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Store-Operated Orai Channels: Structure and Function

Murali Prakriya¹

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Feinberg School of Medicine, Chicago, Illinois, USA

Abstract

In many animal cells, store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels function as an essential route for Ca^{2+} entry. CRAC channels control many fundamental cellular functions including gene expression, motility, and cell proliferation, are involved in the etiology of 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 channel operation for several decades, the discovery of Orai1 as a prototypic CRAC channel protein and STIM1 as the endoplasmic reticulum (ER) Ca^{2+} sensor has led to rapid progress in our understanding of the mechanisms and functions of CRAC channels. It is now known that activation of CRAC channels following ER Ca^{2+} store depletion is governed by several events, which include the redistributions and accumulations of STIM1 and Orai1 into overlapping puncta at peripheral cellular sites, resulting in direct protein–protein interactions between the two proteins. In this chapter, I review the molecular features of the STIM and Orai proteins that regulate the gating and ion conduction mechanisms of CRAC channels.

1. 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. This signaling pathway is widespread in eukaryotic cells and is involved in a host of cellular functions ranging from gene expression to regulation of proliferation. Clinical studies have revealed that patients with mutations in CRAC channels suffer from a devastating immunodeficiency, muscle weakness, and abnormalities in the skin and teeth (Feske, 2009, 2010). Moreover, animal studies have implicated a growing list of possible diseases including allergy (Di Capite, Bates, & Parekh, 2011), multiple sclerosis (Ma, McCarl, Khalil, Luthy, & Feske, 2010; Schuhmann et al., 2010), cancer (Prevarskaya, Skryma, & Shuba, 2011), thrombosis (Varga-Szabo, Braun, & Nieswandt, 2011), and inflammatory bowel disease (McCarl et al., 2010) to loss or gain of CRAC channel activity, highlighting the potential importance of CRAC channels for human health and disease.

The store-operated channels (SOCs) of T lymphocytes and mast cells were the first to be characterized using electrophysiological techniques (Hoth & Penner, 1992; Zweifach & Lewis, 1993). These channels, termed calcium release-activated calcium (CRAC) channels, exhibit high Ca^{2+} selectivity and can be distinguished from other Ca^{2+} -selective channels based on their low unitary conductance and low permeability to large monovalent cations (Prakriya, 2009). CRAC channels are activated through the binding of the ER Ca^{2+} sensors stromal interaction molecule 1 (STIM1) and STIM2 to the CRAC channel proteins Orai1, Orai2, and Orai3 (Hogan, Lewis, & Rao, 2010; Lewis, 2011). The STIM proteins bind to

and directly activate Orai channels, and these two families of molecules can fully reconstitute SOCE in heterologous expression systems, indicating that these proteins are both necessary and sufficient for SOCE.

Discovery of the STIM and Orai proteins prodded tremendous advances in the molecular mechanisms of channel activation, regulation, and ion conduction. We now have a basic framework for how the channel is activated following ER Ca^{2+} store depletion. Human and animal studies have also illuminated the physiological roles of these molecules and their homologues (in mammals Orai2, Orai3, and STIM2) in many tissues. This chapter focuses on the molecular characteristics of STIM and Orai proteins that regulate the activation of CRAC channels and their ion conduction mechanisms.

2. IDENTIFICATION OF THE Orai PROTEINS

Although the first recordings of CRAC currents occurred in the late 1980s (Lewis & Cahalan, 1989), it was not until 2006 that Orai1 was identified as the prototypic CRAC channel protein (Feske et al., 2006; Prakriya et al., 2006; Vig, Beck, et al., 2006; Yeromin et al., 2006). In the intervening period, several candidate molecules including several TRP channels and voltage-gated Ca^{2+} channels were presented as possible candidates as CRAC channel pore (Prakriya & Lewis, 2003, 2004), only to be discarded due to inconsistencies in the pore properties of native CRAC channels and the candidate proteins. Ultimately, efforts that led to the identification of the CRAC channel protein ultimately harnessed the power of high-throughput screening, linkage analysis, and the human genome sequencing project, tools that became available only in the new millennium. An important step in this discovery was the identification of human patients with a severe combined immunodeficiency lacking CRAC channel function in T-cells (Feske, Giltman, Dolmetsch, Staudt, & Rao, 2001; Feske, Prakriya, Rao, & Lewis, 2005; Partiseti et al., 1994). These patients exhibited a devastating immunodeficiency characterized by impaired T-cell activation and effector gene expression (Feske et al., 1996), which confirmed earlier pharmacological and genetic evidence that CRAC channels orchestrate many aspects of lymphocyte development and function (Fanger, Hoth, Crabtree, & Lewis, 1995; Lewis, 2001). Feske et al. (2006) 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. 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 (Feske et al., 2006; Vig, Peinelt, et al., 2006; Zhang et al., 2006). A human ortholog of this protein mapped to the same region on chromosome 12 identified by linkage analysis (Feske et al., 2006). This molecule, named Orai1, is a widely expressed 33-kDa cell surface protein with four predicted transmembrane domains, intracellular N- and C-termini (Fig. 1.1B) and no significant sequence homology to other previously identified ion channels. The human SCID defect was found to arise from a missense mutation in Orai1 (R91W) that abrogated CRAC channel activity (Feske et al., 2006).

