (S112R) after transient transfection in HEK cells. D, avidin-agarose pull-down reveals the presence of TRPV6 (~83 kDa), ANK 3 + 4-TRPV6 (~75 kDa), and +5p2-TRPV6-EGFP (~110 kDa) in the fraction of cell surface biotinylated proteins. Cytosolic EGFP is not biotinylated (not shown). E, M5 is unable to form tetramers as detected by immunoblot analyses of fractions after sucrose gradient centrifugation.
M5, \(-3.3 \pm 0.9 \text{ pA/pF}, n = 5\), which is equivalent to untransfected HEK cells; current densities at \(-80 \text{ mV ramp potential}\). To check whether the inability of M5 to form functional channels is due to alteration of a tetramerization domain, we investigated multimer formation by sucrose density gradient centrifugation. TRPV6-EGFP could be detected as mature glycosylated protein in fractions corresponding to the densities of the trimeric and tetrameric complexes with very little protein in fractions containing the monomeric form (Fig. 6E). Detection of M5, in contrast, was shifted to fractions containing the monomer. Interestingly the secondary structure prediction for the protein was not altered by exchange of the five amino acids in M5 (bioinf.ca.ucl.ac.uk/psipred, Ref. 29). We also created a 138\text{AR} \rightarrow 138\text{EA} point mutant to exchange two non-conserved residues within ANK 3 that due to their position may confer specificity (see Fig. 2A). This two-amino acid exchange, however, did not alter the ability to form functional channels (\(-108 \text{ pA/pF at } -80 \text{ mV}, n = 2\)) and should not affect subunit interaction. It is furthermore unlikely that arginine 140 (Fig. 6A) is critical in the hTRPV6 sequence as murine TRPV6 contains a glycine at this position and was able to co-immunoprecipitate together with human TRPV6 (data not shown). We therefore believe it is the alteration of the two highly conserved leucines at positions 136 and 137 that destroys the ability of the ANK 3 region to interact. Moreover insertion of the TRELY motif after LLARR, resulting in a final protein with five additional amino acids (Fig. 6A, +5), also yielded non-functional channels (\(-3.3 \pm 1 \text{ pA/pF}, n = 4\)) that showed only a very weak interaction with wt-TRPV6 (Fig. 6B). However, insertion of five additional amino acids after ANK 4 (+5p2) restored a stronger interaction with wt-TRPV6 (Fig. 6B), confirming that the sequence and tertiary structure of the ANK 3 and linker region is critical for interaction. Although the +5p2 constructs were able to interact, they alone were also unable to form functional channels. This could be due to a failure of the second interaction site around ANK 5 to continue a molecular zipperpin process that is required for strongest physical interaction necessary to yield functional channels. Using cell surface biotinylation experiments (Fig. 6D) and confocal imaging (not shown), we could not detect qualitative differences in the localization of +5p2 or the \(\Delta\text{ANK 3 + 4}\) construct when compared with wild type, suggesting that the inability to form functional channels is not based on misrouting of these constructs.

**Functional Analysis: Suppressor of Dominant-negative TRPV6 Constructs**—If deletion of the interacting domain indeed prevents functional assembly, then expression of N-terminal domains containing the interaction domain but no functional ion pore should function as a dominant-negative construct when expressed in TRPV6-containing cells. Recording from the stable TRPV6-expressing cell line, we could detect inward calcium currents that were not as large as those after transient TRPV6 expression but showed similar characteristics regarding rectification, reversal potential, and selectivity (Fig. 7A). On average, peak current density was \(-61.5 \pm 12 \text{ pA/pF at } -80 \text{ mV ramp potential}\) (Fig. 7D). These currents were TRPV6-specific and could be significantly reduced by transient expression of a TRPV6 antisense construct (\(-20.5 \pm 10 \text{ pA/pF}\) but not by transient transfection of the vector coding for EGFP alone (\(-71.6 \pm 11 \text{ pA/pF}\)) (Fig. 7D). Expressing the ANK 3-6-EGFP construct also reduced current densities by \(-60\%\) to \(-23.4 \pm 10 \text{ pA/pF}\) (Fig. 7D) 48 h after transfection, confirming the dominant-negative properties of the interaction domain.

