Differential Roles of the C and N Termini of Orai1 Protein in Interacting with Stromal Interaction Molecule 1 (STIM1) for Ca\(^{2+}\) Release-activated Ca\(^{2+}\) (CRAC) Channel Activation*\(^{[3]}\)

Received for publication, January 3, 2013, and in revised form, February 27, 2013. Published, JBC Papers in Press, February 27, 2013, DOI 10.1074/jbc.M113.450254

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Background: STIM1-operated Orai1 channels mediate Ca\(^{2+}\) entry for T cell activation, but the gating mechanism remains elusive.

Results: The C and N termini of Orai1 have differential roles in STIM1-triggered channel activation.

Conclusion: The binding between STIM1 and the Orai1 C terminus docks STIM1 onto the Orai1 N terminus, leading to channel activation.

Significance: This stepwise gating mechanism contributes to ER-PM crosstalk for Ca\(^{2+}\) entry, a fundamental process in cell biology.

The entry of extracellular Ca\(^{2+}\), which is mediated by Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels, is essential for T cell activation and the normal functioning of other immune cells. Although the molecular components of CRAC channels, the Orai1 pore-forming subunit and the STIM1-activating subunit have been recently identified, the gating mechanism by which Orai1 channels conduct Ca\(^{2+}\) entry upon Orai1-STIM1 interaction following Ca\(^{2+}\) store release remains elusive. Herein, we show that C-terminal truncations or point mutations prevented Orai1 from binding to STIM1 and subsequent channel opening. In contrast, an Orai1 mutant with an N-terminal truncation interacted with but failed to be activated by STIM1. Moreover, Orai1 channels with C-terminal disruption, but not N-terminal truncation, could be gated by fused functional domains of STIM1. Interestingly, the channel activities of Orai1 mutants carrying either an N-terminal or a C-terminal truncation were restored by a methionine mutation at the putative gating hinge, the conserved Gly-98 site in the first transmembrane segment (TM1) of Orai1. Collectively, these results support a stepwise gating mechanism of STIM1-operated Orai1 channels; the initial binding between STIM1 and the C terminus of Orai1 docks STIM1 onto the N terminus of Orai1 to initiate conformational changes of the pore-lining TM1 helix of Orai1, leading to the opening of the channel.

The first observation that severe combined immunodeficiency (SCID) syndrome can be related to defective calcium release-activated calcium (CRAC)\(^{2}\) channels was made in 1996 and linked this condition to a failure of T cell activation and cytokine production (1). This T cell dysfunction was attributed to diminished store-operated Ca\(^{2+}\) entry through CRAC channels (2), which appears to influence Ca\(^{2+}\)-dependent gene expression via the calcineurin/nuclear factor of activated T-cells (NFAT) pathway (3–5) and is the result of a point mutation (R91W) within the Orai1/CRACM1 gene coding for the pore-forming subunit of CRAC channels in the plasma membrane (PM) (6–9).

Endogenous CRAC channels in T lymphocytes and mast cells bear unique biophysical properties, including high Ca\(^{2+}\) selectivity, low unitary conductance, voltage-independent slow gating kinetics upon Ca\(^{2+}\) release from the endoplasmic reticulum (ER), and Ca\(^{2+}\)-dependent inactivation through various mechanisms (10–12). Following Ca\(^{2+}\) store (ER) depletion, STIM1, an ER Ca\(^{2+}\) sensor with a single transmembrane (TM) segment, migrates to ER-PM junctions and activates CRAC channels for Ca\(^{2+}\) entry (13–17). Mutations (e.g. E136X) in the human STIM1 gene can also lead to SCID (18). The functional roles of the STIM1 and Orai1/CRACM1 proteins in and beyond the immune system have been progressively unveiled via gene

* This work was supported by the 2012 Texas A&M-Weizmann Program (to S. L. Z. and X. P.), the Research Grants Program Grant 110598, Scott & White Memorial Hospital (to S. L. Z.), the Kruse Family Centennial Chair Fund, Scott & White Memorial Hospital (to L. K.), the National Basic Research Program of China (973 Program; Grant 2010CB833702 to J. H.), and National Natural Science Foundation of China (NSFC) Grant 31000515 (to C. H.).