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

STIM1 was identified in 2005 as an ER Ca^{2+} sensor in two laboratories working independently through the use of RNAi screens for regulators of SOCE in *Drosophila* S2 (Roos et al., 2005) and mammalian cells (Liou et al., 2005). The molecule is a 77-kDa single-pass ER membrane protein, with a luminal N-terminal domain containing the signal peptide and C-terminal domain in the cytosol (Lewis, 2011). Both regions exhibit several domains critical to STIM1 function, including a sterile alpha motif (SAM) and two EF hands in the N-terminus (Fig. 1.1A) and three coiled-coil domains, a Ser/Pro-rich region, and a Lys-rich region in the C-terminus. 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 (Baba et al., 2006; Liou et al., 2005; Muik et al., 2008; Navarro-Borelly et al., 2008; Wu, Buchanan, Luik, & Lewis, 2006; Zhang et al., 2005). 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 (Baba et al., 2006; Liou et al., 2005; Muik et al., 2008; Navarro-Borelly et al., 2008; Wu et al., 2006; Zhang et al., 2005). The EF-hand and SAM domains (EF-SAM) mediate critical roles in this process. EF-hand *STIM1* mutants with impaired Ca^{2+} -binding form puncta and activate CRAC channels independently of ER Ca^{2+} store depletion (Liou et al., 2005; Zhang et al., 2005); the absence of Ca^{2+} binding in these mutants essentially tricks the molecules into responding as if stores are depleted. Moreover, deletion of the SAM domain abrogates oligomerization and puncta formation in response to store depletion (Baba et al., 2006), indicating that this domain mediates a critical role in initiating the *STIM1* conformational response to store depletion. 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-coil domain (Baba et al., 2006; Huang et al., 2006; Liou, Fivaz, Inoue, & Meyer, 2007; Park et al., 2009; Yuan et al., 2009). 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* fulfills 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

The conclusion that Orai1 forms the Ca^{2+} -selective pore of the CRAC channel came quickly following the identification of this molecule. One line of evidence came from studies showing that overexpression of Orai1 together with *STIM1* in HEK293 cells produced large currents (Mercer et al., 2006; Peinelt et al., 2006; Soboloff et al., 2006) with characteristics consistent with native CRAC channels including high Ca^{2+} selectivity, low Cs^+ permeability, and a narrow pore (Mercer et al., 2006; Peinelt et al., 2006; Yamashita, Navarro-Borelly, McNally, & Prakriya, 2007). Additional studies showed that other properties including Ca^{2+} block of Na^+ currents and pharmacological responses to 2-aminoethyl diphenyl borate (2-APB) and La^{3+} were similar to those seen for native CRAC channels (Yamashita et al., 2007; Yeromin et al., 2006). A second line of evidence came from mutation of a highly conserved acidic residue in Orai1 (E106D), which 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 (Prakriya et al., 2006; Vig, Beck, et al., 2006; Yeromin et al., 2006). In the ensuing years, cysteine-scanning studies identified the pore-lining residues of the CRAC channel (McNally, Yamashita, Engh, & Prakriya, 2009; Zhou, Ramachandran, Oh-Hora, Rao, & Hogan, 2010) and the crystal structure of the *Drosophila* Orai protein complex was determined, firmly establishing the Orai proteins as the Ca^{2+} -selective pores of CRAC channels. 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 function similarly in producing store-operated Ca^{2+} entry when coexpressed with *STIM1* in HEK293 cells (Gross et al., 2007; Gwack et al., 2007; Lis et al., 2007) and are widely expressed in most tissues (Gross et al., 2007; Gwack et al., 2007; Wissenbach, Philipp, Gross, Cavalie, & Flockerzi, 2007). However, Orai1 remains the best-studied CRAC channel protein and appears to be the predominant isoform mediating SOCE in most cells. By contrast, there is no direct genetic evidence for a role of Orai2 or Orai3 channels in any cell type yet.

5. THE CRAC CHANNEL IS ESSENTIAL FOR LYMPHOCYTE ACTIVATION

A well-established function of CRAC channels is the generation of long-lasting $[Ca^{2+}]_i$ elevations essential for Ca^{2+} -dependent gene transcription and cytokine production in lymphocytes. Evidence supporting such a role has come from several lines of study. SKF96365, an imidazole compound that inhibits CRAC channels, was also found to block IL-2 production in T-cells with similar efficacy (Chung, McDonald, & Gardner, 1994). Likewise, nanomolar concentrations of La^{3+} block I_{CRAC} , the $[Ca^{2+}]_i$ rise, and the induction of T-cell activation markers such as CD25 and CD69 in response to CD3- or thapsigargin stimulation (Aussel, Marhaba, Pelassy, & Breittmayer, 1996). Subsequent genetic studies provided compelling evidence that these pharmacological effects occur through blockade of CRAC channels. For example, mutant Jurkat T-cells lacking I_{CRAC} displayed severely attenuated production of cytokines such as IL-2 (Fanger et al., 1995). More recently, several studies of human patients have shown that severe immunodeficiencies arise from mutations in CRAC channels that render them inactive (Feske, 2010; Feske et al., 2001, 2006, 2005; Le Deist et al., 1995; Partiseti et al., 1994). The abrogation of CRAC channel function in these cells results in the elimination of Ca^{2+} elevations necessary to drive nuclear translocation of NFAT (Feske et al., 2001), an important and widely expressed transcription factor involved in cytokine gene expression (Hogan, Chen, Nardone, & Rao, 2003). Collectively, these studies established the critical role of CRAC channels for antigen-mediated T-cell activation and kindled strong interest in understanding the molecular basis of CRAC channel function.

