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| Citation | Jin, Xiangshu, Jason Touhey, and Rachelle Gaudet. 2006. “Structure of the N-Terminal Ankyrin Repeat Domain of the TRPV2 Ion Channel.” Journal of Biological Chemistry 281 (35): 25006–10. https://doi.org/10.1074/jbc.c600153200. |
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Structure of the N-terminal Ankyrin Repeat Domain of the TRPV2 Ion Channel

The transient receptor potential (TRP) channels are a large family of cation channels that are important in processes ranging from sensory signaling to magnesium homeostasis (1). TRP channels, like many voltage- and ligand-gated channels, have six membrane-spanning segments and are thought to function as tetramers. TRP channels also have large N- and C-terminal cytosolic regions containing putative protein interaction and regulatory motifs. The TRP channel family is divided into six subfamilies based on sequence identity and function, including the TRPV subfamily (named after the vanilloid receptor, TRPV1); there is little homology in the N- and C-terminal cytosolic regions between subfamilies.

The six mammalian TRPV channels are important in sensory and pain perception and in calcium homeostasis. At the molecular level, TRPV1 and TRPV2 are both activated at noxious temperatures (>43 °C and >52 °C, respectively) (2, 3). TRPV1 is also gated by exogenous ligands such as capsaicin and resiniferatoxin (reviewed in Ref. 4). TRPV3 is activated by warm temperatures (>32–39 °C) and camphor (5–7), whereas TRPV4 senses osmotic stimuli, heat (>27 °C), and phorbol esters (8–10). The more distant TRPV homologs TRPV5 and TRPV6 are regulated by intracellular Ca<sup>2+</sup> levels and mediate trans-epithelial Ca<sup>2+</sup> transport in the kidney and intestine (11).

The N-terminal region of TRPV channels contains ankyrin repeats, which are likely to be essential for channel function as their deletion in TRPV1 impairs activation (12). Furthermore, the TRPV5 and TRPV6 ankyrin repeats were implicated in the tetrameric assembly of the channels (13, 14). Ankyrin repeats are 33-residue sequence motifs often involved in protein-protein interactions and present in many eukaryotic and prokaryotic proteins with functions that include signaling, cytoskeleton integrity, transcription, and cellular localization (15, 16).

Determining the three-dimensional structures of TRPV channel domains is an important step in understanding the molecular basis of gating and regulatory mechanisms. We report the structure of N-terminal cytosolic ankyrin repeat domain (ARD) of the TRPV2 ion channel. The TRPV2-ARD is monomeric in solution and its three-dimensional structure consists of six ankyrin repeat structural motifs, of which only four could be identified by sequence motif searches. Comparison with other ankyrin repeat structures highlights several unusual structural features of the TRPV2-ARD. The structure provides a context for data on the functional roles of the N-terminal regions in TRPV ion channel physiology.

EXPERIMENTAL PROCEDURES

Cloning and Protein Expression—The TRPV2-ARD coding sequence (two constructs, residues 62–326 and 75–321) was amplified from the rat TRPV2 cDNA (provided by Michael Caterina) and inserted into the Ndel and NotI sites of pET21-C6H. pET21-C6H was generated by ligating a short double-stranded oligonucleotide (5′-TCGACACTAGTGACGTCGC-GGCCGCTCATCATCACCATCATGATA-3′ and 5′-GGGCTTCAATGATGATGTGATGATGAGCGCCGCGAC-GTCACTAGTG-3′) in the Sall and NotI sites of pET21a (Novagen). The resulting coding sequences include a C-terminal AAAHHHHHHH-tag. The proteins were expressed in Escherichia coli BL21(DE3) in LB medium and induced at A<sub>600</sub> = 0.5 with 75 μM isopropyl β-D-thiogalactopyranoside at room temperature for 8–16 h. Selenomethionine (Se-Met)-substituted protein was expressed with feedback inhibition of methionine synthesis in BL21(DE3) grown in fully supplemented M9 minimal medium with Se-Met.

