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Permeation and gating mechanisms in store-operated CRAC channels

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1. ABSTRACT

Ca^{2+} is a ubiquitous signaling messenger mediating many essential cellular functions such as excitability, exocytosis and transcription. Among the different pathways by which cellular Ca^{2+} signals are generated, the entry of Ca^{2+} through store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels has emerged as a widespread mechanism for regulating Ca^{2+} signaling in many eukaryotic cells. CRAC channels are implicated in the physiology and pathophysiology of numerous cell types, underlie several disease processes including a severe combined immunodeficiency syndrome, and have emerged as major targets for drug development. Although little was known of the molecular mechanisms of CRAC channels for several decades, the discovery of Orai1 as a prototypic CRAC channel pore-subunit, and the identification of STIM1 as the ER Ca^{2+} sensor, have led to rapid progress in our understanding of many aspects of CRAC channel behavior. This review examines the molecular features of the STIM and Orai proteins that regulate the activation and conduction mechanisms of CRAC channels.

2. INTRODUCTION

Store-operated calcium entry (SOCE) is the process by which the emptying of ER calcium stores causes influx of calcium across the plasma membrane. SOCE is found in nearly all animal cells, with the upstream activation signal triggering store release and the temporal-spatial characteristics of the resulting calcium influx varying from cell to cell and between different physiological stimuli. The store-operated channels (SOCs) of T lymphocytes and mast cells were the first to be characterized using electrophysiological techniques. These channels, termed calcium release-activated calcium (CRAC) channels, are characterized by extremely high Ca^{2+} selectivity, low unitary conductance, and low permeability to large monovalent cations. Although CRAC channels have been investigated for more than two decades, the molecular components of this pathway were identified only recently. It is now known that STIM1, a protein in the endoplasmic reticulum (ER) membrane, is responsible for sensing the drop in ER calcium (1, 2), and that Orai1 is a prototypic pore-forming subunit of the CRAC channel (3-

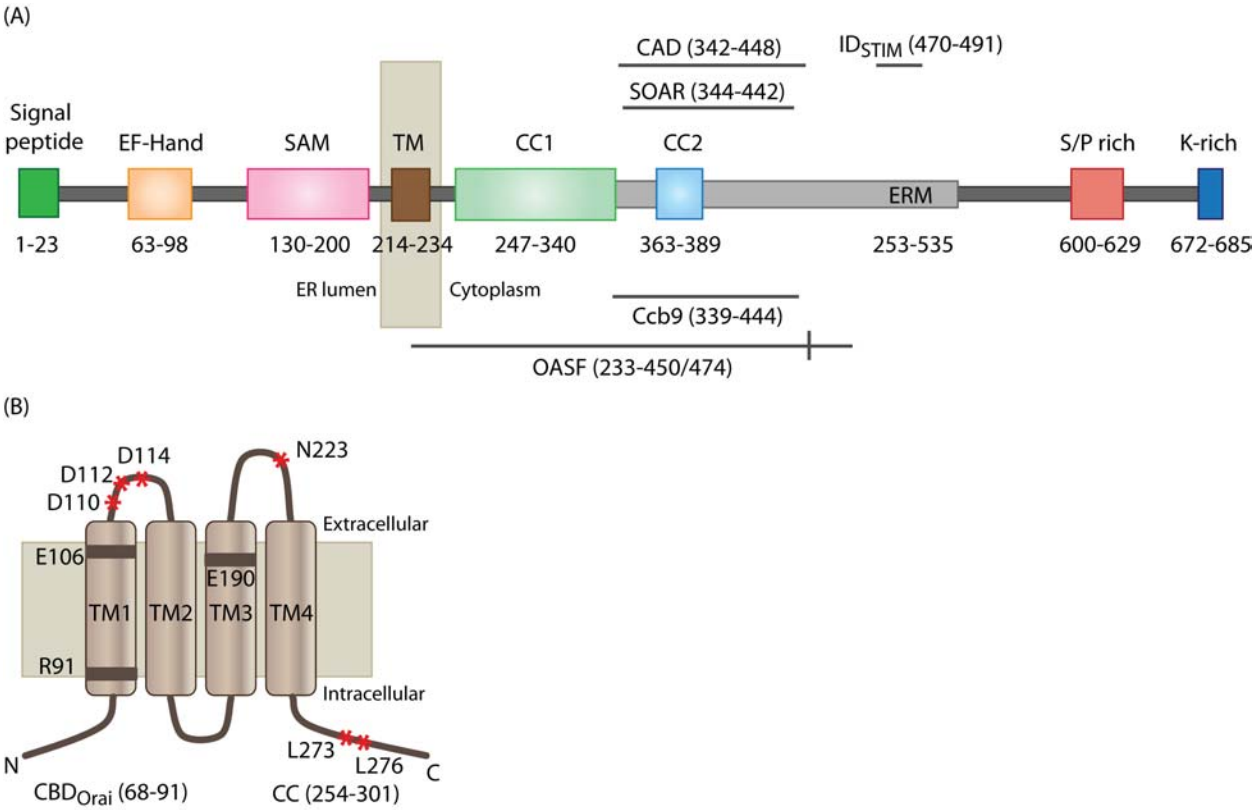

Figure 1. Predicted topology and critical residues of STIM1 and Orai1. A. Functional domains within STIM1. Important domains that control the activity of STIM1 are shown. These include including EF-hand and sterile alpha motif (SAM) domains in the ER lumen and two coiled-coil domains (CC1 and CC2), an ezrin, radixin, moesin (ERM) domain, a serine-proline rich region (S/P rich) and a polybasic domain (K-rich) on the cytosolic side. The minimal functional units of STIM1 (CAD, SOAR, Ccb9 and OASF) that constitutively activate CRAC channels are also shown. IDSTIM is the region of STIM1 required for inactivation of CRAC channels. B. Predicted transmembrane topology of Orai1. Several critical residues identified from mutagenesis studies are highlighted. E106 is the Ca2+-selectivity site. Acidic residues in the I-II loop (D110, D112 & D114) regulate La3+ block. An inherited mutation (R91W) results in non-functional channels and immunodeficiency in human patients. The N-terminus of Orai1 has a CAM binding domain (CBDOrai) and the C-terminus of Orai1 contains a predicted coiled-coil domain (CC) that contains critical residues for STIM1 binding (L273 & L276).