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

Studies of the isolated STIM1 EF-hand SAM domain in solution indicate that the dissociation constant of Ca^{2+} binding is $\sim 500 \mu M$ (Stathopulos, Li, Plevin, Ames, & Ikura, 2006; Zheng et al., 2011), which is consistent with the range of Ca^{2+} concentrations known to exist in ER lumen (Demaurex & Frieden, 2003; Luik, Wang, Prakriya, Wu, & Lewis, 2008). Similar measurements for STIM2 indicate a lower affinity Ca^{2+} -binding affinity (Zheng et al., 2011), consistent with the ability of STIM2 to form puncta more readily in resting cells (Brandman, Liou, Park, & Meyer, 2007). Measurements of the dependence of $[Ca^{2+}]_{ER}$ on puncta formation indicate an apparent $K_{1/2}$ of $210 \mu M$ for STIM1 and $406 \mu M$ for STIM2 (Brandman et al., 2007), confirming that STIM2 needs significantly lower level of store depletion than STIM1 for puncta formation. This difference likely explains why STIM2, but not STIM1, promotes constitutive activation of SOCE when overexpressed with the Orai proteins (Bird et al., 2009; Brandman et al., 2007).

Structural studies of the isolated luminal domain fragments indicated that Ca^{2+} unbinding from the N-terminal EF-hand triggers the unfolding and aggregation of the luminal domain, resulting in the appearance of dimers and higher-order multimers (Stathopulos et al., 2006). This finding suggested that Ca^{2+} store depletion leads to the formation of higher-order oligomers of STIM1 (Stathopulos et al., 2006), a hypothesis that was confirmed in follow-up studies using FRET with full-length STIM1 (Covington, Wu, & Lewis, 2010; Liou et al., 2007). STIM1 oligomerization is an early step in the channel activation process, occurring well before STIM1 redistribution to the plasma membrane (Liou et al., 2007; Luik et al., 2008). 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 dimerizer leads to puncta formation and activation of SOCE independently of ER store depletion (Luik et al., 2008). Thus, STIM1 oligomerization serves as a critical upstream activation switch that unfolds all

subsequent steps of the channel activation process. These features are described in detail in Chapter 3 of this book.

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 (Baba et al., 2006; Liou et al., 2005; Luik, Wu, Buchanan, & Lewis, 2006; Zhang et al., 2005). Accumulation of STIM1 near the plasma membrane causes ER tubules to move toward the plasma membrane and STIM1 appears to facilitate this process (Wu et al., 2006), indicating that store depletion rearranges the ER, with STIM1 facilitating this change. Consistent with the idea that changes in $(\text{Ca}^{2+})_{\text{ER}}$ (and not cytoplasmic (Ca^{2+})) directly regulate this process, depletion of ER Ca^{2+} stores with TPEN, a low affinity membrane permeant Ca^{2+} buffer which is not expected to affect cytoplasmic (Ca^{2+}) , also causes STIM1 and Orai1 puncta formation (Gwozdz, Dutko-Gwozdz, Zarayskiy, Peter, & Bolotina, 2008). Luik and colleagues further 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} (Luik et al., 2008). 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, the appearance of STIM1 near the surface of the cell precedes the development of I_{CRAC} by ~ 10 s (Wu et al., 2006), indicating that STIM1 translocation is required for CRAC activation, but that the activation also requires additional steps.

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 (Liou et al., 2007; Muik et al., 2008), 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 (Liou et al., 2007), 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 overexpressing STIM1 alone: when coexpressed with Orai1, STIM1 Δ K accumulates into puncta to the same extent as that seen in full-length STIM1 and supports the normal extent of puncta formation (Park et al., 2009), though activation of I_{CRAC} is still delayed compared to full-length STIM1 (Li et al., 2007). Because the polybasic tail is not found in *C. elegans* or *Drosophila* STIM proteins, these results suggest that the polybasic domain is a vertebrate adaption that facilitates STIM1 migration to the plasma membrane but is not absolutely essential for Orai channel activation. It is also tempting to postulate in this context that the accumulation and stability of STIM1 at the ER–PM junctions are influenced by the strength of the STIM1 plasma membrane-binding interactions, with weaker interactions diminishing the efficacy of STIM1 migration and binding to the plasma membrane.

In contrast to STIM1 oligomerization, 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 (Baba et al., 2006; Grigoriev et al., 2008; Honnappa et al., 2009). STIM1 also coimmunoprecipitates with the MT-associated proteins, EB1 and EB3, indicating that STIM1 is closely associated with MTs (Grigoriev et al., 2008). The mechanistic features and functional relevance of this association, however, remain nebulous. The tubulovesicular STIM1 movements cease upon store depletion, and STIM1 that accumulates into puncta no longer colocalizes with MTs (Baba et al., 2006; Grigoriev et al.,

2008). Nocodazole, which depolymerizes MTs and therefore would be expected to severely impair STIM1 association with microtubules, does not affect puncta formation or even SOCE (Baba et al., 2006; Grigoriev et al., 2008), although it does eliminate the tubulovesicular STIM1 movements. Likewise, depletion of cellular ATP eliminates the tubulovesicular movement but does not impact puncta formation (Chvanov et al., 2008). 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 suggest 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 (Liou et al., 2007; Park et al., 2009).

7. CONFORMATIONAL CHANGES IN STIM1

Some reports have suggested that internal electrostatic interactions between different regions of STIM1 are critical for the transition from resting STIM1 oligomers to their active state (Korzeniowski, Manjarres, Varnai, & Balla, 2010). An acidic region in the CC1 domain was found to interact with a basic region in the CC2 domain to mask the active site of STIM1 that interacts with Orai1 (termed the CAD domain). Oligomerization appears to favor the removal of this internal autoinhibition to reveal the CAD and polybasic domains, thereby permitting productive interactions between CAD and Orai proteins leading to channel activation (Calloway, Holowka, & Baird, 2010; Korzeniowski et al., 2010). Similar conclusions were drawn by Muik et al. (2011) who made use of an intramolecular STIM1 FRET sensor to show that the cytoplasmic region of STIM1 that interacts with Orai1 switches from a closed to an open configuration upon interaction with Orai1. 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 (Muik et al., 2011). These findings, however, have recently been called into question by more recent evidence indicating that mutations that would be expected to disrupt the electrostatic interactions have no effects on STIM1 function (Yu, Sun, Hubrack, Selvaraj, & Machaca, 2013). Moreover, the recent structures of the CAD domain are not readily compatible with interactions between acidic and basic STIM1 regions (Yang, Jin, Cai, Li, & Shen, 2012). Thus, the molecular mechanisms underlying dimer formation and stimulus-induced conformational changes in STIM1 remain to be clarified.