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\(^{2}\) The abbreviations used are: CRAC channel, Ca\(^{2+}\)-release-activated Ca\(^{2+}\) channel; STIM1, stromal interaction molecule 1; TM, transmembrane segment; PM, plasma membrane; ER, endoplasmic reticulum; CAD, CRAC activation domain; SOAR, STIM1 Orai-activating region; TG, thapsigargin; eGFP, enhanced green fluorescent protein; dOrai, Drosophila Orai.
silencing and knock-out techniques applied in cultured cells, tissues, and animal models (19–21). STIM2, the other human STIM molecule, may be involved in the regulation of the basal cytosolic Ca\(^{2+}\) level (22). The biophysical properties of Ca\(^{2+}\) channels formed by overexpressed Orai2/CRACM2 or Orai3/ CRACM3, the two other human Orai proteins, are similar to that of Orai1 channels (23). However, their physiological functions have not been systematically characterized.

The full-length human Orai1 protein (301 amino acid residues) contains four TM helices and cytosolic N and C termini (6, 24, 25). Recent structural studies revealed that the *Drosophila* CRAC channel is composed of six Orai subunits (26). Based on site-directed mutagenesis studies, a conserved glutamate residue, Glu-106, in the first extracellular loop of Orai1 is critical for Ca\(^{2+}\) selectivity (7–9). Intracellular Ca\(^{2+}\) store depletion has been shown to strengthen the dynamic interaction between the STIM and Orai proteins for channel activation (7). STIM1 was suggested to form oligomers when Ca\(^{2+}\) is dissociated from its N-terminal EF-hand motif located in the ER lumen (27–29). STIM1 then directly binds to Orai1 through its cytosolic C terminus (30, 31). A small Orai1-binding region (called the CRAC activation domain, CAD (30), STIM1 Orai-activating region, SOAR (32), or Orai1-activating small fragment, OASF (33)) was identified in the STIM1 C terminus by mutagenesis studies. The molecular structure of SOAR was recently determined by x-ray crystallography studies (PDB ID codes 3TEQ (hSOAR) and 3TER (ceSTIM1-CCR)) (34). Removing the entire N or C terminus of Orai1 impairs channel function, and both fragments appear to interact with STIM1 despite a much lower binding affinity of the Orai1 N terminus to STIM1 (30–32, 35–37). However, the mechanisms by which the STIM1-Orai1 interaction triggers channel opening and the detailed contributions of the individual cytosolic regions of Orai1 to channel gating are still unclear. In the present study, we differentiated the contributions and roles of the N and C termini of Orai1 in channel activation and demonstrated the dominance of a methionine mutation at a conserved glycine site (i.e. the putative gating hinge) for channel opening with either full-length or truncated Orai1. Based on these results, we propose a three-step gating model for STIM1-operated Orai1 channels.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning and Mutagenesis**—The generation of pcDNA3/humanSTIM1, pcDNA5/Myc-humanOrai1, and enhanced green fluorescent protein (eGFP)-tagged human Orai1 was described previously (15, 38, 39). mCherry-tagged CAD (residues 342–448 of full-length human STIM1) was created by ligating the PCR-amplified CAD fragment into the XhoI and KpnI sites of the pmCherry-C1 vector (Clontech). Human Orai1-SS-eGFP was received as a gift from Dr. T. Xu, Dr. P. Xu, and Dr. M. Prakriya. pcDNA3.1/*Drosophila* Orai (dOrai) was created by subcloning *Drosophila* Orai from the pAc5.1/V5-His B vector to the pcDNA3.1 (+) vector (25). Fifty human Orai1 mutants, seven Orai1-SS-eGFP mutants, and one dOrai mutant were tested in this study; the cloning protocols are summarized in the supplemental Experimental Procedures.