5). STIM1 binds to and directly activates Orai1 and these two molecules appear both necessary and sufficient to reconstitute SOCE (6-9).

Since the discovery of STIM1 and Orai1, substantial progress has occurred in our understanding of the molecular mechanisms of channel regulation and ion conduction. Genetic and biochemical approaches have also illuminated the physiological roles of these molecules and their homologs (in mammals Orai2, Orai3, and STIM2) in mediating SOCE in a variety of cell types. This work has given rise to a basic framework for the molecular choreography of STIM and Orai-mediated SOCE. This review focuses on the molecular characteristics of STIM and Orai proteins that regulate the choreography of channel activation and the conduction mechanisms in CRAC channels.

3. STIM1 IS THE ER CA^{2+} SENSOR FOR SOCE

STIM1 (Stromal interaction molecule 1) was identified as a critical regulator of SOCE independently in two laboratories through the use of RNAi screens for regulators of SOCE in *Drosophila* S2 (10) and mammalian cells (2). STIM1 is a 77KDa single-pass ER membrane protein, with luminal n-terminal domain containing the signal peptide and c-terminal domain in the cytosol (11). The n-terminal domain consists of a sterile alpha motif (SAM) and two EF hands (Figure 1A), whereas the c-terminal domain contains two coiled-coiled domains (12), a Ser/Pro-rich region, and a Lys-rich region at the end of the C-terminus (11). Whereas *Drosophila* has a single STIM gene, mammals have two closely related genes, *STIM1 and STIM2,* which differ significantly in their C-terminal region. In resting cells, STIM1 is largely localized in the bulk ER (1, 2, 8, 9, 13, 14). ER Ca^{2+} store depletion triggers unbinding of Ca^{2+} from the luminal EF-hand, which ultimately results in the redistribution of STIM1 from the bulk ER into puncta located in close apposition to the plasma membrane (1, 2, 8, 9, 13, 14). The EF-hand and SAM domains (EF-SAM) mediate critical roles in this process, as EF-hand STIM1 mutants with impaired Ca^{2+} binding form puncta and activate CRAC channels independently of ER Ca^{2+} store depletion (1, 2). Moreover,

deletion of the SAM domain abrogates puncta formation in response to store depletion (13). The cytosolic C-terminal portion is essential for the redistribution of STIM1 oligomers to ER-PM junctions and subsequent CRAC channel activation occurs through a critical channel interaction domain encompassing the second coiled-coiled domain (13, 15) (6, 16, 17). In this manner, changes in Ca^{2+}-binding at the N-terminus are coupled to SOCE initiation through protein-protein interactions in the STIM1 C-terminus. Thus, STIM1 fulfils two critical roles in the activation process of CRAC channels: sensing the depletion of ER Ca^{2+} stores, and communicating store depletion to CRAC channels located in the plasma membrane.

4. ORAI1 IS THE PORE FORMING SUBUNIT OF THE CRAC CHANNEL

Orai1 was identified in 2006 as a prototypic CRAC channel. An important milestone in its identification was the discovery of human patients with a severe combined immunodeficiency lacking CRAC channel function in T-cells (18-20). These patients exhibited a devastating immunodeficiency characterized by impaired T cell activation and effector gene expression, which confirmed earlier pharmacological and genetic evidence that CRAC channels orchestrate many aspects of lymphocyte development and function (20, 21). Feske *et al*, took advantage of a partial reduction in Ca^{2+} entry in the heterozygotes in the patient pedigree to localize the source of the defect to a small region in chromosome 12 with ~70 genes (22). Simultaneously, genome-wide RNAi screens for genes involved in SOCE in drosophila S2 cells carried out by three groups identified a novel gene as a critical mediator of *Drosophila* SOCE (22-24). A human ortholog of this protein mapped to the same region on chromosome 12 identified by linkage analysis (22). This molecule, named Orai1 by Feske *et al*, is a widely expressed 33 kDa cell surface protein with four predicted transmembrane domains, intracellular N- and C- termini (Figure 1B) and no significant sequence homology to other previously identified ion channels. The human SCID defect was found to arise from a single point mutation in Orai1 (R91W) that abrogated CRAC channel activity (22).

Two important lines of evidence indicate that Orai1 is an essential pore subunit of the CRAC channel. First, overexpression of Orai1 together with STIM1 in HEK293 cells produces enormous CRAC currents (25-27), indicating that over-expression of these proteins is sufficient to recapitulate a current with the characteristics of native CRAC channels. These characteristics include high Ca^{2+} selectivity and low permeability to Cs^{+}, a narrow pore diameter of approximately 3.8 A □, Ca^{2+} block of Na^{+} currents, and pharmacological responses to 2-APB and La^{3+} (25, 26) (28). Second, mutations of highly conserved acidic residues in Orai1, including E106 and E190 significantly diminished the Ca^{2+} selectivity of the CRAC channels, and altered a wide range of properties intimately associated with the pore, including La^{3+} block, the voltage-dependence of Ca^{2+} blockade, and Cs^{+} permeation (3-5, 28). These studies definitively identified Orai1 as a key component of the CRAC channel pore and, further, yielded a list of candidate residues regulating ion selectivity of the channel. Mammalian cells express two other closely related homologues, Orai2 and Orai3 that differ primarily in their C-terminal and the 3-4 loop sequence. All three isoforms appear to function similarly in producing store-operated Ca^{2+} entry when co-expressed with STIM1 in HEK293 cells (29-31) and are widely expressed in most tissues (29, 31, 32).

5. OLIGOMERIZATION AND REDISTRIBUTION OF STIM1 TO THE ER-PLASMA MEMBRANE JUNCTIONS

Recent molecular, functional, and structural studies have yielded significant insight into the mechanisms by which STIM1 senses and communicates ER Ca^{2+} store depletion to Orai channels. The Ca^{2+}-binding affinity of the isolated STIM1 EF-hand SAM domain fragment is ~500 μM (33, 34), consistent with the high concentrations of Ca^{2+} known to exist in the lumen of the ER (35, 36). Structural studies of the isolated luminal domain fragments indicate that Ca^{2+} unbinding from the N-terminal EF-hand unfolds and aggregates the luminal domain, resulting in the appearance of dimers and higher-order multimers (33). This finding led to the hypothesis that Ca^{2+} store depletion leads to the formation of higher order oligomers of STIM1 (33). Subsequent FRET studies with full-length STIM1 confirmed oligomerization as a critical step of the channel activation process (15, 35). STIM1 oligomerization is an early step in the channel activation process, occurring well before STIM1 redistribution to the plasma membrane (15). The critical role of STIM1 oligomerization for CRAC channel activation is underscored by the finding that artificially oligomerizing engineered STIM1 in which the luminal domain is deleted and replaced with the FRB-FKBP dimerizing domains, leads to puncta formation and activation of SOCE independently of ER store depletion (35). Thus, STIM1 oligomerization serves as a critical upstream activation switch that unfolds all subsequent steps of the channel activation process.