8. STIM1 DIRECTLY BINDS TO Orai1

The mechanistic basis for store-dependent activation of CRAC channel activation involves direct binding of STIM1 and Orai1. Direct binding of the two proteins has been demonstrated by several methods using coimmunoprecipitation (Parvez et al., 2008; Penna et al., 2008; Vig, Beck, et al., 2006; Yeromin et al., 2006), FRET microscopy (Muik et al., 2008; Navarro-Borelly et al., 2008), and pull downs (Park et al., 2009). Orai1 and STIM1 associate even in a system of only purified components in solution (Park et al., 2009; Zhou, Ramachandran, Oh-Hora, Rao, & Hogan, 2010), 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.

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 (Fig. 1.1). 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. They also found that deletion of a large stretch of this domain that includes the coiled-coil motifs (amino acids 231–535) eliminated constitutive activity of this fragment (Huang et al., 2006). 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. Subsequently, several groups identified a minimal region in STIM1 encompassing the second and the third CC domains as the critical element required for binding and activating Orai (Kawasaki, Lange, & Feske, 2009; Muik et al., 2009; Park et al., 2009; Yuan et al., 2009). This region is variously called the CRAC activation domain (CAD) (Park et al., 2009), STIM1–Orai1-activating region (SOAR) (Yuan et al., 2009), or Ccb9 (Kawasaki et al., 2009) and includes the amino acids 342–444. The structure of this domain has recently been solved, revealing an R-shaped dimeric module with the main functional domains (CC2 and CC3), forming a hair-pin motif (Yang et al., 2012). Whether this dimeric module represents the active state structure of CAD and how this differs from its resting-state configuration is not known yet; nevertheless, this high-resolution structure provides several testable hypotheses for dissecting the structural underpinnings of STIM1 function.

Interestingly, the regions involved in STIM1–Orai1 binding overlap significantly with the domains found to be important for induced STIM1–STIM1 oligomerization. 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, do not self-associate *in vivo*, and fail to activate Orai1. 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 and puncta formation overlaps with the CAD/SOAR region. One critical mutation identified by their analysis, A369K, exhibited enhanced resting-state oligomerization, colocalization with Orai, and constitutive CRAC channel activity, while at the same time nearly eliminating enhancement of oligomerization upon store depletion (Covington et al., 2010). A second mutation, A376K, caused STIM1 to constitutively self-associate and form puncta, but eliminated colocalization 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 (Covington et al., 2010), a prediction borne out in the crystal structure of the CAD domain. Collectively, these results suggest that residues important for Orai1 binding are also important for STIM oligomerization.

9. Orai1 DOMAINS INVOLVED IN STIM1 BINDING

Structure–function and binding studies have identified two STIM1-binding sites on Orai1, located one each on the C- and N-termini. Early studies, which deleted the Orai1 C-terminus, found that CRAC channel activation and STIM1–Orai1 FRET following store depletion were eliminated, suggesting the presence of an important STIM1-binding site in this region (Li et al., 2007; Muik et al., 2008; Park et al., 2009). *In vitro* pull-down assays with isolated fragments of Orai1 subsequently indicated that the Orai1 C-terminal domain fragments encompassing the region between residues 254–301 associate with the cytosolic domains of STIM1 (Muik et al., 2009; Park et al., 2009; Zhou, Meraner, et al., 2010), 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 a coiled-coil domain, and two point mutations in this region (L273S and L276D) completely disrupt Orai1–STIM1 interaction (Muik et al., 2008; Navarro-Borelly et al., 2008), likely due to disruption of the tertiary structure of the Orai1 CC domain (Frischauf et al., 2009). Interestingly, the recent crystal structure of Orai1 reveals that the C-terminal helices of neighboring subunits form an antiparallel coiled-coil domain and directly interact with each other, with residues L273 and L276 of one subunit contacting L276 and L273 residues of the conjugate subunit (Hou, Pedi,

Diver, & Long, 2012). The study speculates that the hydrophobic interface formed by the antiparallel CC domains opens to interact with the CAD domain during STIM1 binding. An alternate possibility suggested by the structure is that the antiparallel domains stay together even when bound to STIM1, and that the STIM1-binding site on the CRAC channel is formed collectively by the combined surface of the antiparallel coiled-coil domain. These possibilities remain to be formally tested.

Mutations of other nearby hydrophobic residues (F279 and L282) have no effect on I_{CRAC} or STIM1–Orai1 FRET (Muik et al., 2008; Navarro-Borelly et al., 2008). Thus, the role of the hydrophobic residues in the middle of the coiled-coil 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-coil domain does not reduce STIM1–Orai1 FRET or SOCE (Calloway, Vig, Kinet, Holowka, & Baird, 2009). Neutralizing all six residues, however, diminished STIM1–Orai1 FRET and SOCE (Calloway et al., 2009), suggesting that the charged residues are important for functional SOCE. The structural basis of these results remains unclear, but it seems possible that elimination of all charged residues destabilizes the alpha helix.

