

# Divide and Conquer: High Resolution Structural Information on TRP Channel Fragments

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Understanding how proteins facilitate signaling and substrate transport across biological membranes is an important frontier of structural biology. Membrane proteins are the doors and windows of cells: many membrane proteins are gates of entry into or exit from cells or cellular compartments, and others allow cells to sense their environment. One important multifunctional family of membrane proteins is the transient receptor potential (TRP) family of ion channels. TRP channels have recently been the subject of multiple structural analyses, both low resolution electron microscopy studies (reviewed by Moiseenkova-Bell and Wensel in this issue [p. 239]) and the divide and conquer approach of determining high resolution crystal structures of channel fragments, reviewed here.

## Introduction

TRP channels form a large family of cation channels that can be activated by diverse signals, including chemical ligands and/or temperature or mechanical stimuli (Ramsey et al., 2006; Venkatachalam and Montell, 2007). Most TRP channels are also modulated by various intracellular signals, including calcium, phosphoinositides, and other lipid metabolites. TRP channels are mostly found in the animal kingdom (organisms with a nervous system), consistent with their prominent role in sensory perception. They are distributed into seven subfamilies according to sequence and function (Montell, 2005): TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPN (NOMPC), TRPP (polycystin), and TRPV (vanilloid). Of note, TRPN channels are found in most animal genomes but excluded from mammalian ones.

The structural biology of ion channels is an important and expanding research endeavor. Mechanistic understanding of ion channel function is central to our understanding of neurobiology and many other physiological processes. Furthermore, ion channels are important targets for drug development. With the rapidly increasing number of structures of ion channels and their fragments (Minor, 2007), including

structural studies of TRP channels (Gaudet, 2008b), there is an opportunity to leverage this structural information in studies of TRP channel function and physiology. TRP channel biologists and physiologists may want to brush up on structural biology approaches, and an excellent starting point is a recent primer on structural biology for neurobiologists (Minor, 2007). Conversely, structural biologists benefit from integrating knowledge on TRP channels and general channel physiology in planning their experiments. TRP channels are challenging structural biology targets, and the more that is known about their molecular properties, the more likely we will be to succeed in obtaining valuable three-dimensional structures.

In structural biology, the aim is to understand proteins at several levels: What are their structural and functional modules? How is the modular architecture integrated to drive their molecular mechanisms? How are these proteins incorporated into larger assemblies? How do these assemblies regulate protein function in a cellular context? Furthermore, the integration of structural and physiological approaches enables us to advance from static three-dimensional structures to the description of dynamic processes like conformational changes and ligand interactions.

Determining the high resolution structure of complete TRP channels remains a major challenge. One alternative and complementary strategy is to divide and conquer: determine crystal structures of isolated domains of TRP channels. The resulting information can then be pieced together and integrated with biochemical and physiological data to advance our understanding of TRP channel function. Below, I describe how the divide and conquer approach can be implemented, illustrate some recent results obtained with fragments of TRPV and TRPM channels, and pinpoint some challenges that lie ahead in moving from this piecemeal approach to the ultimate goal of obtaining a full molecular-level description of TRP channel function.

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Abbreviations used in this paper: ARD, ankyrin repeat domain; TRP, transient receptor potential.

## TRP Channels as Modular Proteins

TRP channel subunits are rather large, ranging from  $\sim$ 700 to more than 2,000 amino acid residues, and have six membrane-spanning segments with an extended pore loop between the fifth and sixth segment. This transmembrane domain arrangement is homologous to that of other ion channels in a large superfamily that includes voltage-gated calcium channels and Shaker potassium channels (Venkatachalam and Montell, 2007). All members of this superfamily are believed to assemble as tetramers of the six-segment transmembrane domain, with a central ion permeation path. Most if not all TRP proteins can homotetramerize to form functional channels, and several also have the ability to heterotetramerize, thus increasing the permutations of possible functional units (for a recent review see Schaefer, 2005).