Measurements of the Ca^{2+}-affinity of the isolated EF-hand SAM fragments indicate that STIM2 has a lower affinity for Ca^{2+} binding than STIM1 (34). Consistent with this *in vitro* evidence, STIM2 forms puncta more readily in resting cells (37) and measurements of the dependence of $(Ca^{2+})_{ER}$ on puncta formation indicate that significantly greater store depletion is required for STIM1 puncta formation, with an apparent $K_{1/2}$ of 210 μM for STIM1 and 406 μM for STIM2 (37). These results likely explain why STIM2 has been found in several studies to promote constitutive activation of SOCE when over-expressed with the Orai proteins (37, 38).

Perhaps the most striking feature of STIM1 behavior is its redistribution from the bulk ER in resting cells with full stores, to the plasma membrane where it accumulates into discrete puncta (1, 2, 12, 13). Accumulation of STIM1 near the plasma membrane causes ER tubules to move toward the plasma membrane and STIM1 appears to facilitate this process (14), indicating

that store depletion rearranges the ER, with STIM1 facilitating this change. Consistent with the idea that changes in $(Ca^{2+})_{ER}$ (and not cytoplasmic (Ca^{2+})) directly regulate this process, store depletion with TPEN, which would not be expected to affect cytoplasmic (Ca^{2+}) directly, causes STIM1 and Orai1 puncta formation (39). Luik and colleagues (2008) showed that the accumulation of Cherry-STIM1 at the plasma membrane exhibits the same dependence on ER Ca^{2+} concentration as activation of $I_{CRAC,}$ (35). This is consistent with the notion that channel activation requires a local interaction between STIM1 and Orai subunits which can only occur following the redistribution of STIM1 from the bulk ER to the periphery. Moreover, Wu *et al* 2006 showed that appearance of STIM1 near the surface of the cell precedes the development of I_{CRAC} by ~10 s, indicating that STIM1 translocation is required for CRAC activation, but that the activation also requires an additional step (14).

Although STIM redistribution is triggered by the same initial conformational change that causes oligomerization – with both steps requiring unbinding of Ca^{2+} from its N-terminal EF-hand, these processes are distinct and fully separable. Redistribution occurs with a lag of tens of seconds following STIM1 oligomerization (8, 15), indicating that the two steps are kinetically separable. Additionally, truncation of a basic region at the extreme C-terminus of STIM1 attenuates redistribution of the truncated STIM1 to peripheral sites without affecting STIM1 oligomerization (15), indicating that the molecular determinants of these processes are distinct. Interestingly, the deleterious effect of removing the K-rich C-terminal region on STIM1 redistribution is only seen in cells over-expressing STIM1 alone: when co-expressed with Orai1, STIM1ΔK accumulates into puncta to the same extent as that seen in full-length STIM1 (6), though activation of I_{CRAC} delayed compared to full length STIM1 (47). This suggests that a key factor for the accumulation and stability of STIM1 into puncta at the ER-PM junctions is the overall avidity of STIM1-binding sites in the plasma membrane.

Relatively little is known about the mechanisms controlling STIM1 redistribution to peripheral puncta. Studies with fluorescently-labeled STIM1 (CFP- or YFP-) indicate that STIM1 is at least partially associated with microtubules (MTs) and moves rapidly along tubulovesicular structures that overlap with MTs in resting cells with replete stores (13, 40, 41). These movements cease upon store depletion, and the STIM1 that accumulates into puncta no longer co-localizes with microtubules (13, 40). STIM1 also co-IPs with the MT-associated proteins, EB1 and EB3, indicating that STIM1 and EB proteins are in some way associated (40). The functional relevance of this association, is however, murky. Nocodozole, which depolymerizes microtubules, eliminates the tubulovesicular movement, but does not affect puncta formation or even SOCE (40) (13). Likewise, depletion of cellular ATP eliminates the tubulovesicular movement, but does not impact puncta formation (42). Thus, the relationship between MT association and CRAC channel activation currently remains mysterious. One possibility is that STIM1 exhibits two forms of movement, one along MTs that is powered by motors, and second diffusive mode of migration that is MT-independent. The available data suggests that SOCE is driven solely by the diffusive mode of STIM1 mobility, but this would be predicted to limit its movement to relatively short distances, a prediction that appears borne out by limited diffusional mobility of STIM1 (6, 15).

Recent studies indicate that internal electrostatic interactions between different regions of STIM1 are critical for the transition from resting STIM1 oligomers to their active state (43). An acidic region in the CC1 domain appears to interact with a basic region in the CC2 domain to mask the active site of STIM1 that interacts with Orai1 (called the CAD domain, see below). Oligomerization appears to favor the removal of internal autoinhibition to reveal the CAD and polybasic domains, thereby permitting productive interactions between CAD and Orai proteins leading to channel activation (43, 44). Similar findings were described by Muik *et al.*, who made use of an intramolecular STIM1 FRET sensor to show that the a minimal STIM1 domain that interacts with Orai1 switches from a closed to an open configuration upon interaction with ORAI1 (45). They suggested that the closed confirmation of STIM1 is stabilized by coiled-coil interactions within the C-terminal region of STIM1 and interaction with ORAI1 opens up STIM1 to expose its active Orai1 binding site (45).

6. STIM1 BINDS ORAI1

Several laboratories have shown that the molecular basis of the channel activation process involves direct binding of STIM1 and Orai1. STIM1 and Orai1 co-immunoprecipitate each other (4, 5, 46, 47), and Orai1 also appears capable of co-immunoprecipitating STIM2 (47). Orai1 and STIM1 associate even in a system of only purified components in solution (7), indicating that the interaction between these proteins is strong enough to persist in a variety of chemical environments, and can form even in detergent-solubilized extracts.