In addition to the well-described interaction at the C-terminus of Orai1, studies using a system of purified components have revealed a second interaction site on Orai1, located at the N-terminus. A purified peptide consisting of the cytosolic N-terminal region corresponding to the region 68–91 interacts with CAD in coimmunoprecipitation and split-ubiquitin assays (Park et al., 2009). Further, GST pull-down assays have shown direct interaction of a purified N-terminal fragment 65–87 with purified STIM1-ct and the STIM1 C-terminal fragment 233–498 (Zhou, Meraner, et al., 2010). However, because these biochemical assays test for interaction in solution, it is unclear whether these results apply to full-length molecules in live cells.

Functional studies have indicated differing roles for the C- and N-terminal sites. In contrast to the complete elimination of STIM1 binding seen in the C-terminal mutations, N-terminal deletions were found to retain significant levels of STIM1–Orai1 binding, and yet, these deletions abrogated SOCE and I_{CRAC} (Li et al., 2007; Lis, Zierler, Peinelt, Fleig, & Penner, 2010; Muik et al., 2008; Park et al., 2009). A construct with only 73–84 deleted fails to support CRAC activity when coexpressed with STIM1 (Park et al., 2009). Yet, N-terminal deletion mutants localize to the plasma membrane, and upon store depletion form puncta (Li et al., 2007) and support increases in Orai1–STIM1 FRET (Muik et al., 2008). These data suggested that the functional effects of N-terminal deletions on CRAC activity may not be due solely to decreased binding to STIM1. However, a more recent study from our lab indicates that the N-terminus contributes significantly to the overall stability of STIM1–Orai1 binding with deletions and mutations at this site strongly diminishing Orai1 recruitment into puncta and STIM1–Orai1 binding (McNally, Somasundaram, Jairaman, Yamashita, & Prakriya, 2013). We also found that completely deleting the C-terminal site resulted in nonfunctional channels even when CAD was directly tethered to the Orai1 C-terminus, indicating that the C-terminal site has a role in gating in addition to its known role in binding STIM1. Thus, the C- and N-terminal STIM1-binding sites are both essential for multiple aspects of Orai1 function including STIM1–Orai1 association, Orai1 trapping, and channel gating.

10. CRAC CHANNEL SUBUNIT STOICHIOMETRY

There is strong evidence that the Orai subunits interact with each other to form a multisubunit complex (Gwack et al., 2007; Lis et al., 2007; Navarro-Borelly et al., 2008;

Penna et al., 2008; Vig, Beck, et al., 2006; Zhou, Meraner, et al., 2010). However, attempts to evaluate the stoichiometry of this interaction from biochemical and functional assays have proven controversial. Gwack et al. (2007) reported that purified Orai1 comigrates with STIM1 in glycerol-gradient centrifugation, and that this fraction runs as monomers and dimers on denaturing SDS-PAGE. Maruyama et al. (2009) reported that purified Orai1 is 3× larger than a tetramer, and likewise Park et al. (2009) found that purified Orai1 elutes in a 290-kDa complex. While these studies reaffirmed that Orai exists in a higher-order oligomer, they did not provide an easily interpretable Orai1 stoichiometry.

Two labs applied the subunit-counting approach wherein channel stoichiometry is evaluated by counting the number of photobleach steps of GFP fused to Orai1 monomers (Ulbrich & Isacoff, 2007). With this approach, these studies concluded that Orai1 channels stably bound to soluble STIM1-ct have 4 Orai1 copies per channel complex (Ji et al., 2008; Penna et al., 2008). However, in the absence of STIM1, the two groups came to differing conclusions. Penna et al. (2008) reported that most GFP–Orai1 complexes bleach in only two steps. Likewise, Orai3 channels gated directly by the small molecule, 2-APB, were found to bleach mostly in two steps (Demuro et al., 2011). These results were interpreted in favor of model in which Orai exists as a dimer in the resting state, with STIM1 assembling the Orai1 dimers to form functional, tetrameric channels. In contrast, Ji et al. (2008) found that coexpression of STIM1-ct did not affect the number of bleach steps, which occurred in three or four steps in both conditions (Ji et al., 2008). In an alternate approach, Madl et al. (2010) 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. 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). In yet another approach, Mignen, Thompson, and Shuttleworth (2008) exploited the ability of pore mutants of Orai1 (e.g., E106Q) to suppress CRAC channel activity through a dominant-negative effect (Mignen et al., 2008). They found that the ability of (monomeric) Orai1-E106Q to inhibit I_{CRAC} is eliminated when coexpressed with tandem wt Orai1 constructs containing four protomers (Mignen et al., 2008). Taken together, these studies agreed that the active channel is a tetramer, although they reached different conclusions on the resting-state stoichiometry. All of these studies, however, suffer from the caveat that while they indicate that the minimal stoichiometry must be at least four subunits, they do not rule out the possibility that the stoichiometry may in fact be more than four.

The most definitive evidence for the subunit stoichiometry of the CRAC channel has come from the recent crystal structure of the *Drosophila* Orai1 channel (Hou et al., 2012). This structure of the closed channel (crystallized in the absence of STIM1) revealed a multimeric complex composed of six Orai subunits arranged in a threefold axis of symmetry. The hexameric stoichiometry is consistent with the published evidence from size-exclusion and light scattering studies showing a complex of 290 kDa (Park et al., 2009). Thus, the structure of the purified Orai complex has produced a model that differs from all previous studies of stoichiometry. It remains unclear, however, whether the purified hexameric complex exhibits the canonical properties of CRAC channels including high Ca^{2+} selectivity and low permeability to Cs^{+} . Additional studies are clearly needed to evaluate the findings predicted from the structure, and electrophysiological and molecular approaches should provide healthy debate on this issue.