The transmembrane domain of TRP proteins spans  $\sim$ 300 residues and is connected at the N and C termini to large intracellular regions containing protein-interaction and regulatory motifs with distinctive features for each TRP subfamily (for a recent review see Gaudet, 2006). For instance, ankyrin repeats are ubiquitous ligand-interaction motifs that are found in the N-terminal cytosolic region of TRPC, TRPV, TRPA, and TRPN channels. As an example, Fig. 1 illustrates the relationship between the ankyrin repeats and other regions of TRPV channels. As a contrasting example, the overall domain structure of a TRPM channel is depicted in Fig. 2. TRPM channels do not have ankyrin repeats but instead have a large,  $\sim$ 700-residue N-terminal intracellular region homologous only to other TRPM channels. C-terminal to the transmembrane domain, TRPM channels have a coiled-coil domain. In some TRPM channels, an enzymatic domain then follows the coiled-coil domain: TRPM6 and TRPM7 have an  $\alpha$ -kinase domain (Nadler et al., 2001; Runnels et al., 2001), and TRPM2 has a NUDIX domain that interacts with ADP-ribose nucleotides (Perraoud et al., 2001).

How can this modular domain structure of TRP channels be leveraged in structural biology? A fundamental element of a successful divide and conquer approach to protein structure determination is to properly identify the boundaries of TRP channel domains to enable the expression and purification of these domains in isolation. The word “domain” is often used rather loosely by non-structural biologists (and sometimes even structural biologists) to describe any fragment a protein—often, the terms “segment,” “region,” or “motif” would be more appropriate. The structural biology definition of a domain is a compact globular structure that can fold autonomously and originates from early structural studies of immunoglobulins (Wetlaufer, 1973). This definition implies that (1) a domain is large enough to have a unique three-dimensional fold, and (2) a domain can often fold on its own in isolation from the rest of the protein. This second point is the key to a divide and conquer approach because by identifying the proper domain boundaries of a

region of interest, it can then be isolated for structural studies. Furthermore, a protein domain is a self-contained unit that can interact with other molecules or other parts of the protein. In a divide and conquer approach, one can therefore still obtain information about relevant regulatory interactions by determining structures of domains with their respective ligands. From a genomics perspective, a domain can also evolve new functionalities and be swapped in and out of genes during evolution by duplication or deletions (Moore et al., 2008).

The divide and conquer approach to TRP channel structural biology has thus far yielded structures of two different types of domains, ankyrin repeats from TRPV channels and a coiled-coil from a TRPM channel. Both types of domains are found not just in TRP channels, but also in many other protein families. At first glance, that might lead one to think that the resulting structures are old news; after all, there are many published structures of ankyrin repeats and coiled-coils (for recent reviews see Gaudet, 2008a and Grigoryan and Keating, 2008). But in both cases, structures of some of their representatives in TRP channels have yielded surprises. The next two sections describe the lessons we have thus far learned from structures of TRP channel ankyrin repeats and coiled-coils.