Purification of TRPV2-ARD—Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM...
imidazole, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, and 0.1% Triton X-100) with 0.2 mg/ml lysozyme and lysed by sonication. The cleared lysate was loaded onto a nickel-nitrilotriacetic acid column (Qiagen), washed, and eluted using a step gradient (60, 100, and 250 mM imidazole). Fractions containing TRPV2-ARD were pooled, concentrated, and loaded on a Superdex 200 16/60 column (GE Healthcare) in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. Fractions containing TRPV2-ARD were pooled, diluted to 75 mM NaCl, loaded on a Resource-Q column (GE Healthcare), and eluted using a linear NaCl gradient. Pure fractions of TRPV2-ARD (75–321) were pooled and dialyzed against 20 mM HEPES, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM DTT, and concentrated to 5 mg/ml. TRPV2-ARD (62–326) dialyzed against 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5% glycerol, and 1 mM DTT and concentrated to ~10 mg/ml. Typical yield was 5–10 mg/liter of culture. Protein samples were judged adequate if ~95% pure on Commassie-stained gels. Near 100% Se-Met incorporation was confirmed by mass spectroscopy.

Size Exclusion Chromatography and Light Scattering—Size exclusion analyses were performed using a Superdex 200 10/30 column (GE Healthcare), loading 100–500 µl of protein at concentrations ranging from 100 to 350 mM in the corresponding storage buffer. Size exclusion chromatography-multi-angle laser light scattering measurements were performed on the Superdex 200 10/30 column coupled to DAWN multi-angle light scattering and Optilab differential refractive index detectors (Wyatt Technologies). Data acquisition and analysis were performed using Astra 4.0 software (Wyatt Technologies).

Crystallization of TRPV2-ARD—Crystals were grown by hanging drop vapor diffusion with a 1:1 ratio of protein and reservoir solution in each drop. Crystal form I (native and Se-Met-substituted) grew at room temperature from 1.2 M lithium acetate, 0.1 M MES, pH 6.5. Crystal form II grew at 4 °C from 1.2 M lithium acetate, 0.1 M MES, pH 6.0. Crystal form III (62–326) grew at 4 °C from 9% PEG-8000, 0.1 M sodium citrate, pH 5.0, 5–7% xylitol. All crystals were flash-frozen after cryo-protection with 20% glycerol in the corresponding reservoir solution.

Data Collection and Structure Determination—X-ray diffraction data were collected at 100 K. Native and Se-Met derivative data of TRPV2-ARD crystal form I were collected at the Advanced Photon Source Structural Biology Center ID-19 beamline with the SBC CCD2 detector and data on TRPV2-ARD crystal form III were collected at the Advanced Photon Source BM8 beamline using an ADSC Q315 CCD detector. Both were processed with HKL2000 (17). Data on TRPV2-ARD crystal form II were collected on a MicroMax007 x-ray generator equipped with an R-AXIS IV++ detector and processed with CrystalClear (Rigaku/MSC Inc.). The TRPV2-ARD structure (crystal form I; 2.2 Å resolution) was determined using FIGURE 1. The TRPV2-ARD structure. A, primary structure of TRPV2. Ankyrin repeats are shown in labeled gray boxes; starred repeats are not recognized by sequence motif searches. B, left: Coomassie-stained gel of ~2.5 µg of each of the three final protein samples used in crystallization, constructs 62-326, 75-321 (Se-Met substituted), and 75-321. Right: representative size exclusion chromatography trace of construct 75–321 on a Superdex 200 10/30 column.

Arrows indicate elution volumes of molecular mass standards (molecular mass in kDa). C, ribbon diagram of TRPV2-ARD showing each ankyrin repeat in a distinct color. D, superposition of TRPV2-ARD structures. For clarity, only one chain from each crystal form is shown. Regions showing the most variability are colored green, gold, and blue for crystal forms I, II, and III, respectively. E, a large number of aromatic residues (shown in purple) are concentrated on the solvent-exposed surface of fingers 2 and 3.
Se-Met single-wavelength anomalous diffraction phasing with SOLVE (18) and DM (19). The TRPV2-ARD crystal form II (to 1.6 Å) and III (to 3.1 Å) structures were determined by molecular replacement with the TRPV2-ARD crystal form I structure as a search model using MOLREP (20). Structures were refined with TLS in REFMAC (21). Data, phasing, and refinement statistics are provided in supplemental Table 1.