11. HOW MANY STIM MOLECULES DOES IT TAKE TO ACTIVATE CRAC CHANNELS?

To examine the functional stoichiometry of CRAC channel activation by STIM1, Li et al. (2011) used tandem constructs to determine the effect of STIM:Orai1 ratio on CRAC channel activity (Li et al., 2010). 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 (Li et al., 2010). 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 (Li et al., 2010). 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 than optimal, I_{CRAC} is diminished but not eliminated.

In an alternate approach, Hoover and Lewis varied the relative expression of full-length STIM1 and Orai1 that were fused to mCherry and GFP to study the functional requirements of Orai1 activation as well as trapping at the ER-PM junctions (Hoover & Lewis, 2011). Their results confirmed that activation is graded with increasing STIM1 concentration, but in contrast to the findings using concatenated constructs, this study found that the dependence on STIM1 concentration is highly nonlinear (Hoover & Lewis, 2011). Maximal CRAC current activation requires the binding of two STIM1 molecules per Orai1 subunit and declines sharply with diminishing STIM1 such that the minimal stoichiometry for trapping Orai proteins into puncta fails to evoke significant activation (Hoover & Lewis, 2011). These results were interpreted in terms of a model in which once an optimal STIM threshold is reached, individual channels open abruptly to their fully active state in an all-or-none manner due to high cooperativity of channel opening. At face value, this model is consistent with findings indicating that the slow increase in I_{CRAC} following store depletion occurs from the stepwise recruitment of closed channels to a very high P_o state (Prakriya & Lewis, 2006). However, whether this type of modal gating occurs due to STIM1 binding or represents an intrinsic property of Orai proteins remains to be resolved.

Interestingly, there is strong evidence that the STIM1:Orai1 ratio affects not only STIM1-dependent activation but also calcium-dependent fast inactivation (CDI; Mullins, Park, Dolmetsch, & Lewis, 2009; Scrimgeour, Litjens, Ma, Barritt, & Rychkov, 2009). An acidic region in the C-terminus of STIM1 (amino acids 470–491) is critical for this gating process although the precise mechanism by which this domain confers fast inactivation remains unclear (Derler et al., 2009; Lee et al., 2009; Mullins et al., 2009). STIM1/Orai1 ratio also affects permeation to Ba^{2+} and Sr^{2+} , as well as inhibition by the compound, 2-APB (Scrimgeour et al., 2009). 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.

12. STRUCTURAL BASIS OF SELECTIVITY AND PERMEATION

CRAC channels are widely noted for their exquisite Ca^{2+} selectivity ($P_{\text{Ca}}/P_{\text{Na}} > 1000$), which places them in a unique category of highly Ca^{2+} -selective channels together with voltage-gated Ca^{2+} (Ca_v) channels (Hoth & Penner, 1993). 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 (DVF) solutions

(Bakowski & Parekh, 2002; Lepple-Wienhues & Cahalan, 1996; Prakriya & Lewis, 2002), 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+} ($K_i \sim 20 \mu\text{M}$ at -100 mV) (Bakowski & Parekh, 2002; Hoth & Penner, 1993; Lepple-Wienhues & Cahalan, 1996; Prakriya & Lewis, 2002, 2006; Su, Shoemaker, Marchase, & Blalock, 2004). 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 (Prakriya & Lewis, 2006; Yamashita et al., 2007). 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 (Lansman, Hess, & Tsien, 1986; Sather & McCleskey, 2003). In contrast to Ca_v channels, however, CRAC channels are virtually impermeable to the large monovalent cation, Cs^+ ($P_{\text{Cs}}/P_{\text{Na}} < 0.1$). 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^+ (Prakriya & Lewis, 2006; Yamashita et al., 2007).

To identify the Orai1 residues that are directly exposed to the ion conduction, one study applied the substituted cysteine accessibility method (SCAM) (McNally et al., 2009). 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 (Karlin & Akabas, 1998). The results of this study indicated that residues in TM1 flank the pore and ruled out TM3 and, specifically, E190 as pore-lining residues (McNally et al., 2009). Similar conclusions were reached in an independent study that examined the pattern of disulfide cross-linking of Cys residues introduced into Orai1 (Zhou, Ramachandran, et al., 2010). The SCAM study also indicated that the TM1–TM2 loop segments interact tightly with both large ($>8 \text{ \AA}$) and small ($<3 \text{ \AA}$), and 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 (McNally et al., 2009). Strong Cd^{2+} reactivity of several residues in TM1 indicated that the centrally located TM1 helices are close to one another and therefore line a narrow pore, a feature that is likely to account for the low permeability of the CRAC channel to large cations ($>3.8 \text{ \AA}$) and its low unitary conductance. 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 toward building a structural model of the open pore and were largely confirmed by the recent crystal structure of the *Drosophila* Orai protein (Hou et al., 2012).