**RESULTS**

**Orai1 Mutants with C-terminal Truncations Fail to Recruit the CAD Domain to the Peripheral Regions of the PM**—To elucidate how STIM1 interacts with Orai1 for CRAC channel activation and to determine the role of the individual intracellular termini of Orai1 in this process, a series of Orai1 truncations and Orai1 point mutants was systematically generated (Fig. 1). First, the C terminus of Orai1 was incrementally removed up to the predicted membrane boundary of the TM4 segment. The eGFP-tagged Orai1-ΔC1 (1–276) and Orai1-ΔC2 (1–266) truncations, in which a predicted coiled-coil domain (36) was

![FIGURE 1. Schematic diagram of the Orai1 mutants with truncations or point mutations described in the present study.](image-url)
deleted, were well expressed in the peripheral regions of the PM of HEK293A cells (supplemental Fig. S1A). However, the majority of eGFP-Orai1-ΔC3 (1–256) failed to incorporate into the PM (supplemental Fig. S1A), most likely due to protein misfolding. In parallel, the L273S point mutation was introduced into the C terminus of eGFP-Orai1. The residue equivalent to this leucine in the Drosophila Orai proteins was recently shown to contribute to a hydrophobic patch, packing an antiparallel coiled-coil motif between the C termini of two Drosophila Orai subunits in a hexamer (26). The serine substitution at the 273 site was reported to prevent the STIM1–Orai1 interaction, possibly by disrupting the short coiled-coil motif within the C terminus of Orai1 (36). In the present study, we showed that HEK293A cells overexpressing STIM1 plus Orai1-L273S exhibited diminished store-operated Ca\(^{2+}\) entry as compared with control cells (Fig. 2, B and D). In another set of eGFP-tagged Orai1 truncations, the N termini were progressively truncated. Orai1-ΔN1-(74–301) lacked the first 73 nonconserved residues, and Orai1-ΔN2-(81–301) bore part of the putative interface for a second physical contact with STIM1. Both were dominantly expressed in the peripheral regions of the PM (supplemental Fig. S1A). However, eGFP-Orai1-ΔN3-(88–301) was not stably presented on or near the PM (supplemental Fig. S1A). Thus, Orai1-ΔC3 and Orai1-ΔN3 were not followed for further study.

To evaluate the ability of the individual Orai1 mutants to interact with STIM1, the mutants were co-expressed with mCherry-tagged SOAR/CAD fragments of STIM1 (30, 32) in HEK293A cells. Instead of using full-length STIM1 or the entire cytosolic portion of STIM1 (38, 41), SOAR/CAD was selected in the present study for its higher binding affinity to Orai1 (30, 32). Confocal imaging showed that CAD was evenly distributed in the cytosol when expressed alone (Fig. 2A). However, CAD co-localized with the Orai1 channels, surrounding the peripheral regions of the PM, when co-expressed with wild-type (WT) Orai1. It is worth noting that many transfected cells did not survive, presumably due to uncontrollable Ca\(^{2+}\) entry resulting from the CAD–Orai1 interaction and persistent CRAC channel activation. Nevertheless, cell survival was improved by introducing the E106A point mutation to Orai1, leading to nonconducting channels with an altered selectivity filter. The majority of CAD molecules were associated with Orai1–E106A along the peripheral regions of the PM, without any detectable change in cell morphology (Fig. 2A). Interestingly, CAD was not coupled to C-terminally truncated Orai1 (Orai1–E106A–ΔC1 or Orai1–E106A–ΔC2; Fig. 2A) but was able to co-localize with N-terminally truncated Orai1 (Orai1–E106A–ΔN2; Fig. 2A). This observation is consistent with several recent studies (30, 32, 35, 36).

**Both Termini of Orai1 Are Essential for Channel Activation upon Store Depletion**—In a series of parallel studies, the original set of Orai1 mutants without the E106A mutation was co-expressed with full-length STIM1 in HEK293A cells for functional analysis. Thapsigargin (TG), a sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump inhibitor, was applied to passively deplete the Ca\(^{2+}\) store for CRAC channel activation, and the intracellular Ca\(^{2+}\) mobilization associated with TG-triggered store-operated Ca\(^{2+}\) entry was recorded at the single-cell level. In contrast to the confocal imaging data described above, mutations or truncations at either terminus of Orai1 markedly reduced channel activity (Fig. 2, B–D). This result is consistent with the conclusions from a few recent studies (30, 32, 35, 36), suggesting that both cytosolic regions of Orai1 are essential for channel function. To rule out the possibility that the fusion of eGFP to Orai1-ΔN2 prevented the contact between STIM1 and the remaining N terminus of Orai1-ΔN2 and thereby silenced the channel, another series of studies was performed in a set of Orai1 proteins with a simple Myc tag for identification. The Myc-Orai1-ΔN2 mutant co-expressed with STIM1 also failed to respond to store depletion induced by TG (supplemental Fig. S1, B and C). On the other hand, eGFP-Orai1-ΔN1 exhibited similar channel activities as compared with the full-length WT channels (Fig. 2, C and D). However, a reduction of channel activities in two Orai1 truncations similar to our eGFP-Orai1-ΔN1 has been reported (32, 35). This discrepancy may be due to the differences in tags/tag locations of these constructs.