### Lessons from Ankyrin Repeats

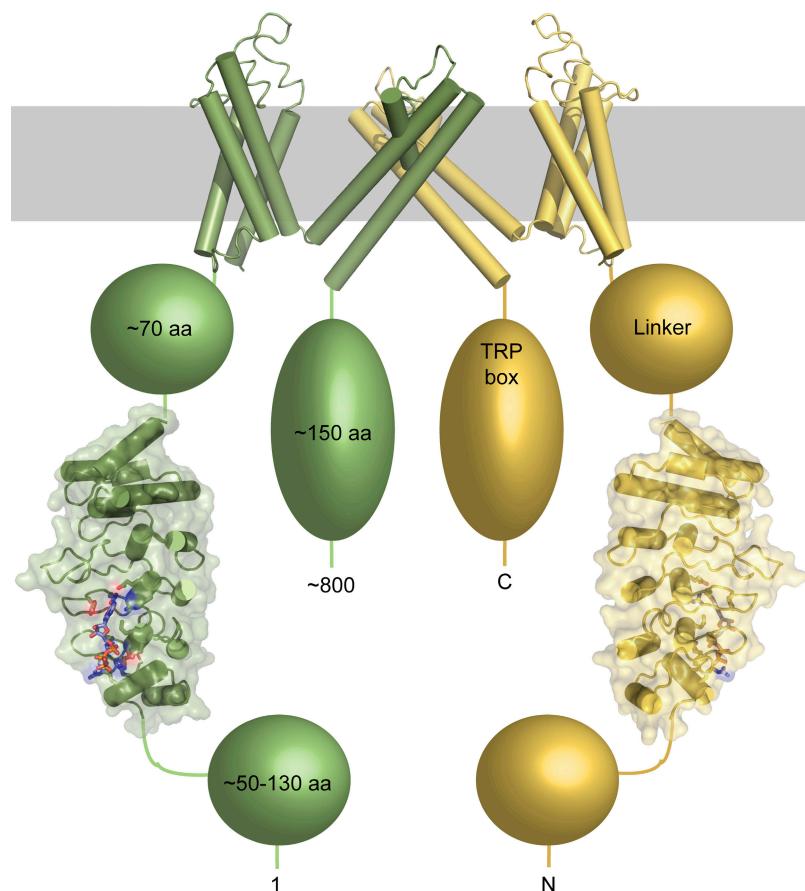
Mammalian TRPV channels are divided into two subgroups: TRPV1 through TRPV4 mediate responses to many sensory stimuli, including heat, low pH, neuropeptides and chemical ligands, whereas TRPV5 and TRPV6 are expressed in the kidney and gut, respectively, and are involved in calcium homeostasis (Venkatachalam and Montell, 2007). Several TRPV channels are polymodal detectors. For example, TRPV1 is activated not only by noxious heat, but also by capsaicin and low extracellular pH. The intracellular N-terminal region of TRPV proteins contains six ankyrin repeats, short sequence motifs often involved in protein–protein interactions (Gaudet, 2008a). The isolated TRPV ankyrin repeat domains (ARDs) do not oligomerize, suggesting that the ARDs interact with regulatory factors instead (Phelps et al., 2008).

Ankyrin repeat sequences span  $\sim$ 33 residues and fold into a structural motif consisting of two  $\alpha$ -helices folding back onto each other to form a helical hairpin, followed by a long hairpin loop that extends roughly perpendicular to the helical axes. Multiple such structural motifs are stacked side by side with their helices nearly parallel to each other to form an ARD, with the number of repeats ranging from 3 to  $>30$  (Gaudet, 2008a). The structures of several TRPV ARDs have now been published: rat TRPV1 (Lishko et al., 2007), both rat (Jin et al., 2006) and human TRPV2 (McCleverty et al., 2006), and mouse TRPV6 (Phelps et al., 2008), and their folds are very similar to each other, consistent with their sequence homology. The TRPV ARDs have six ankyrin repeat motifs,

with atypical long finger loops and a pronounced twist between the fourth and fifth repeat, such that the helices of repeats 1–4 and 5–6 are no longer nearly parallel to each other (Fig. 1). Both the long loops and the unusual twist break the regularity of the repeats, giving the TRPV ARDs a unique shape. Because both the long loops and the inter-repeat twist are caused by residues that diverge from the ankyrin repeat sequence consensus but are conserved in TRPV proteins, it is expected that this unique shape will be observed in all TRPVs (Phelps et al., 2008).

The unique shape of the TRPV ARDs, while of interest to structural biologists investigating repeat proteins and protein folding and design, is not particularly informative about the role of the ARD in TRPV channel function. However, when hundreds of chemicals were screened to optimize the TRPV1-ARD crystallization conditions, it was observed that the presence of ATP altered the crystal shape, likely by changing the packing interactions between protein molecules. This new crystal form diffracted to higher resolution, allowing structure determination and refinement. The resulting electron density map indicated that an ATP molecule was indeed bound to the TRPV1-ARD (Fig. 1) (Lishko et al., 2007) on the concave surface that is typically occupied by ligand in ARD–ligand complexes (Gaudet, 2008a). Biochemical assays demonstrated that both ATP and calcium calmodulin bind to this same binding surface in a competitive manner—the binding of one excludes the binding of the other. Another clue that the TRPV1-ARD interaction with ATP is physiologically relevant is that it is conserved in the chicken homologue (Phelps et al., 2007), indicating that it is better conserved than capsaicin sensitivity because chicken TRPV1 is insensitive to capsaicin (Jordt and Julius, 2002). In electrophysiology experiments, intracellular ATP prevented desensitization to repeated applications of capsaicin, whereas calcium calmodulin plays an opposing role and was required for desensitization (Lishko et al., 2007). The accumulated data lead to a model for the calcium-dependent regulation of TRPV1 via the competitive interactions of ATP and calmodulin at the N-terminal binding site. In summary, the crystallographic determination of the TRPV1-ARD structure has lead to the fortuitous discovery of a regulation mechanism for TRPV1. It will be interesting to see whether this mechanism is conserved in other TRPV ion channels.