The crystal structure of *Drosophila* Orai added new insights into the structural basis of permeation in CRAC channels (Hou et al., 2012). The structure confirmed that TM1 flanks the ion conduction pathway, and with the exception of F99, the pore-lining residues observed in the structure matched the residues found from cysteine accessibility, including E106, V102, L95, and R91 (Hou et al., 2012). In the middle of the TM1 segment, the X-ray crystal structure found that the side chain of F99 projects into the pore (Hou et al., 2012). By contrast, the cysteine accessibility study indicated that the pore-lining residue at this position originates from G98 (McNally, Somasundaram, Yamashita, & Prakriya, 2012; McNally et al., 2009). The reason for this discrepancy between the cysteine scan and the crystal structure could indicate a possible structural alteration caused by introducing the Cys mutation but may also reflect a difference between the structures of closed and open channels that were the basis of the results in the X-ray crystallography and accessibility studies, respectively. The closed structure revealed a Ca^{2+} ion density a few angstroms above the predicted selectivity filter formed by E106 rather than at the selectivity filter itself (Hou et al., 2012). In addition, this density overlaps significantly with the electron density

for lanthanide ions, a result that disagrees with functional evidence indicating that lanthanide ion binding occurs at the outer mouth of the pore rather than at the selectivity filter in open channels (McNally et al., 2009; Yeromin et al., 2006). These differences could be reconciled by supposing that the energy profiles for ion-binding sites significantly differ in the open state resulting in different positions for the ion densities in the open channel from what was seen for the closed channel. The crystal structure provides a framework for investigating selectivity and permeation in CRAC channels but clearly much more needs to be understood before the structure can be incorporated into plausible models for selectivity and permeation. Ultimately, the channel structures of both the closed and open states are required to illuminate the dynamics of the steps of CRAC channel ion transport cycle.

13. STIM1 REGULATES CRAC CHANNEL ION SELECTIVITY

A surprising functional aspect of CRAC channels revealed by analysis of the ion selectivity of a mutant Orai1 channel is that STIM1 not only controls CRAC channel gating but also bestows many fundamental features that have historically defined the fingerprint of the CRAC channel pore (McNally et al., 2012). A variety of substitutions at the pore-lining residue, V102, including substitutions to Cys, Ala, Ser, and Thr, produce constitutively open channels that are open even in the absence of bound STIM1 (McNally et al., 2012). The ion selectivities of the STIM1-bound and -free channels, however, are strikingly different. STIM1-free V102C mutant channels exhibit poor Ca^{2+} selectivity and allow permeation of Cs^+ and several other large cations that are normally impermeable through CRAC channels (McNally et al., 2012). Enabling the mutant channels to interact with STIM1, however, restores high Ca^{2+} selectivity to the poorly selective STIM1-free channels. In effect, the aberrant ion selectivity of the STIM1-free mutant channels is corrected following STIM1 binding (McNally et al., 2012). These changes are accompanied by alterations in the pore geometry, specifically, a significant narrowing of the pore to state that more closely resembles the dimensions seen for wt Orai1 channels. Critically, this tuning of Orai1 ion selectivity by STIM1 is not unique to the V102C mutant channels but is also seen in wild-type Orai1 channels as the amount of STIM1 bound to Orai1 is increased (McNally et al., 2012), suggesting that the V102X mutations merely unmask a native intermediate channel activation state due to a leaky gate.

These surprising results reveal that STIM1-free Orai1 channels are intrinsically poorly Ca^{2+} selective. Instead, the distinguishing characteristics of CRAC channels including high Ca^{2+} selectivity, low Cs^+ permeability, and a narrow pore are bestowed to the otherwise poorly Ca^{2+} -selective Orai1 channels by STIM1. How does STIM1 modulate Orai1 ion selectivity? Given that the Orai1 N-terminus bears a STIM1-binding site in close proximity to the pore-forming TM1 segment (Fig. 1.1A), it is not difficult to envision that STIM1 binding to the N-terminus could exert powerful effects on the energetic stability of the selectivity filter.

The finding that STIM1 regulates Orai1 selectivity has many important implications for the nature of Ca^{2+} signals arising from opening of Orai1 channels under different conditions and even the identity of native cation currents activated by non-STIM1–Orai1 activators. The ability of CRAC channels to conduct Na^+ under certain conditions may expand their potential functions to include novel modes by which they encode and process cellular information. Because emerging evidence suggests that CRAC channels, aside from activation by STIM1, can also be activated in a STIM1-independent fashion by other ligands, including the small molecule, 2-APB (DeHaven, Smyth, Boyles, Bird, & Putney, 2008; Peinelt, Lis, Beck, Fleig, & Penner, 2008; Schindl et al., 2008; Yamashita, Somasundaram, & Prakriya, 2011; Zhang et al., 2008), and the Golgi Ca^{2+} -ATPase, SPCA2 (Feng et al., 2010), these findings raise the possibility that Orai1 channels may function

either as highly Ca^{2+} -selective channels or nonselective channels depending on the nature of the upstream activation signal.

In addition, the tight coupling of permeation and gating found for CRAC channels provides an alternative perspective on ion channel gating that contradicts conventional ion channel postulates on the separation of gating and selectivity. The picture that emerges is of a hydrophobic gate (V102) located in close proximity to the selectivity filter (E106); the proximity of the two structures likely results in variety of conformational alterations in the selectivity filter during gating. Further, these findings reinforce the emerging viewpoint that there is much more happening in the vicinity of the selectivity filter in ion channels than initially imagined (Contreras, Srikumar, & Holmgren, 2008; Thompson & Begenisich, 2012).

14. REGULATION OF CRAC CHANNELS BY CALCIUM

14.1. Calcium-dependent potentiation

In response to a switch from an external solution containing millimolar concentrations of Ca^{2+} to one containing only monovalents (DVF solution), the initial spike of Na^+ current slowly decays over tens of seconds (Lepple-Wienhues & Cahalan, 1996; Prakriya & Lewis, 2002). Conversely, restoring extracellular Ca^{2+} results in a slow recovery of the Ca^{2+} current (Lepple-Wienhues & Cahalan, 1996; Prakriya & Lewis, 2002; Su et al., 2004; Zweifach & Lewis, 1996). The rate and degree of recovery of the divalent current also depends on the identity of the divalent carrying the current (Su et al., 2004). This gating process has been named CDP to reflect the enhancement of channel activity induced by calcium ions and has been well-characterized in immune cells (Prakriya & Lewis, 2002; Zweifach & Lewis, 1996). The underlying gating process of this effect appears to be mediated by a change in the number of active channels that are either recruited (during CDP) to a high open probability (P_o) or closed (during depotentiation) from a high P_o state to a very low P_o state (Prakriya & Lewis, 2006).