Biochemical association and *in situ* FRET evidence indicates that the association of STIM1 and Orai1 only occurs in response to store depletion (4) (8, 9). N-terminally labelled STIM1 (YFP-STIM1) does not produce FRET with CFP-labelled Orai1, before or after store depletion, indicating that STIM1 c-terminus is the active site for the association (8). Consistent with this interpretation, deletion of the c-terminal domain eliminates colocalization with Orai1 and channel activation following store depletion (48).

Deletion and serial truncations have been used to identify the region of STIM1 required for Orai1 activation. The c-terminal domain consists of two putative coiled coils, but the rest of the sequence bears little similarity to any known motifs (Figure 1). Huang *et al* (2006) found that expressing just the cytoplasmic portion of STIM1 (STIM1-ct) was sufficient to activate SOCE, though not to the same extent as full-length STIM1 (16). They also found that deletion of the first half of the c-terminal domain (del231-

535) of STIM1-ct eliminated constitutive activity of this fragment (16). Baba *et al* (2006) found that deleting either 249-390 or 391-end in full-length STIM1 reduced SOCE, suggesting that these regions likely contribute to Orai1 activation (13). Subsequently, several groups analyzed a series of STIM1-ct fragments and identified a critical region of STIM1 encompassing the second CC domain as the minimal sequence required for binding and activating Orai (6) (49) (17) (50). Park *et al* (2009) identified 342-448 as the minimal region required to constitutively activate Orai1, and named this region CAD (CRAC Activating Domain) (6). They showed that the constitutive activity evoked when this fragment was co-expressed with Orai1 has the hallmarks of CRAC activity seen when full-length STIM1 is co-expressed with Orai1, including high Ca^{2+} selectivity, low permeability to Cs^{+}, and inhibition by lanthanum. Likewise, Yuan *et al* (2009) found that the STIM1 fragment spanning 344-442 could constitutively activate CRAC, and named this second region SOAR (STIM Orai Activating Region) (17). Muik *et al* (2009) found that a fragment corresponding to 233-450/474 (Orai-activating small fragment OASF) was sufficient for constitutive CRAC activity (49). And finally, Kawasaki *et al*, found constitutive activation with a fragment that they refer to as Ccb9 that encompasses the region 339-444 (50). Collectively, these studies indicated that the channel activation domain in STIM1 consists of a short ~100 a.a. region encompassing the second CC domain.

Interestingly, the regions involved in STIM-STIM binding are also important for STIM-Orai binding. Muik *et al* (2009) tested a series of STIM1 c-terminal fragments and found that OASF fragments homomerized *in situ* and fragments shorter than OASF lacking key elements of the CAD/SOAR domain are monomeric on native gels, don't self-associate *in vivo*, and fail to activate Orai1 (49). Covington *et al* 2010 directly investigated the regions of the STIM1 C-terminus important for store-depletion induced oligomerization and found that the critical region for oligomerization & puncta formation overlaps with the CAD/SOAR region (51). One critical mutation identified by their analysis, A369K, exhibited enhanced resting-state oligomerization, co-localization with Orai, and constitutive CRAC-channel activity, while at the same time nearly eliminating enhancement of oligomerization upon store-depletion (51). A second mutation, A276K, caused STIM1 to constitutively self associate and form puncta, but eliminated co-localization with Orai and CRAC channel activity before or after store depletion. Their results predicted that both residues lie on a hydrophobic face of the alpha helix in CC2 (51). Collectively, these results suggest that residues important for Orai1 binding are also important for STIM oligomerization.

7. ORAI1 DOMAINS INVOLVED IN STIM1 BINDING

In Orai1, the critical STIM1 interaction site appears to be localized in the c-terminus. Deletion of the Orai1 C-terminus results in the elimination of CRAC channel activation and the increase in STIM1-Orai1 FRET upon store-depletion (48) (8) (6). *In vitro* pull-down assays with isolated fragments of Orai1 indicate that the Orai1 c-terminal domain fragments encompassing the region between residues 254-301 associate with the cytosolic domains of STIM1 (6) (7) (49), demonstrating that the STIM1 C-terminus directly interacts with the Orai1 C-terminus. As in STIM1, the critical binding motif in the Orai1 C-terminus is predicted to be a coiled-coil domain, and two point mutations in this region (L273S and L276D) have been shown to completely disrupt Orai1-STIM1 interactions (8, 9), likely due to disruption of the tertiary structure of the Orai1 CC domain (52). Interestingly, mutations of other nearby hydrophobic residues (F279 and L282) have no effect on I_{CRAC} or STIM1-Orai1 FRET (8) (9). Thus, the role of the hydrophobic residues in the middle of the coiled-coiled region is uncertain. Consistent with this region being an amphipathic helix with the hydrophobic face being responsible for STIM1 binding, neutralization of the aspartates (D284-287-291N) or glutamates (E272-275-278Q) in the coiled-coiled domain doesn't reduce STIM1-Orai1 FRET or SOCE (53). Neutralizing all these residues, however, does decrease S1-O1 FRET and SOCE (53), most likely because eliminating all the negative charge in this region destabilizes the alpha helix.

In addition to the interaction at the C-terminus, STIM1 is also reported to weakly bind to the N-terminus. Park *et al* (2009) found that a purified peptide consisting of the cytosolic n-terminal region corresponding to the region 68-91 interacts with CAD in co-immunoprecipitation and split-ubiquitin assays (6). Consistent with these findings, Zhou *et al* (2010) used a GST-pulldown assay to show that a purified n-terminal fragment 65-87 can interact with purified S1-ct and the S1 c-terminal fragment 233-498 (7). However, because these biochemical assays test for interaction in solution, it unclear whether these results apply to full-length molecules in live cells.