Ankyrin repeats are also found in other TRP channels, including TRPA, TRPN, and TRPC channels. The TRPC and TRPV channels have few repeats and irregular sequences (Phelps et al., 2007, 2008), whereas TRPA and TRPN channels have many regular repeats (for a recent review see Gaudet, 2008a). As was done in the case of TRPV channels, the abundant information on ankyrin repeats from both natural and designed proteins can be



**Figure 1.** The ankyrin repeats of TRPV channels. Diagram shows the topology of TRPV channels with the relative position of the ankyrin repeats illustrated with the structure of the TRPV1 ARD. Only two of the four subunits are shown for clarity in yellow and green, respectively; the subunits in front and in back of the plane of the page are omitted. The transmembrane domains are illustrated using the homologous structure of the Shaker potassium channel (Long et al., 2005). The N- and C-terminal segments of unknown structure are depicted with shapes that approximate their relative size. ATP and ATP-interacting side chains are shown as sticks and colored according to atom type, and a transparent surface representation highlights the surface complementarity of the ATP and its binding site. The approximate size of those protein segments in numbers of amino acid (aa) residues is indicated for the green subunit. The transmembrane and ARDs of TRPV channels are each  $\sim$ 250 amino acid residues. TRPV subunits typically are  $\sim$ 800-residues long.

leveraged to study the role of ankyrin repeats in other TRP channels. Of particular interest is TRPA1, which transduces pain signals in response to irritants like mustard oil (Bandell et al., 2004). Irritants covalently attach to the thiol group of several cysteines in TRPA1's 17 ankyrin repeats to activate the channel (Hinman et al., 2006; Macpherson et al., 2007), and structures of the ankyrin repeats will be useful to decipher how this chemical modification can lead to channel opening. Allicin, a compound found in garlic, activates TRPV1 through the chemical modification of a single cysteine, C157, in the ARD of TRPV1 (Salazar et al., 2008). Cysteine 157 is buried in the protein core between repeats 1 and 2, implying that its chemical modification requires a fairly large conformational change (Gaudet, 2008b; Salazar et al., 2008). Similarly to the TRPA channels, TRPN channels have large numbers of ankyrin repeats with sequences very close to ankyrin repeat motif consensus, although little is known about the biological roles of these repeats. TRPC channels have few repeats (likely four or five), which have weak similarity to ankyrin repeat consensus. The structure of TRPC channel ankyrin repeats is therefore likely to have some unusual kinks and loops, as was observed in TRPV channels.

#### Lessons from Coiled-Coils

The TRPM channels have coiled-coil domains in their C-terminal cytosolic region. Biophysical studies (Tsuruda et al., 2006) have validated the existence of these coiled-coils in all but one of the eight mammalian TRPM channels (TRPM1 was not validated in this study) and demonstrated that these coiled-coils can form homotetrameric assemblies, which is consistent with the expected tetrameric state of functional TRPM channels.