CDP appears to be directly dependent on the degree of pore occupancy by divalent ions, and divalents with greater permeability cause a greater degree of potentiation (Prakriya & Lewis, 2002; Su et al., 2004). This finding suggests that the divalent binding site that causes CDP is in the pore. Likewise, in cells over-expressing STIM1 with different Orai proteins, DeHaven et al. (2007) showed that the extent of potentiation depends on the identity of the divalent ion according to the sequence: $\text{Ca} > \text{Ba} \gg \text{Mg}$ (Orai1 and Orai2), or $\text{Ca} \sim \text{Ba} \gg \text{Mg}$ (Orai3) (DeHaven et al., 2007). These divalent preferences mirror that of the selectivity sequence of CRAC channels: $\text{Ca} \gg \text{Ba} > \text{Mg}$ for Orai1 and Orai2, and $\text{Ca} \sim \text{Ba} > \text{Mg}$ for Orai3. Although these results narrow the location of the CDP site to somewhere in the permeation pathway, other critical mechanistic and structural aspects of the phenomenon are unclear. In Orai1, the E106D Orai1 mutation decreases divalent permeability and also decreases depotentiation of Na and Cs DVF solutions (Yamashita et al., 2007), 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 also regulated by STIM1. Data from Scrimgeour et al. (2009) show 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. 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 (Scrimgeour et al., 2009). 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.

14.2. Calcium-dependent 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 (Fierro & Parekh, 1999; Hoth & Penner, 1993; Zweifach & Lewis, 1995). 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 binding to the N-terminus of Orai1 (Derler et al., 2009; Lee et al., 2009; Mullins et al., 2009). 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 (Scrimgeour et al., 2009). This also suggested that multiple STIM1 must likely bind the CRAC channel to evoke fast inactivation. In 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 due 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 (Derler et al., 2009). 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 (Derler et al., 2009; Lee et al., 2009; Mullins et al., 2009). Mullins et al. (2009) showed that a fragment (termed ID_{STIM}) 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. 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 Orai1.

Assuming that the expression levels are similar for all three Orai isomers, the available data suggest that the extent of fast inactivation varies among the Orai orthologs in the sequence Orai3>Orai2>Orai1 (Frischauf et al., 2011; Lee et al., 2009; Lis et al., 2007). 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 (Lee et al., 2009) and other results suggesting that nonconserved regions in the N-terminus are important (Frischauf et al., 2011). A major caveat in these studies is that the relative expression of the different Orai mutations and chimeras was not systematically controlled. Given recent evidence indicating that the relative STIM:Orai expression ratio has a profound effect on the rate and degree of fast inactivation (Scrimgeour et al., 2009), 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 (Yamashita et al., 2007). Diminished inactivation is not due to differences in channel expression or because of lower Ca^{2+} permeability of mutant channels (Yamashita et al., 2007). 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 (Yamashita et al., 2007). 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, Jung, Ribalet, and Gwack (2010) showed that mutations in the loop 2–3 region greatly decrease fast inactivation and enhance SOCE and I_{CRAC} amplitudes. Overexpressing 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 (Srikanth et al., 2010). More tests are needed to elucidate if this peptide truly comprises the inactivation gate and determine how mutations in the selectivity filter might alter its function.

15. CONCLUSIONS

The identification of the STIM and Orai protein families has produced dramatic advances in many aspects of CRAC channel function. In particular, we now have a firm mechanistic framework for understanding how CRAC channels are activated by depletion of intracellular Ca^{2+} stores, and we know the key structural elements involved in ion permeation and selectivity. However, there remain many broad unresolved issues. A major unknown is the gating mechanism of the channel: how does STIM1 binding to Orai1 open the pore? As described above, the structure of the closed channel is now available, many critical steps of STIM1 activation have been described in considerable detail, and recent studies are beginning to dissect the conformational changes that occur during gating. We can therefore expect the coming years to provide steady progress on the possible structural mechanisms of CRAC channel gating. Another unknown relates to the mechanisms and functions of the “other” Orai and STIM proteins, Orai2, Orai3, and STIM2. The study of these noncanonical CRAC channel proteins is currently constrained by total lack of genetic evidence in both animals and human for the physiological roles of these proteins. However, recent evidence highlighted in the chapters in this volume from Shuttleworth, Trebak, and their colleagues reveals fascinating differences in the behavior of these proteins from those of canonical CRAC channels, including but not limited to store-independent regulation and altered ion conduction properties. The development of genetic tools such as specific knockouts and/or transgenic mice should further open the door for providing new information on the physiological roles of these proteins. Finally, although some work has occurred in the development of small molecular drugs against CRAC channels, the mechanism and targets of most CRAC channel inhibitors remain unclear. Recent breakthroughs in solving the crystal structures of the CRAC channel proteins should influence structure-based drug design and hopefully provide a new generation of CRAC channel blockers with therapeutic potential.

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Figure 1.1.

(A) A schematic representation of STIM1 and its key functional domains. These domains include: Sig, signal peptide; SAM, sterile alpha motif; TM, transmembrane domain; CC, coiled-coil domain; CAD, CRAC activation domain; ID_{STIM1}, inactivation domain of STIM1. (B) The predicted topology of Orai1. The STIM1-binding domains are shaded in pink. Key residues (mentioned in the text) are labeled.