Furthermore it has been shown that mutating a critical aspartate within the pore of rabbit TRPV5 or mouse TRPV6 channels abolishes calcium permeation (21, 22). Interestingly inserting the same mutation (D542A) in the human TRPV6 sequence abolished ion permeation completely, and we used this EGFP-tagged pore mutant construct as an independent dominant-negative construct. Indeed transient expression of the full-length, EGFP-tagged TRPV6 pore mutant (PM) reduced the peak current densities of the stable cell line to \(-17 \pm 3.8 \text{ pA/pF}\) (Fig. 7, B and D). We reasoned that if we deleted the interaction domain within the pore mutant construct, we should be able to suppress its dominant-negative effect. Deletion of the 79-aa region (113-191) encompassing the interacting domain from this construct (\(\Delta\text{3 + 4-PM}\) indeed restored current densities to those of control cells (\(-60.7 \pm 17 \text{ pA/pF}\); Fig. 7, C and D), thus confirming that the interaction domain indeed mediates the dominant-negative effect. To check localization of the latter two constructs we imaged EGFP fusion protein fluorescence in transfected HEK 293 cells and detected signals in the plasma membrane region (Fig. 7E, arrows). Because much of the fluorescence signal was detected in Golgi and endoplasmic reticulum structures, we cannot rule out that the peripheral fluorescence arises from structures just below the plasma membrane. However, cell surface biotinylation experiments (Fig. 6D) showed that both wild-type and \(\Delta\text{ANK 3 + 4-TRPV6}\) protein traffic to some degree to the cell surface.

**Interaction Studies Using Bacterial Two-hybrid Screens**—To verify the interactions seen in the co-immunoprecipitation experiments with full-length TRPV6 as one partner and to specify whether interactions occur between identical or different N-terminal sequences, we used analogous constructs in a bacterial two-hybrid approach. Here protein interactions between the bait protein, which is coupled to the \(\alpha\)-cl repressor protein, and the target protein coupled to the \(\alpha\)-subunit of RNA polymerase allow expression of reporter genes that enable colonies to grow on carbenicillin-agar and express \(\beta\)-galactosidase. Table I summarizes the results obtained with this method. We con-
Subunit Assembly of TRPV6 Channels

Table I

Defining interaction with bacterial two-hybrid screens

pBTL and pTRG are the bait and target vectors containing the indicated N-terminal (NT) or C-terminal (CT) TRPV6 fragments. Interactions were defined as − if < 100 colonies, + if > 100 colonies, ++ if > 300 colonies, or +++ if > 1000 colonies grew on reporter gene selection plates (see "Experimental Procedures"). pBT-LGF2 and pTRG-Gal11 are positive controls.

| pBTL aa | ANK | pTRG Interaction |
|---------|-----|-----------------|
| hTRPV6 NT 1–329 | 1–6 | hTRPV6 NT 1–328 |
| 1–191 | 1–4 | 116–191 3 + 4 |
| | | 116–191 |
| | | 116–163 |
| | | 158–191 |
| | | 192–328 5 + 6 |
| 116–191 | 3 + 4 | 116–191 3 + 4 |
| | | 116–163 3 |
| | | 158–191 4 |
| | | 192–328 5 + 6 |
| | | 192–230 5 ++ |
| | | 231–328 6 |
| | | 280–238 |
| | | 192–329 5 + 6 |
| hTRPV6 CT 582–725 | hTRPV6 CT 582–725 |
| pBTL All TRPV6 pBTL constructs |
| pBT-LGF pTRG pTRG constructs |
| pBTL-LGF2 pTRG-Gal11P |

firmed the N-terminal region within amino acid residues 116–191 (ANK 3 and ANK 4) as one interacting domain; in addition we can specify this particular region as a self-interacting and possible multimerization domain (see Fig. 4C). Using this system, further subdivision of this region showed that ANK 3 alone could interact with ANK 3 + 4. In addition to the region around ANK 3, we also detected a strong self-interaction of 192–230 (ANK 5) with itself and also a heterotypic interaction between ANK 5 and ANK 3 (see Table I). Positive interactions were independent of the Bacteriomatch vector used. No transactivation between the different TRPV6-containing constructs shown in Table I and pTRG or pBTL vectors alone was seen. The construct containing only ANK 1 + 2 showed transactivation with pTRG vector and was not further examined. We also did not detect an interaction between C-terminal constructs, confirming the result from the co-immunoprecipitation experiment.

We also positively retested the interaction of 116–191 with itself in a novel Bacteriomatch reporter strain that grows on minimal medium (His drop-out) with too little divalent ions for detecting ion-dependent interactions, suggesting that, in contrast to the Zn2+ dependence of the potassium channel Shal and Shaw T1 tetramerization domain (23), multimerization via ANK 3 is independent of divalent cations.