RESULTS

Domain Boundary Determination—We identified the ARD boundaries of TRPV2 using sequence alignments, a sequence motif search algorithm (SMART (22)) and secondary structure predictions (NPS@ (23)). Additional pairs of α-helices are predicted to precede and link the ankyrin repeat sequence motifs. We therefore postulated that the ARD extended further toward the N terminus, from residues 75 to 321 (Fig. 1A). A similar prediction was recently published for TRPV6 (14). This N-terminal boundary coincides with the beginning of the high sequence identity region within the TRPV subfamily. Hence, a minal boundary coincides with the beginning of the high sequence identity region within the TRPV subfamily. Hence, a

Solution Characterization of Oligomeric State—TRPV proteins are tetrameric ion channels (24, 25), and the ankyrin repeats of TRPV5 and TRPV6 have been shown to be important for channel tetramerization (13, 14). We therefore investigated the oligomeric state of the isolated TRPV2-ARD. Both size exclusion chromatography and multi-angle laser light scattering (26) indicate that the TRPV2-ARD is monodisperse and monomeric in solution at protein concentrations up to 350 μM (size exclusion chromatography apparent molecular mass of 29 kDa and light scattering molecular mass of 27.3 kDa, versus calculated molecular mass = 28,628 Da for construct 75–321; Fig. 1B and data not shown).

Overall Structure of TRPV2-ARD—We determined the structure of the TRPV2-ARD in three crystal forms (supplemental Table 1), and except where noted, the 1.6-Å TRPV2-ARD structure from crystal form II was used for analyses (Fig. 1C). The structure comprises six repeating structural repeats consisting of a pair of anti-parallel α-helices with an intervening “finger” loop. All six repeats therefore resemble the ankyrin repeat structural motif, with two short anti-parallel helices followed by a finger loop projecting outward from the helical axes at a ~90° angle, resulting in a characteristic L-shaped cross-section. The helical hairpins stack together such that the inner helices and fingers form a concave surface. This assembled structure has been likened to a cupped hand: the loops form the fingers, hence the name, and the exposed surface of the inner helices forms the palm. This concave palm surface is often a site of protein-protein interactions in other ankyrin repeat proteins (15, 16).

Conformational Differences of TRPV2-ARD in Three Different Crystal Forms—Within the three crystal forms of the TRPV2-ARD, five independent observations of the structure are available for comparison. The overall structures are similar, with pairwise root mean squared deviation values ranging from 0.31 to 2.0 Å for all Ca atoms, although several local conformational differences were observed. The most different conformations are superimposed in Fig. 1D, displaying marked differences within the long fingers 1–3 and the N terminus of outer helix 5, indicating additional flexibility afforded by this unusually long helix. The flexibility suggested by this conformational variability supports the idea that these three fingers may play important roles in interacting with other proteins. A number of bulky aromatic residues, Phe208, Tyr209, and Phe210, are located on the concave face of fingers 2 and 3 (Fig. 1E). This extended patch of solvent-exposed aromatic residues forms a potential hydrophobic interacting surface.

Comparison with Other Ankyrin Repeat Structures—A comparison of the TRPV2-ARD structure with canonical ankyrin repeat sequences reveals that, although several consensus ankyrin repeat residues are highly conserved in the six repeats (Fig. 2A), the TRPV2-ARD has several unusual structural features. In a typical ankyrin repeat structure, such as that of the 12 C-terminal repeats of ankyrin, the repeats stack very regularly, with only a slight counterclockwise twist of ~2–3° along the stacking axis (27). In contrast, the overall structure of the TRPV2-ARD can be seen as two adjacent stacks of repeats. Repeats 1–4 stack with an average twist of ~2°, similar to ankyrin, but a large twist of ~21° between repeats 4 and 5 leads to a distortion of the concave palm-finger surface often used for protein-protein interactions by ankyrin repeat-containing proteins (Fig. 2B). The length of the repeats is quite variable and generally longer than the 33 residues of canonical ankyrin repeats, 43, 47, 45, 36, 49, and 33 residues, respectively, in TRPV2 (note that the last repeat does not have an associated finger; Fig. 2A). Insertions within ankyrin repeat domains have been previously observed in other structures and are most often found in the finger region, although other insertion locations have also been observed (15, 16). In TRPV2-ARD, insertions are found not only in fingers 1–3 but also in outer helices 5 and 6. Although the lengths of the inner helices are typical of a canonical ankyrin repeat, 7 residues on average (15), outer helices 5 and 6 are signif-
Sequence conservation of the ARD of mammalian TRPV proteins. A, sequence alignment of the ARD of mammalian TRPV proteins generated using ClustalW (36). Secondary structure elements are depicted schematically above the sequence. Black asterisks denote conserved aromatic residues forming a solvent-exposed patch; arrowheads point to known TRPV1 phosphorylation sites (see "Discussion" for details). B, the sequence conservation level of mammalian TRPV channels mapped onto the TRPV2-ARD structure. A color gradient from dark green to white represents the most conserved to most variable residues, respectively. The patch of conserved aromatic residues on fingers 2 and 3 is contoured with a dashed line. Left, the concave surface, slightly rotated from the orientation in previous figures to better show the conservation of residues on helices. Right, the convex surface formed by the outer helices, a ~90° rotation from the view on the left.