Deletions that remove much of the Orai1 n-terminus abrogate SOCE and I_{CRAC} (48) (8) (6) (54). A construct with only 73-84 deleted fails to support CRAC activity when co-expressed with STIM (6). Yet, N-terminal deletion mutants localize to the plasma membrane, and upon store-depletion form puncta (48) (54) and support increases in Orai1-STIM1 FRET at a similar rate as WT Orai1 (8), suggesting that the interaction at the Orai1 C-terminus is unaffected. These data suggest that the functional effects of n-terminal deletions on CRAC activity may not be due solely to decreased binding to STIM1. Interestingly, fusing the 2 copies of the activation domain of S1 (336-485) to the c-terminus of an Orai1 n-terminal deletion mutant (del 1-90) failed to rescue function (55). Since this manipulation essentially dramatically increases the local concentration of STIM1, the lack of channel function in this mutant is not due to a decrease in the local concentration of STIM1 (8). However, it is possible that binding to the C-terminus is required to properly orient STIM1 for channel activation. Several studies have also analyzed the functional behavior of chimeras in which the N-terminus or portions thereof were swapped between the three Orai isoforms (54) (56). These experiments confirm the importance of the n-terminal region as a key

determinant of channel activity, but whether these effects on channel function arise due to alterations in channel gating, permeation, or STIM1 binding to Orai1 remain unclear.

8. CRAC CHANNEL SUBUNIT STOICHIOMETRY

Biochemical studies indicate that Orai subunits interact to produce both homomultimers and heteromultimers, suggesting that functional CRAC channels exits in multi-subunit complexes (5, 7, 29, 30, 46). Attempts to evaluate the stoichiometry of this interaction from purely biochemical assays, however, have been largely unsuccessful. Gwack *et al* (2007) reported that purified Orai1 co-migrates with STIM1 in glycerol-gradient centrifugation, and that this fraction runs as monomers and dimers on denaturing SDS-PAGE (29). Maruyama *et al* (2009) reported that purified Orai1 is 3x larger than a tetramer (57), and likewise, Park *et al* (2009) found that purified Orai1 elutes in a 290kDa complex (6). While these studies reaffirmed that Orai exists in a higher order oligomer, they did not, however, provide an easily interpretable Orai1 stoichometry.

In a different approach, the number of Orai1 copies in the CRAC channel complex was measured by counting the number of single-molecule photo-bleach steps of GFP per complex. Two labs applied this approach and determined that in the presence of STIM1-ct, which has been shown to constitutively bind and activate Orai1, there are 4 copies of Orai1 per channel complex (46, 58). When STIM1-ct is absent, however, Penna *et al* (2008) reported that most GFP-Orai1 complexes bleach in only 2 steps (46). Based on this result, they suggested that STIM1 assembles Orai1 dimers to form functional, tetrameric channels. In contrast, Ji *et al* (2008) found that co-expression of STIM1-ct did not affect the number of bleach steps, which occurred in 3 or 4 steps in both conditions (58). Moreover, they found that when tandem constructs containing only one GFP molecule are used, the number of photobleaching steps per complex decreased as expected depending on the number of Orai1 copies in the tandem construct. Since the cells were fixed for this experiment, it could be argued that fixation may have artificially induced STIM1 binding resulting in four Orai1 subunits per channel. However, in a control experiment, Orai1-Orai1 FRET was unaltered by fixation, arguing that fixation doesn't impact the stoichiometry of Orai1 (58). In a more recent study, Madl *et al* used a combination of photobleaching and single molecule brightness analysis on the mobile fraction of Orai1 in resting cells and concluded that Orai1 predominantly diffuses as a tetramer (59). They also showed that FRET between Orai1 dimers was unaltered upon store-depletion suggesting that the stoichiometry of Orai1 was independent of its association with STIM1 (59). Nonetheless the number of Orai1 copies per complex prior to STIM1 binding and CRAC activation remains controversial and a topic of active debate.

In another approach, Mignen *et al* (2008), exploited the ability of pore mutants of Orai1 (e.g., E106Q) to exert a powerful dominant-negative effect on CRAC channel activity without affecting surface expression (30, 60). They found that the ability of (monomeric) Orai1-E106Q to inhibit I_{CRAC} is eliminated when co-expressed with tandem wt Orai1 constructs containing four protomers (60). Moreover, a tandem tetramer containing only one copy of E106Q gave no current (full rather than partial inhibition), strongly suggesting that the functional channel is a tetramer (60). The results of this functional approach, thus, agree with the other studies indicating that active CRAC channels are tetrameric.

9. STIM:ORAI RATIO FOR OPTIMAL CRAC ACTIVITY

Given that both STIM and Orai must oligomerize to form the active CRAC channel complex, a key question is how many STIM1 molecules are required for channel activation. Since it is difficult to measure STIM1-Orai1 binding and CRAC activation simultaneously, more creative approaches were employed to answer this question. Li *et al* (2011) used tandem constructs to determine the effect of STIM:Orai1 ratio on CRAC channel activity (55). In their approach, tandem constructs with varying number of Orai1 protomers were fused to a STIM1 region containing the minimal activation domain (336-485, called S). Their results indicated that for tandem constructs in which the S:Orai1 ratio was 1:1 or 1:2, addition of a tandem S-S construct increased I_{CRAC} magnitude, but for complexes where the ratio is 2:1, the exogenous expression of S-S had no impact on current magnitude (55). Moreover, (when expressed alone) I_{CRAC} was largest for constructs with a 2:1 S-Orai1 ratio, and decreased as the STIM1-Orai1 ratio decreased (55). Their data suggested that a 2:1 STIM1:Orai1 ratio gives optimal CRAC activity. If each complex contains 4 copies of Orai1, then the active complex would have 8 copies of STIM1. Crucially, their data showed that if the STIM1:Orai1 ratio is less then optimal, I_{CRAC} is diminished but not eliminated.

Interestingly, data in Scrimgeour *et al* (2009) indicates that the STIM1:Orai1 ratio affects not only STIM1-dependent activation, but several additional gating processes as well (61). By varying the transfection ratio of full-length STIM1 and Orai1 in HEK293 cells, this study found that increasing the STIM1:Orai1 ratio increases fast inactivation rate and its extent, permeation of the ions Ba^{2+} and Sr^{2+}, as well as inhibition by the compound, 2-APB (61). Effects on Ba^{2+} and Sr^{2+} permeation were likely due to modification of calcium-dependent potentiation (CDP, see below). These results suggest that in addition to serving as a ligand to promote channel activity, STIM1 likely serves as a mobile subunit, influencing many key properties of the channel.