Together, the results from this set of experiments suggest that the C terminus of Orai1 is the key element responsible for the initial engagement between STIM1 and Orai1. It appears that this step may stabilize the STIM1–Orai1 binding complex and allow the subsequent physical contact between STIM1 and the N terminus of Orai1 because STIM1 and the N terminus of Orai1, as measured by *in vitro* pulldown assays, have a very low binding affinity (30, 31). The binding may not occur *in vivo* unless STIM1 is adjacent to the Orai1 N terminus. In other words, the requirement for the C terminus in Orai1 channel activation may be substituted by an alternative mechanism in

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which STIM1 is physically placed proximal to the N terminus of Orai1 to facilitate their communication.

Differential Roles of the Intracellular Domains of Orai1 in Channel Gating—To test the above hypothesis, Orai1-SS-eGFP, which contains two tandem STIM1 cytoplasmic activation domains (residues 336–485, designated as the S domain) tethered to the C terminus of Orai1, was utilized (42, 43). This fusion protein, as reported previously (42, 43), is constitutively active and conducts a Ca\(^{2+}\) current with similar biophysical properties as that of endogenous CRAC channels (Fig. 3, A and C–E). Mutations in both termini of Orai1 were individually introduced into Orai1-SS-eGFP, and the corresponding channel mutants were examined for their function via [Ca\(^{2+}\)]\textsubscript{i} introduced into Orai1-SS-eGFP, and the corresponding channel activation.

![Image](https://via.placeholder.com/150)

**FIGURE 3.** Tethering the functional domains of STIM1 to Orai1 bypasses the requirement for the C terminus, but not the N terminus, of Orai1 for channel activation. A and B, representative averaged [Ca\(^{2+}\)]\textsubscript{i}, traces from HEK293A cells expressing eGFP (n = 14), full-length (FL) Orai1-SS-eGFP (n = 3), Orai1-E106A-SS-eGFP (n = 15), Orai1-L273S-SS-eGFP (n = 10), Orai1-ΔC1-SS-eGFP (n = 8), or Orai1-ΔN2-SS-eGFP (n = 16) are shown. Solution exchanges are indicated. Disruption of the N terminus (ΔN2), but not the C terminus (L273S or ΔC1), of Orai1-SS-eGFP abolished spontaneous Ca\(^{2+}\) entry. C, the summary of averaged resting [Ca\(^{2+}\)]\textsubscript{i} levels from cells expressing eGFP (37 cells) or Orai1-SS-eGFP proteins: full length (22 cells), E106A (74 cells), L273S (29 cells), ΔC1 (26 cells), ΔN1 (18 cells), ΔN2 (69 cells), ΔN1-ΔC1 (20 cells), and ΔN2-ΔC1 (77 cells). D, representative time course of inward Orai1-SS-eGFP currents, measured at −110 mV, is shown. E, corresponding I-V curves are presented for the time points indicated in D. Ch: choline, F, Orai1-ΔN2-SS-eGFP is mainly expressed in the peripheral regions of the PM. Scale bar, 10 µm. G and H, representative time course and I-V relationship of Orai1-ΔN2-SS-eGFP currents are shown. Traces in E and H are leak-subtracted; the observed residual current after 10 µM Gd\(^{3+}\) (CRAC channel blocker) treatment was considered as the leak. I, break-in inward current densities (picoamperes/picofarads (pA/pF)) were averaged from cells transfected with wild-type Orai1-SS-eGFP (full-length, 9 cells), Orai1-E106A-SS-eGFP (7 cells), Orai1-L273S-SS-eGFP (4 cells), Orai1-ΔC1-SS-eGFP (7 cells), Orai1-ΔN2-SS-eGFP (10 cells), or Orai1-ΔN2-ΔC1-SS-eGFP (7 cells). *p < 0.01 in comparison with full-length Orai1-SS-eGFP.
ΔC1-SS-eGFP did not (Fig. 3, C and J). In conclusion, linking the functional S domain of STIM1 to Orai1 bypasses the requirement for the C terminus but not the N terminus of Orai1 in channel gating.

