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CRACking the structure of Orai

New work reveals the intramolecular properties of a store-operated, calcium influx channel

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Calcium (Ca^{2+}) is well recognized as a key second messenger in a variety of eukaryotic signal transduction pathways, and nature has developed a number of regulatory mechanisms to mediate its cellular influx and efflux and its homeostasis within the cell. Stimulus-evoked release of Ca^{2+} stored within internal organelles (i.e., endoplasmic and/or sacroplasmic reticulum) is known to trigger the subsequent influx of external Ca^{2+} as a means to replenish the internal store, and a flurry of studies over the past 5–10 y have demonstrated that this influx process involves two main molecular players; STIM1, a transmembrane Ca^{2+} sensor protein localized to the internal storage organelle and Orai, an integral plasma membrane protein that mediates Ca^{2+} entry in response to store depletion. Cahalan and coworkers initially identified STIM1 as a novel Ca^{2+}-binding protein through use of a comprehensive siRNA-based screen of candidate molecules in Drosophila S2 cells whose loss could impact evoked Ca^{2+} entry.[1] A year later, Orai was discovered through genetic mapping studies of a novel mutation associated with impaired lymphocyte function and severe immunodeficiency in patients,[2] and was subsequently found to be an integral plasma membrane component of the calcium influx channel. The latter studies revealed that lymphocyte dysfunction was associated with a loss of evoked calcium influx in these cells, which correlated with a single Arg to Trp amino acid substitution in the first predicted transmembrane segment of Orai1. More recently, investigators have reported that Orai protein monomers are able to co-assemble to form a Ca^{2+}-selective cation channel that may be activated by interaction with STIM1.

In light of Orai's role as a novel Ca^{2+} entry channel, Long and colleagues set out to examine the physical makeup and structure of functional Orai channels through the use of high resolution crystallographic imaging techniques. To do so, the investigators first performed extensive biochemical screening of several dozen Orai protein orthologs cloned from multiple species to identify isoforms with optimal detergent solubility and the capability to assemble into uniform protein multimers. Based on results from this initial phase, the Orai protein from *Drosophila melanogaster* was chosen as the most suitable candidate for crystallization trials. *Drosophila* Orai (dOrai) shares 73% primary sequence identity with human Orai1, and the majority of this amino acid identity is contained within the predicted transmembrane segments. Following expression and purification of an optimized form of the dOrai protein (amino acids 132–341), the authors utilized a fluorescence-based Na^+ influx assay in the absence of external divalent cations to demonstrate that purified dOrai could form cation conductive channels following reconstitution into lipid vesicles. Importantly, creation of a non-conducting pore mutant (K163W) equivalent to the disease-causing, genetic mutation in human Orai1 (i.e., R91W) prevented Na^+ influx, whereas a V174A mutation produced constitutively active dOrai channels, as expected from studies examining Orai1. Gadolinium, a

well-known pore blocker of mammalian Orai1 channels, inhibited cation flux in the constitutively active dOrai V174A mutant, but did not interfere with Na^+ entry into liposomes following addition of monensin, an established Na^+ ionophore. These results demonstrated that the engineered form of dOrai could assemble into functional cation channels, thereby raising confidence that structural information obtained from these multimers would reflect the properties of a competent channel complex.

Crystals containing holo-dOrai channel complexes were found to diffract X-rays at a resolution of 3.35Å, which was sufficient to identify secondary and tertiary structures and the positions of amino acid side chains within a single channel. Following refinement of the crystallographic data, a single channel complex was found to contain six dOrai subunits arranged in a circular pattern around a central axis. Each subunit was observed to contain four α-helical transmembrane segments (TM1–4), with both N- and C-termini located intracellularly. Six TM1 segments (one from each subunit) were positioned as a tight concentric ring around the central axis of the holo-channel, forming a tubular ion conduction pathway. TM2 and TM3 segments from each subunit were observed to form a second ring of α-helices surrounding the TM1 segments, and both these inner and middle rings were encompassed by an outer ring comprised of the remaining TM4 segments. When viewed from the external surface, the holo-channel thus appears as an assembly of three concentric rings of transmembrane helices (i.e., inner, middle and outer), with each ring displaying its own type of symmetry