**DISCUSSION**

In this study we defined two N-terminal interaction domains of TRPV6 channels and analyzed their role in subunit assembly. Our results demonstrated that one specific N-terminal ANK repeat is critical in allowing tetrameric subunit assembly of functional TRPV6 channels. Within the 328-amino acid-containing N-terminal region, 25 core amino acid residues (116–140) of the third ANK repeat are critical for initiating subunit assembly; deletion of these amino acid residues prevented TRPV6-TRPV6 association and generation of functional channels. Even small variations to the sequence of ANK 3 by either inserting five additional amino acid residues or by replacing 136LLARR140 with 136TREG140 did not rescue biochemical and functional interaction between subunits (Fig. 6A). All modified full-length constructs showed EGFP fluorescence, appeared to localize to some degree to the plasma membrane, and as shown for the largest deletion as well as the +5p2 construct could be pulled down by non-permeabilized cell surface biotinylation experiments, suggesting that the proteins are synthesized and targeted correctly. Correct targeting should be expected as functional expression and targeting of TRPV5 and TRPV6 apparently require an interaction between a C-terminal sequence and the S100A10-annexin 2 complex (24), a site that is still present in all deletion and mutant constructs. Deleting the interaction domain from single TRPV6 subunits did not alter the degree of cell surface expression; tetramer formation thus does not appear to be essential for trafficking to the cell surface.

![Fig. 8. Zippering model of TRPV6 subunit assembly with ANK 3 as the anchor and ANK 5 as the stabilizing region. Intracellular C-terminal regions have been left out for clarity.](https://example.com/fig8)

**Experimental Procedures**

pBT-LGF2 and pTRG-Gal11 are positive controls.
Moreover the ANK 3 + 4 domain, when added to the γ1 subunit of voltage-gated calcium channels, conferred TRPV6 binding in intact cells. In contrast to the tetramerization domain T1 from voltage-gated potassium channels where T1 from Kv1.3 and Kv1.4 as well as from Shaker is not essential for channel formation (25–29), we could not detect TRPV6-specific calcium currents after deletion of 79, 50, or 25 amino acids encompassing the interaction domain or after deletion of the entire N terminus (Δ1–330) or the region containing five ANK repeats and the partial sequence of ANK domain 6 (Δ1–254).

As shown by protein interaction studies using a bacterial two-hybrid approach (Table 1), the 50 amino acid residues containing ANK 3 and the adjacent linker region are sufficient for a homotypic interaction, a finding that is supported by enabling multimerization of EGFP after addition of the ANK 3 + 4 region. Our results using the bacterial two-hybrid system furthermore argue for a direct interaction that is not dependent on another protein expressed in eucaryotic cells. In the two-hybrid screen we also detected a homotypic interaction between ANK 5 + 6 and ANK 5, but not ANK 6, as well as a heterotypic interaction between ANK 5 + 6 and ANK 3 but not between ANK 5 + 6 and ANK 4. In bacteria, the full-length N-terminal constructs did not interact with each other (see Table I), which may be because ANK 5 folds back upon ANK 3, thus preventing ANK 3-ANK 3 interaction. Obviously, in eucaryotic cells, protein folding is more complex, and the full-length N-terminal construct could be co-immunoprecipitated with TRPV6. A second interaction site downstream of ANK 3 (ANK 4–6 (aa 158–328)) could also be detected by co-immunoprecipitation of short recombinant proteins with the TRPV6 full-length channel (Fig. 3A). However, because deletion of ANK 3 or a five-amino acid exchange within ANK 3 caused loss of both self-association and TRPV6 ionic currents, the second N-terminal interaction site encompassing the putative fifth ANK repeat must be a subordinate site in vivo. Our data give rise to a model where subunit assembly may resemble a zipperpering process where ANK 3-ANK 3 (or linker region) interaction is the stringent requirement before any other interaction. Both interaction domains (ANK 3 and ANK 5) and possibly hydrophobic interactions within the transmembrane domains may then be needed for formation of the most stable functional complex (Fig. 8), a theory that is supported by the partial biochemical but not functional rescue of the interaction in the +5p2 construct. In contrast to the tetramerization via T1 seen in voltage-gated potassium channels where Zn$^{2+}$ ions are an important cofactor (23), tetramerization of TRPV6 via ANK 3 appears not to require a divalent ion. Not much is known about a role for ankyrin repeats in multimerization of proteins; so far only for the chloroplast protein SRP43 it has been shown that its third and fourth ankyrin repeats are involved in dimerization of the protein (30). It will be interesting to investigate whether ANK repeats of other TRP family members govern their subunit assembly.

Our studies suggest that it is the tertiary structure of the third ANK repeat and its downstream linker region, the specific sequence, and most likely the spacing of the first interaction domain in relation to the second interaction site as well as to the first transmembrane domain and pore region that govern functional tetrameric assembly of TRPV6 (Fig. 8). This could explain how a rather common motif like the ANK repeats can nevertheless confer specificity upon interaction between proteins. TRPV6 and TRPV5 are identical in their sequence of the second helical segment of ANK 3 as well as in the distance between ANK 3 and S1; functional heteromeric channels containing both TRPV5 and TRPV6 subunits as shown by Hoenderop et al. (5) are consistent with our results.

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