**DISCUSSION**

Implications for the Structure of Other TRPV Channels—Experimental evidence exists that repeat 1 of TRPV5 (residues 64–77) and repeat 3 of TRPV6 (residues 116–140) are important in tetrameric channel assembly (13, 14). Although both our solution studies and the dissimilar packing interactions in different crystal forms indicate that TRPV2-ARD does not oligomerize, it is possible that the distantly related TRPV5- and TRPV6-ARDs tetramerize to promote channel assembly. Alternatively, the TRPV5- and TRPV6-ARD may interact with additional cellular factors to promote channel assembly. Another hypothesis is that these ARDs promote channel assembly by interacting with other regions within the TRPV5 and TRPV6 channels. Additional studies with the isolated TRPV5 and/or TRPV6-ARD could discriminate between these hypotheses.

Implications for the Regulation of TRPV Channels—Functional and biochemical studies have implicated the N-terminal domain of TRPV channels in several regulatory mechanisms involving both phosphorylation events and the binding of regulatory proteins. Phosphorylation and dephosphorylation are common mechanisms by which ion channel function is regulated. Protein kinase A phosphorylates several residues within TRPV1, including Ser116 and Thr370, and thereby prevents desensitization of the channel (28, 29), and Src kinase phosphorylates Tyr200 in human TRPV1 (Tyr199 in rat (30)). Based on the TRPV2-ARD structure, TRPV1 S116 is predicted to be on inner helix 1 and Tyr199 on the concave face of finger 2, and either phosphorylation event could therefore regulate the binding of regulatory factors to the concave face of the TRPV1-ARD. Thr370 is just past the proposed C-terminal boundary of sequence in the inner helices and the hydrophobic core of the palm supports our proposal that all TRPV channels have a six-repeat ARD. Also, the aromatic residues on the surface of fingers 2 and 3 are well conserved, particularly within the TRPV1–4 channels (Fig. 3). In contrast, the residues with the highest degree of sequence variability are located within the first ankyrin repeat, the solvent-exposed surface of the outer helices and the fingers of the ARD. The tip of finger 3, which seems very flexible according to our structural studies, also has particularly high sequence variability. Thus this finger is likely to adopt different conformations in the other TRPV channels and could be a major determinant of specificity in the regulatory interactions of TRPV channels.
the TRPV1-ARD, likely in a linker region between the ARD and the following domain. Thus Thr$^{370}$ phosphorylation could possibly regulate the spatial relationship of the two domains.

A binding site for the SH3 domain of PACSIN3 was recently identified at residues 132–144 of TRPV4, which is a proline-rich region immediately preceding the predicted ARD (Fig. 3 (31)). The TRPV1 N terminus, in particular residues 200–414, encompassing the last three ankyrin repeats and ~50 additional residues preceding the channel domain, was found to associate with synaptotagmin IX and snapin, two components of SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor)-dependent exocytosis in excitable cells (32). Two-hybrid screens have also identified proteins that interact with the N terminus of TRPV2, including the recombinase gene activator and acyl-CoA binding domain protein 3 (ACBD3) (33–35). The identification of the ARD binding sites can help design further mutagenesis or structural studies of the TRPV-ARD interactions with these identified ligands.

The TRPV2-ARD structure reported here will therefore serve as a scaffold for future investigations of the molecular mechanisms regulating TRPV ion channels.

Acknowledgments—We thank current and former members of the Gaudet laboratory for help and discussions, particularly Deborah de Jong for her contributions to protein purification and crystallization, Piotr Sliz for help with data collection, and Steve C. Harrison and Catherine Dulac for discussions. Use of the Advanced Photon Source Structural Biology Center beamlines was supported by United States Department of Energy Contract No. W-31-109-ENG-38, and use of the Advanced Photon Source beamlines was supported by Award RR-15301 from the National Center for Research Resources at the National Institutes of Health.

Note Added in Proof—The human TRPV2-ARD structure has been determined by another group and is in press elsewhere (37).

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