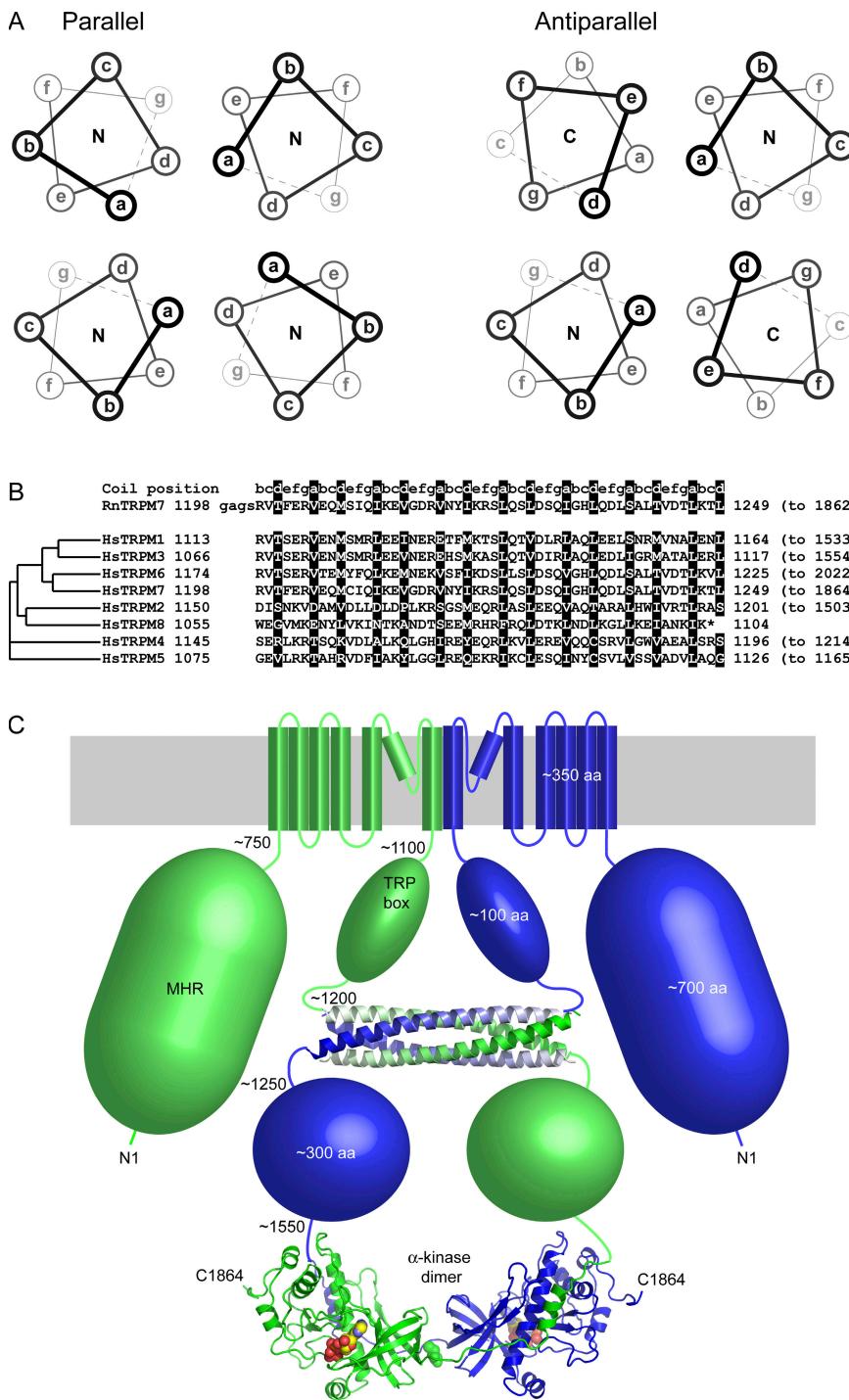
Coiled-coils are protein interaction and assembly motifs forming  $\alpha$ -helices that zip up together in a helical coil conformation (for a recent review see Grigoryan and Keating, 2008). Coiled-coils are found in many protein families, including transcription factors, cellular and viral membrane fusion proteins, and ion channels. Coiled-coil motifs are identified in protein sequences by their characteristic recurring pattern of aliphatic residues alternating every third then fourth residue to form seven-residue repeats. The sequence patterns are a reflection of the regularity of three-dimensional coiled-coil structures (Fig. 2 A). Within each repeat of seven amino acids, routinely labeled *a* through *g*, residues *a* and *d* are usually aliphatic and form the internal core of the coiled-coil. Residues *e* and *g* are generally polar or charged and interact with each other across strands, often dictating the specificity of assembly through electrostatic interactions. Residues *b*, *c*, and *f* tend to lie on the outside surface and have less influence on coiled-coil interactions.