Orai1-G98M, Which Contains a Methionine Substitution at the Putative Gating Hinge Site, Forms Constitutively Active Channels—Based on our recent findings from the gain-of-function Orai1 mutants, Orai1-G98D and Orai1-G98P, we speculated that the Gly-98 site in the middle of the Orai1 TM1 segment serves as a gating hinge, controlling the opening of the channel after the contact between STIM1 and Orai1 (40). However, we also found that G98D mutants exhibited cellular toxicity, which is most likely attributed to the misfolding of Orai1-G98D and ER stress in association with the destabilization of its transmembrane helices by the negatively charged aspartate (Fig. 4, A–C). To address this issue, the original glycine residue was replaced with each of the other 19 amino acid residues. Eleven individual substitutions at the 98 site, all with considerably elongated side chains, were able to constitutively open the corresponding channel mutants (Fig. 4, E–G). Correlating the results of these G98X mutants with the structural differences in the side chains and backbones of their 98 sites, our current data suggest that long side chains extruding from the 98 site are likely to add certain tension, pushing the pore-lining TM1 into an “open” configuration in the absence of STIM1. Among the mutants, the Orai1-G98M mutant mediated the most store-independent Ca2+ entry (Figs. 4G and 5B) and spontaneous current without the co-expression of STIM1 (Fig. 5, C and D). Similar to Orai1-G98D and Orai1-G98P, the current conducted by Orai1-G98M was nonselective and was blocked by Gd3+ (Fig. 5, C and D). However, unlike Orai1-G98D, the Orai1-G98M protein was well tolerated in transfected cells and predominantly distributed to the peripheral regions of the PM (Figs. 4D and 5, E and F). Therefore, the G98M mutation was utilized to unveil the function of the individual Orai1 cytosolic domains in channel gating in the following studies.

Endogenous CRAC channels formed by Orai1 and dOrai proteins in human T cells and Drosophila S2 cells, respectively, share very similar electrophysiological properties (25, 45). If the Gly-98 residue represents the gating hinge for Orai1 channel activation, the equivalent glycine in the Drosophila Orai proteins (Gly-170, Fig. 6A) should display channel-gating properties as well. To test this glycine hinge hypothesis, the Drosophila Orai-G170M mutant was generated and expressed in HEK293A cells. As expected, dOrai-G170M channels conducted spontaneous Ca2+ entry as well as unprompted current in a Gd3+ (10 μM)-sensitive manner (Fig. 6, B–E) as compared with the wild-type dOrai channels, which showed nondetectable channel activity (Fig. 6, B–E).

G98M Can Gate Truncated Orai1 Channels without the Reputed STIM1-binding Sites—We speculated that STIM1 triggers conformational changes in the pore-lining TM1 above the N terminus of Orai1 for channel activation after its recruitment by the Orai1 C terminus and binding to the N terminus. Therefore, a study with gain-of-function mutations at the gating hinge, which force TM1 into an open configuration, was conducted to circumvent the requirement for STIM1 or STIM1-binding sites within both termini of Orai1 for channel
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![Image Diagram](https://via.placeholder.com/150)

**Figure 5.** G98M is a novel gain-of-function Orai1 mutation. A and B, representative [Ca\(^{2+}\)] entry through Orai1-G98M channels is shown. C and D, representative time course and corresponding I-V curves of STIM1-independent, nonselective cationic currents mediated by Orai1-G98M channels are presented. The red trace in D shows currents from a representative control cell expressing WT eGFP-Orai1 alone, which was recorded under the same conditions as G98M (the black trace). Ch: choline. E and F, fluorescent images (green + differential interference contrast) of HEK cells expressing eGFP-Orai1-G98M (E) or eGFP-Orai1-G98D (F) indicate that the Orai1-G98M proteins are less toxic to host cells than the Orai1-G98D proteins. Scale bar, 10 \(\mu \text{m}\).