10. MOLECULAR DETERMINANTS OF CRAC CHANNEL PERMEATION AND SELECTIVITY

Perhaps the most prominent hallmark of CRAC channels, noted in virtually all biophysical studies of these channels, is their extraordinarily high Ca^{2+} selectivity. CRAC channels are amongst the most Ca^{2+} selective channels known ($P_{Ca}/P_{Na} > 1000$), which places them in a

unique category of highly Ca^{2+} selective channels together with voltage-gated Ca^{2+} (Ca_v) channels (62). Interestingly, high Ca^{2+} selectivity is only manifested in Ca-containing solutions, CRAC channels readily conduct a variety of small monovalent ions (Na^+, Li^+, and K^+) in divalent-free solutions (63-65), indicating that high Ca^{2+} selectivity is not an intrinsic feature of the CRAC channel pore but arises due to ion-ion and ion-pore interactions. This is clearly revealed by the blockade of monovalent currents by micromolar concentrations of Ca^{2+} (K_i~ 20 μM at -100 mV) (62-67). Occupancy by a single Ca^{2+} ion appears sufficient to block the large monovalent conductance, and, as expected for a binding site within the pore, Ca^{2+} block is voltage-dependent (28, 66). These characteristics are qualitatively reminiscent of the properties of L-type Ca_v channels, in which Ca^{2+} ions similarly bind tightly to a high-affinity binding site within the pore to occlude Na^+ flux (68, 69). In contrast to Ca_v channels, however, CRAC channels are virtually impermeable to the large monovalent cation, Cs^+ (P_{Cs}/P_{Na}<0.1). An early interpretation of this finding, supported by subsequent molecular studies, is that the pore of the CRAC channels is significantly narrower than Ca_v channels, resulting in steric inhibition of electrodiffusion of Cs^+ (28, 66).

Elucidation of the molecular basis of the CRAC channel's unique permeation properties began in earnest with the identification of the Orai and STIM proteins. Because permeation and gating properties of ion channels are typically shaped by the arrangement and chemistry of the pore-lining residues, several efforts focused on determining the important residues of the ion conduction pathway. Early efforts used site-directed mutagenesis of conserved acidic amino acids in the predicted transmembrane (TM) domains to identify the molecular determinants of Ca^{2+} selectivity. These studies resulted in the identification of several residues that influenced ion permeation, including E106 in TM1, E190 in TM3, and D110, D112, and D114 in the TM1-TM2 linker region of human Orai1 (Figure 1) (3-5) (28). Mutations of these residues resulted in a wide range of effects including loss of Ca^{2+} selectivity, increase in Cs^+ permeation, changes in the voltage-dependence of Ca^{2+} block, and for the TM1-TM2 loop residues, diminished La^{3+} block (3-5, 28). Based on these findings, early models of the CRAC channel pore concluded that TM1 and TM3 flank the ion conduction pathway with the Glu residues at 106 and 190 and the Asp residues in the TM1-TM2 loop forming coordinating sites for the conducting ions (5, 70).

While the mutagenesis studies provided a list of candidate residues that might regulate Ca^{2+} selectivity, these studies did not establish whether the residues were directly exposed to the ion conduction pathway or merely stabilized the pore. To address this issue, we recently applied the substituted cysteine accessibility method (SCAM) (71) to more directly illuminate the identity of pore-lining residues of active CRAC channels. In this approach, residues in the pore-lining region are mutated individually to Cys and the sensitivity of the mutated channels to blockade by aqueous thiol-labeling reagents, such as MTS reagents is assessed (71). The new information from this study confirmed that E106 and the TM1 segment flank the pore, but indicated that E190 and the TM3 residues are not pore-lining, forcing a revision of the prevailing model of the channel pore (72). Similar conclusions were reached in an independent study that examined the pattern of disulfide cross-linking of Cys residues introduced into Orai1 (73). From the SCAM study, we also found that the TM1-TM2 loop segments interact tightly with both large (> 8 Å) and small (< 3 Å) as well with positively-charged and negatively-charged probes, suggesting that these loops form an outer vestibule with sufficient flexibility to accommodate ions of different size and charge (72). Strong Cd^{2+} reactivity of several residues in TM1 indicated that the centrally located TM1 helices must be close to one another and therefore line a narrow pore, a feature that might be responsible for the low permeability to only small ions (< 3.8 Å) and the low unitary conductance of CRAC channels. Moreover, differences in the accessibility of probes of different sizes showed that the pore narrows sharply at the base of the vestibule, near the Ca^{2+} binding site formed by E106. These results provided the first step towards building a structural model of the open pore.

The revised model of Ca^{2+} selectivity at a single-locus (E106) is qualitatively reminiscent of the single-locus models proposed for voltage-gated Cav channels and the TRPV5/6 channels (74, 75). However, the architecture of the CRAC channel pore exhibits fundamental differences from Ca_v channels in many other critical respects. CRAC channels appear to have a long narrow pore flanked by the TM1 segment more or less entirely along the permeation pathway, but Ca_v channels are reported to have a wide pore with a inner vestibule large enough to accommodate very large thiol-reagents and one or more hydrated ions (76, 77). These differences in pore architecture may be responsible for the differences in permeability to large cations and conductance of the two channel types.

11. REGULATION OF CRAC CHANNELS

11.1. Calcium-dependent potentiation (CDP)

A distinctive feature of native CRAC channels is that in response to a switch from an external solution containing mM concentrations of Ca^{2+} to one containing only monovalents (divalent-free solution or DVF)), the initial spike of Na^+ current slowly decays over tens of seconds (63, 64). Conversely, restoring extracellular Ca^{2+} results in a slow recovery of the Ca^{2+} current (63, 64, 78) (67). The rate and degree of recovery of the divalent current also depends on the identity of the divalent carrying the current (67). This gating process has been named calcium-dependent potentiation, and has been well-characterized in immune cells (63, 78). The underlying gating process of this effect appears to mediated by a change in the number of active channels, that are either recruited (during CDP) to a high open probability (P_o) or shutoff (during depotentiation) from a high P_o state to a very low P_o state (66).

CDP appears to be directly dependent on the degree of pore occupancy by divalent ions, suggesting that

divalents with greater permeability cause a greater degree of potentiation (63, 67). This suggests that the divalent binding site that causes CDP is in the pore. In a heterologous system, HEK cells with STIM1 and Orai overexpressed and activated by IP_3 and BAPTA in the pipette, DeHaven *et al* (2007) showed that the extent of depotentiation in DVF was greater if the divalent in the previous solution was Ca > Ba >>> Mg for Orai1 and Orai2, and Ca~ Ba >>Mg for Orai3 (79). These divalent preferences mirror that of the selectivity sequence of CRAC channels: Ca >> Ba > Mg for Orai1 and Orai2, Ca ~ Ba > Mg for Orai3. Thus CDP is likely mediated by divalent binding somewhere in the permeation pathway. In Orai1, the E106D Orai1 mutation decreases divalent permeability and also deceases depotentiation of Na and Cs DVF solutions (28), suggesting that the Ca^{2+} selectivity and CDP sites are in close proximity.