Although the above description may suggest that coiled-coil structures are predictable, this is currently not the case (Grigoryan and Keating, 2008). Coiled-coil struc-

tures have been observed that contain anywhere between two and seven helical strands, and strands can associate in either parallel or antiparallel orientations. The number and orientation of the strands in a coiled-coil assembly cannot be predicted. Small variations in coiled-coil sequences, as little as one residue, can change the observed assembly mode (Grigoryan and Keating, 2008). Furthermore, some strands preferentially form homo-oligomers, whereas others form specific hetero-oligomers. Repeat residues *e* and *g* play important roles in dictating specificity, but in ways that cannot yet be predicted easily. In summary, the identification of coiled-coil repeats can enable the prediction of which residues are most likely to mediate affinity (*a* and *d*) and specificity (*e* and *g*), and which residues may have little influence on assembly (*b*, *c*, and *f*). But the nature of the resulting assembly cannot be predicted with certainty.

TRPM6 and TRPM7 are two closely related TRPM family members that are important in magnesium uptake and homeostasis (Schlingmann et al., 2007). The crystal structure of the TRPM7 coiled-coil was recently determined to high resolution (Fujiwara and Minor, 2008). It forms a homotetrameric coiled-coil, consistent with the predicted tetrameric functional channel. But surprisingly, it is an antiparallel tetrameric coiled-coil, with two strands going in one direction and two strands going in the opposite direction (Fig. 2 C). This is striking because it breaks the fourfold rotational symmetry that is expected for the transmembrane domain of the TRPM7 channel, with the four subunits related by 90° rotations around an axis perpendicular to the plane of the membrane. The antiparallel topology was confirmed in solution using cross-linking experiments (Fujiwara and Minor, 2008). It will be important to further confirm that the antiparallel topology observed for the isolated coiled-coil domain is also present in intact TRPM7 channels, although sequence analyses do strongly support an antiparallel topology for the TRPM7 coiled-coil and closely related TRPM channels (Fujiwara and Minor, 2008).

The TRPM7 coiled-coil is followed by an atypical  $\alpha$ -kinase domain. The structure of that kinase domain, determined in 2001 (Yamaguchi et al., 2001), showed a domain-swapped dimer where the two subunits are held together by an exchange of their N-terminal helices (Fig. 2 C). The  $\sim$ 80-Å distance between the C termini of two antiparallel strands of the TRPM7 coiled-coil structure matches well the  $\sim$ 90-Å distance between the N termini of one kinase dimer. Therefore, by breaking the fourfold symmetry of the transmembrane domain, the antiparallel coiled-coil may allow two kinase dimers to exist side by side. This observation lends further support to the physiological relevance of the unexpected oligomer symmetry observed in both structures. It also prompts a word of caution regarding electron microscopy analyses of TRP channel structures. Symmetry averaging is routinely used to improve the signal to



noise when analyzing electron microscopy images. However, one can no longer assume that a TRP channel obeys fourfold rotation symmetry throughout the tetrameric assembly.

TRPM6 is the closest TRPM7 homologue that also has a C-terminal  $\alpha$ -kinase domain, with a sequence identity of 53% at the protein level overall and 69% in the coiled-coil domain. TRPM6 and TRPM7 have been reported to form heterotetramers when coexpressed in the same cells (Chubanov et al., 2004). It will be interesting to follow up

on the structural studies of the two TRPM7 C-terminal domains with structural and/or biochemical studies to investigate whether the TRPM6 and TRPM7 coiled-coils can form heterotetramers, and whether the TRPM6 and TRPM7 kinase domains can form heterodimers. The results could suggest possible stoichiometries and topologies available to TRPM6/TRPM7 heterotetramers.