Compared with the structure of K^+-selective channels, the ion conduction pathway of the dOrai channel displays several unique features. These include a series of six Glu residues at the outer mouth of the conduction pathway, each located within an individual TM1 segment, whose carboxylate side chains project centrally into the aqueous pore to form a horizontal ring with an aperture ~6Å in diameter. The possibility that this narrow opening represents a major binding site/selectivity filter for permeant cations entering from the external solution was examined by soaking the dOrai crystals in solutions containing millimolar concentrations of either Ca^{2+} or Ba^{2+}; this approach revealed distinct electron densities at the level of the Glu ring, consistent with the presence of a bound cation within this structure. Given the dimensions of this narrow aperture, it is likely that Ca^{2+} ions traverse this ring in either a partially or fully dehydrated state. Immediately following this ring of Glu residues, the authors noted a short cylinder of hydrophobic residues that was suggested to provide structural stability to the ion conduction pore, due to the strength of interactions between adjacent amino acids. Located farther along the conduction pathway, the authors observed a section of positively charged residues that line the cytoplasmic end of the pore. The ion conduction pathway of the dOrai channel thus appears to contain three distinct sections, and such features are likely well conserved among various orthologs of Orai, based on amino acid identity/similarity within the four transmembrane segments.

Based on the observed dimensions for the ion conduction pathway, particularly at the positively charged, cytoplasmic end, the hexameric structure resolved by Long and colleagues may represent the holo-dOrai channel in its closed state. Physiologically, Orai channels are known to be gated/activated by interaction with STIM1 following internal Ca^{2+} store depletion and the authors speculate that this gating process may involve a structural rearrangement involving the N-terminal region of dOrai's TM1 segments. Such interaction would dilate the lower, positively charged section of the pore and allow Ca^{2+} ions to flow by electrochemical movement from the external cation binding site through the dilated intracellular opening and into the cytosol. Interestingly, the authors also observed that the crystal structure of the holo-channel contained electron dense material within this lower, positively charged region of the pore, which they interpreted as the presence of a complex anion. The binding of an anion at this location under physiologic conditions may thus help stabilize the pore in a closed state, thereby preventing unwanted Ca^{2+} entry. These speculations are further supported by a parallel crystallographic structure determined for the dOrai K163W mutant, which is equivalent to the non-conducting, human Orai1 mutant R91W responsible for severe immunodeficiency. K163 is a TM1 residue located within the lower, positively charged region of the conduction pore, and in the K163W mutant channel, the authors noted extensive hydrophobic interactions between the substituted Trp side chains in the intact channel. It was hypothesized that the strength of these interactions may prevent this region of the conduction pathway from widening in response to STIM1 binding, and may thus lock the channel in a closed state.

Interestingly, the observed hexameric structure of the holo-dOrai channel determined in this study by crystallography differs from earlier studies reporting that functional Orai channels were most likely tetramers, based on multi-faceted experimental strategies involving chemical cross-linking of channel subunits, electrophysiololgical recordings and single molecule fluorescence photo-bleaching techniques.[3-6] A strategy utilized in several studies has been to express Orai subunits containing either epitope or fluorescent protein tags in a eukaryotic cell background and then examine biochemical and functional parameters of the expressed channels. One potential confounding issue of this approach is whether a cell's endogenous Orai subunits may co-assemble with exogenous, recombinant Orai subunits, resulting in the formation of hexameric channels with "silent" subunits. It is also unclear whether physically connecting recombinant subunits in dimeric, trimeric or tetrameric linear repeats via C-terminus to N-terminus peptide linkers would effectively prevent co-assembly of the repeat with endogenous Orai subunits. If possible, the mixing of endogenous with exogenous Orai subunits could result in an underestimation of the actual subunit stoichiometry in the functional channels under investigation.

Finally, it is striking that Orai channels share several structural features with gap junction channels that mediate electrical and/or chemical communication between adjacent cells. Gap junctions are composed of two hemi-channels or connexons, which are located in the plasma

membrane of adjacent cells and interact with one another to form an aqueous passage between the cells. A connexon is composed of six connexin subunits or protomers, each of which contains four TM segments with N- and C-termini located intracellularly. Based on crystallographic data,[7] the conduction pathway of the connexin26 hemi-channel is formed by the assembly of TM1 helices and the cytoplasmic region of the pore contains a stretch of positively charged residues, similar to the features described for dOrai. In contrast to the Orai channel, however, the narrowest region of the conduction pathway in the connexon26 gap junction is ~14Å and there is no indication that amino acid side chains lining the pathway may extend into the pore to regulate the movement of particular solutes. This wide passage thus makes connexons permeable to a variety of cations and anions, along with small organic molecules up to 1,000 daltons in mass, and differs from the highly Ca2+-selective behavior of Orai channels. A connexon hemi-channel and holo-dOrai channel thus display common structural features with respect to subunit topology and the assembly of subunits into hexameric complexes, yet differ with regards to ion/solute permeability. In view of the gross structural similarities highlighted above, should we consider a connexon as a type of modified Orai channel (or maybe vice versa)?

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Readers interested in STIM1 and Orai/CRAC channels will want to check out the upcoming September/October issue of *Channels* (Volume 7, Issue 5), which will be dedicated entirely to STIM1 and Orai signaling mechanisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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