There is some evidence to suggest that the conformational change responsible for CDP is not merely localized to the pore. Data from Scrimgeour *et al* (2009) shows that increasing the STIM1:Orai1 transfection ratio leads to some surprising effects on I_{CRAC} in Ba^{2+} or Sr^{2+} containing solutions versus Ca^{2+} solutions (61). Specifically, with a STIM1:Orai1 ratio of 4:1, the potentiation of the Ca^{2+} current appeared to be enhanced and depotentiation of the Ba^{2+} current diminished compared to a STIM1:Orai1 of 1:4 (61). Since STIM1 binds to the c-terminal domain (and possibly the n-terminus), and the CDP divalent binding site is likely near E106, which is on the outer mouth of the pore, it is clear that STIM1 must exert its affect on CDP through some long-range allosteric affect.

11.2. Fast inactivation

Fast inactivation is a prominent hallmark of CRAC channels involving feedback inhibition of channel activity by the high local (Ca^{2+}) around individual CRAC channels, resulting in current decay over 100-300 ms steps during hyperpolarizing steps (62) (80) (81). Multiple protein-protein interactions and motifs appear to be involved in this process, including an acidic region of the C-terminal region of STIM1 and calmodulin (CAM) binding to the N-terminus of Orai1 (82) (83) (84). An early indication for a role for STIM1 came from a study showing that increasing the STIM1:Orai1 transfection ratio increases the extent, rate, and calcium dependence of fast inactivation (61). This also suggested that multiple STIM1 must likely bind the CRAC channel to evoke fast inactivation. It light of this finding, much of the available data on fast inactivation is difficult to interpret because it is not clear in these studies whether the STIM1:Orai1 ratio was controlled between the different conditions. For example, mutations of several Orai1 regions (C-terminus, N-terminus, and the II-III loop are reported to affect inactivation, but it is difficult to know if these effects were really to the mutations or simply due to the mutants expressing at a different level than wt Orai1.

Fast inactivation is not affected by mutations of STIM1 in the N-terminus, including the D76A mutation, which renders STIM1 constitutively active (83). However, mutations or deletions of the region ~474-490 in the c-terminal domain of STIM1 significantly affect fast inactivation. In particular, neutralizing a set of negative charges in this region can enhance or inhibit fast inactivation (82) (83) (84). Mullins *et al* (2009) showed that a fragment (termed IDstim) corresponding to 470-491 binds calcium, and for a series of mutants within this region, the extent of fast inactivation correlated with the ability of this region to bind calcium. (82) These results suggest that calcium binding through charged residues in this region may affect STIM1-Orai1 interaction necessary to evoke the conformational change that occurs during fast gating. The exception, however, was EE482-483AA, which had reduced calcium binding affinity but enhanced fast inactivation. Therefore while calcium binding to this region can affect fast inactivation, it is not absolutely essential for fast inactivation. Since ~474-490 is *not* part of any of the identified minimal activation domains (CAD, SOAR, OASF), these data suggest that this region may allosterically modulate the interaction between the minimal binding domain and Orai.

Assuming that the expression levels are similar for all three Orai isomers, the available data suggests that fast inactivation of O3 > O2 > O1 (30) (84, 85). These differences have been exploited in order to determine the regions of Orai involved in regulation of fast inactivation. The results, however are confusing with one study highlighting the importance of three conserved glutamates in the C-terminus (84) and other results suggesting that non-conserved regions in the n-terminus are important (85). Because the relative expression of the different Orai mutations and chimeras were not systematically controlled, and given that the relative STIM:Orai expression ratio has a profound effect on the rate and degree of fast inactivation (61), the differing results from these studies are not easily reconcilable.

Interestingly, mutations in the putative selectivity filter of Orai1 that alter the ion selectivity of CRAC channels also strongly diminish fast inactivation (28). Diminished inactivation is not due to differences in channel expression or because of lower Ca^{2+} permeability of mutant channels (28). The molecular basis of this effect remains unknown. One possibility is that the inactivation gating mechanism is closely coupled to ion permeation, such that mutations that alter permeation also have effects on inactivation gating (28). A second possibility is that the mutations allosterically affect the gating mechanism, which is located elsewhere. Although the location of the inactivation gate is poorly understood, Srikanth *et al* (2010b) showed that mutations in the loop 2-3 region greatly decrease fast inactivation and enhance SOCE and I_{CRAC} amplitudes (86). Over-expressing a 37 amino acid peptide encompassing the 2-3 loop or including the peptide directly in the patch pipette resulted in dramatically reduced CRAC currents. These results were interpreted in terms of a model in which the intracellular loop acts as a blocking peptide to produce open channel blockade at the intracellular mouth of the channel (86). More tests are needed to elucidate if this peptide truly comprises the inactivation gate and determine how mutations in the selectivity filter might alter the its function.

11.3. Regulation during the cell cycle

It has long been known that Ca^{2+} signals regulate cell proliferation by controlling the progression of cells through the cell cycle, specifically by speeding exit from quiescence in the early G1 phase and the G1/S transition (87-89). Ca^{2+} exerts regulatory control over this process through allosteric activation (or inhibition) of several enzymes and by regulating gene expression through the Ca^{2+}-dependent transcription factors (88). Biochemical assays have shed some light on the underlying mechanisms by determining if the activities and/or expression of STIM and Orai are regulated during the cell cycle. One post-translational mechanism that has attracted attention is the modification of CRAC channel activity by phosphorylation of STIM1. Spassova *et al* (2006) identified three possible phosphorylation sites near the extreme c-terminus of STIM1 (90). Truncating STIM1 at position 597 eliminates all of these phosphorylation sites with little or no effect on I_{CRAC} in Jurkat cells and RBL cells (where native S1 was knocked down). Nonetheless, a separate study showed that the phosphorylation state of STIM1 changes during mitosis with accompanying suppression of SOCE (91). They identified several sites in the C-terminus of STIM1 that were detected by a phospho-specific antibody. Truncating STIM1 at 482 rescues SOCE during mitosis, but also decreased SOCE during interphase (91). By systematically neutralizing the putative phosphorylation sites, they found that that phosphorylation of S486 and S668 (between the S/P and K-rich domains) is responsible for most of the suppression of SOCE during mitosis (91). These data suggest that phosphorylation can affect STIM1 activity, and may be used by the cell to regulate SOCE. In contrast to STIM1 phosphorylation during mitosis, recent studies indicate that suppression of SOCE during meiosis is mediated primarily by internalization of Orai1, likely through a caveolin (Cav)- and dynamin-dependent endocytic pathway (92, 93). Thus, changes in both STIM1 and Orai1 proteins are likely involved in the changes in SOCE seen during cell cycle.