The observation that the TRPM7 coiled-coil is antiparallel also brings up the interesting question of whether all TRPMs will have antiparallel coiled-coils or whether

the observed sequence divergence also encodes structural divergence. The signature pattern of a coiled-coil is identifiable in all mammalian TRPM proteins (Fig. 2 B) (Fujiwara and Minor, 2008), but as described above, the topology of a coiled-coil is not readily predicted and would be worth testing experimentally for each TRPM family member. Because small changes in a coiled-coil sequence can tilt the balance to favor parallel versus antiparallel assembly or alter partnering specificity (Grigoryan and Keating, 2008), the presence of coiled-coil assembly domains might promote rapid evolution and divergence of subunit assembly and topology in protein families like the TRPM channels.

Inhibitors of coiled-coil assembly have been selected or designed by optimizing affinity and specificity to compete effectively against the native interactions (Grigoryan and Keating, 2008). One example is an HIV inhibitor that prevents fusion of the virus with the cell membrane (Frey et al., 2006). Therefore, the structure of the TRPM7 coiled-coil, and any future TRPM coiled-coil structure, could be used to design inhibitors of channel assembly and function. Although the isolated TRPM8 coiled-coil had no effect on TRPM8 function, when it was attached to an accessory TM helix to pre-localize it to the plasma membrane, it inhibited channel assembly and function (Tsuruda et al., 2006). This suggests that a molecule that interacts strongly enough to overcome the high local concentration of the native coiled-coil to disrupt its assembly could be an effective inhibitor of TRPM8.

Coiled-coils are also predicted in TRPC channels at either or both the N-terminal intracellular linker between the ankyrin repeats and the transmembrane channel domain and the C-terminal domain (Lepage and Boulay, 2007; Schindl and Romanin, 2007). These TRPC coiled-coil regions have yet to be confirmed through biochemical and/or structural experiments. It will be interesting to see whether future studies of TRPC coiled-coils will also yield new surprises, considering the interesting recent developments in the TRPM coiled-coil structural studies.

#### Future Outlook

To fully understand the molecular basis of TRP channel gating and regulation, high resolution structures of whole TRP channels will be great assets, whether by electron microscopy techniques or x-ray crystallography. TRP channel structures will generate a framework for interpreting biochemical and electrophysiological information accumulated on these channels. X-ray crystallography of membrane proteins like TRP channels poses several technical challenges. One technical challenge is to produce large amounts of detergent-solubilized, biochemically pure TRP channel tetramers. Recent progress in membrane protein crystallography is encouraging, including structures of vertebrate ion channels including Shaker channels produced in the yeast *Pichia pastoris* (Long et al., 2005) and an ASIC channel produced in

baculovirus-infected insect cells (Jasti et al., 2007). A second technical challenge is obtaining crystals of TRP channels suitable for structure determination by x-ray crystallography. Crystallization is still based on trial-and-error methods screening thousands of conditions. Aside from the typical crystallization solution components (buffering and precipitating agents, salts, and other chemical additives), membrane proteins require additional screening with different detergents and/or lipids. Further variables that may prove useful for TRP channels are the addition of chemical and protein ligands, including agonists, antagonists, blockers, and other modulators. These ligands have functional effects on the proteins by changing their conformation, which can in turn influence their crystal-packing interactions to improve crystal growth.

Detailed mechanistic understanding of TRP channel function will be attained by iterating structures and functional experiments using physiological assays. Significant advances have been achieved through studies of channel fragments. But this divide and conquer approach is ultimately conservative in nature: the long-term goal is to view the channel as a whole, and although it is perhaps a more risky approach, tackling the structure of assembled TRP channels will yield information not attainable from the accumulation of fragmented structures. That is because the divide and conquer approach does not directly answer the question of how the local information—the conformational state of the particular fragment under study—is integrated in the context of the whole tetrameric channel to effect changes in TRP channel function. We can expect that structural information on TRP channels will continue to emerge, both in fragments and, hopefully, whole channel structures in the near future. Continued collaboration between physiology and structural biology will be needed to fully appreciate how complex and elegant TRP channels truly are.

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