11.4. Modification of CRAC channel activity by 2-APB

Of the many modes of CRAC channel modulation, the effects of the compound, 2-aminoethoxydiphenylborate (2-APB) have garnered perhaps the widest attention (94). The complex effects of 2-APB illustrate the various ways in which CRAC channel gating can be altered, and its distinct effects on the different Orai isoforms provide a window into differences in gating behaviors of the three Orai isoforms. 2-APB was first described as a membrane-permeable inhibitor of ER-localized IP3 receptors (95). Subsequently, however, several studies demonstrated that the major effect of 2-APB on Ca^{2+} signaling was primarily through its ability to inhibit store-operated Ca^{2+} entry independently of IP3Rs (96, 97). 2-APB elicits opposing effects on the activity of CRAC channels at low and high concentration. Studies in the native CRAC channels of immune cells show that 2-APB potentiates Ca influx through CRAC channels at low concentrations (< 5 μM), but strongly inhibits I_{CRAC} at high concentrations (>20 μM) (96). 2-APB also modulates Ca^{2+}-dependent fast inactivation of CRAC channels, enhancing the rate of inactivation at low doses and completely eliminating it at high doses (96).

To elucidate the molecular mechanisms of these effects, several groups explored the effects of 2-APB on over-expressed Orai channels in HEK293 cells (98, 99) (100-102). These studies described several interesting differences in the responses of the three Orai isoforms to 2-APB. The behavior of Orai1 and Orai2 channels to 2-APB was reminiscent of the effects previously described for native CRAC channels: low concentrations (<10 uM) potentiate channel activity, whereas higher concentrations (>10 uM) potentiate and then slowly inhibit the current (98, 99) (100, 101). Notably, the potentiation by low doses of 2-APB doesn't alter the permeation properties of the channel (99, 102), and the inhibition at >10 uM becomes increasingly difficult to reverse the longer the channel is exposed to the drug (102). Orai2 appears to be only partially inhibited by high doses of 2-APB (100). In contrast to this bimodal behavior, Orai3 channels display strong activation with no obvious inhibition of the store-operated current (98, 99) (100-102). Activation of Orai3 by 2-APB is characterized by a striking change in permeation properties with a notable decrease in Ca^{2+} selectivity, increased permeability to the large monovalent cation Cs^{+}, and widening of the apparently narrow pore of the Orai3 channel (98, 99) (100-102). Interestingly, 2-APB activated non-selective Orai3 currents with the same biophysical properties even in the absence of STIM1 (98, 99) (100-102), indicating that STIM1 is not required for 2-APB activation of Orai3 channels.

Despite the fundamental differences in gating behaviors suggested by these studies, a later study showed that in addition to store-independent activation, 2-APB elicits a range of other effects on Orai3 channels, including STIM1-dependent potentiation at low concentrations (< 10 μM), and persistent inhibition of the store-operated current at high concentrations (> 20 μM) (102). Thus, the key effects of 2-APB seen in Orai1 and native CRAC currents also occur in store-operated Orai3 channels. Inhibition of Orai3 current by high doses of 2-APB is accompanied by elimination of STIM1-dependent fast inactivation and low-dose potentiation, suggesting that 2-APB causes functional uncoupling of STIM1 from Orai3 channels. Interestingly, by studying the rate of direct activation of Orai3 channels by high doses of 2-APB, this study found that Orai3 channels bound to STIM1 resist 2-APB gating while 2-APB suppresses STIM1-gating (102). By measuring the degree of inhibition of store-operated gating and the rate of direct activation in the same cells, they deduced that the rate of direct 2-APB activation of Orai3 channels increased linearly with the degree of STIM1-Orai3 uncoupling, suggesting that 2-APB has to first disengage STIM1 before it can directly gate Orai3 channels. These results established a mutually antagonistic role for STIM1 and 2-APB in the activation of CRAC channels.

The molecular mechanisms of potentiation, inhibition, and direct activation by 2-APB remain unclear. FRET experiments indicate that 2-APB slightly enhances STIM1-Orai and STIM1-Orai3 FRET (9, 102), and the coupling between the c-STIM and and Orai1 (103). Thus, it is possible that potentiation of Orai channels likely involves increased efficiency of channel coupling to STIM1. Such a

mechanism is also supported by previous findings from noise analysis indicating that potentiation by low doses of 2-APB arises from the recruitment of CRAC channels from a silent state to one of high open probability, resembling the stepwise recruitment of closed channels following store depletion (66). Thus, both 2-APB induced potentiation and the opening of CRAC channels by store depletion likely share common mechanisms involving increased binding of STIM1 with Orai channels. The molecular mechanisms of inhibition of store-operated gating and direct activation, however, remain less clear. By analyzing the rates of direct activation of Orai3 channels in their STIM1-bound and STIM1-free states, Yamashita *et al*, have argued that 2-APB functionally uncouples STIM1 from Orai3 channels (102). Such a mechanism is consistent with previous suggestions that inhibition of store-operated gating by 2-APB involves uncoupling of STIM1 from Orai1 (99, 100). It is important to note, however, that the inhibition of exogenously expressed Orai currents is not accompanied by physical dissociation of the two proteins (9, 102). One possibility is that 2-APB either competitively or allosterically displaces STIM1 from the N-terminus of Orai channels thereby inhibiting store-operated gating without interrupting tight STIM1 binding at the C-terminus. Direct tests of this hypothesis are needed to elucidate the complex effects of 2-APB on Orai channels.

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