DISCUSSION

An understanding of the molecular mechanisms behind ion channel gating provides decisive information that can explain the complexity of ion channel function at the cellular and system levels. The present study aims to advance our knowledge on the gating mechanism of CRAC channel, a type of voltage-independent, highly \(\text{Ca}^{2+}\)-selective channel with low single-channel conductance, which is predominant in most nonexcitable cells and plays indispensable roles in \(\text{Ca}^{2+}\) signal transduction (11). Accumulating evidence suggests that CRAC channels are also crucial in refilling the \(\text{Ca}^{2+}\) store/sarcoplasmic reticulum in skeletal muscles during contraction (46), regulating the proliferation and migration of smooth muscle cells (47–50), triggering the hypertrophic growth of cardiomyocytes (51, 52), and modulating \(\text{Ca}^{2+}\) homeostasis in neurons (53, 54).

Mammalian immune cells exert their biological functions through surface receptor activation by utilizing elevated intracellular \(\text{Ca}^{2+}\) conducted by CRAC channels (55). This sustained \(\text{Ca}^{2+}\) signal redirects the cellular behavior and programs the global transcription network of immune cells for activation, proliferation, and cytokine secretion (21, 55). In
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FIGURE 7. G98M restores the channel activity of Orai1 truncated at the N or C terminus. A and B, averaged cytosolic Ca\(^{2+}\) concentrations from HEK cells expressing: (A) Orai1-ΔC2 (n = 11) or Orai1-G98M-ΔC2 (n = 8) or (B) Orai1-ΔN2 (n = 8) or G98M-ΔN2 (n = 7). C, the bar graph presents the resting [Ca\(^{2+}\)]\(_i\) levels in cells transfected with five pairs of Orai1 cDNA constructs, with or without the G98M mutation: L273S (31 and 59 cells), ΔC1 (27 and 46 cells), ΔC2 (23 and 70 cells), ΔN1 (22 and 28 cells), and ΔN2 (36 and 46 cells), respectively. D and E, representative store-independent currents conducted by exogenous Orai1-ΔC2, G98M-ΔC2, Orai1-ΔN2, or G98M-ΔN2 channels without the co-expression of STIM1 are shown. F, comparison of the normalized break-in current amplitude at −110 mV (picoamperes/picofarads (pA/pF)) from the wild type and a series of mutated Orai1 channels to the Orai1-G98M channels with corresponding mutations: full-length (FL, 13 and 19 cells), L273S (6 and 5 cells), ΔC2 (6 and 19 cells), and ΔN2 (6 and 7 cells), respectively. All current traces in this figure are leak-subtracted; the currents after 10 μM Gd\(^{3+}\) treatment were considered as the leak. *, p < 0.01.

FIGURE 8. CAD does not co-localize with the Orai1-G98M-ΔC1 or ΔC2 mutants. Confocal images show the expression of mCherry-CAD (red) alone or in combination with full-length eGFP-Orai1-G98M, eGFP-Orai1-G98M-ΔC1, or ΔC2 (green) in HEK cells. Scale bar, 10 μm. N.A., not available.

1993, Zweifach and Lewis (56) demonstrated that endogenous CRAC channels in T lymphocytes are activated by the depletion of intracellular Ca\(^{2+}\) stores. Recently, a series of studies suggested that the direct physical interaction between the CRAC pore-forming subunits (e.g. Orai1) and an ER Ca\(^{2+}\) sensor (STIM1) is the molecular basis for the “store-dependent operation” of Ca\(^{2+}\) influx (7, 29–33, 35, 36, 57). This classifies CRAC channels as ligand-gated ion channels, which have a relatively large protein ligand (STIM1) as compared with the neurotransmitters, cyclic nucleotides, and ATP that are present in other types of ligand-gated ion channels. Thus, it is essential to understand the nature of the interaction between STIM1 and Orai1 and how this interaction triggers the opening of Orai1 channels. In the present study, we propose a three-step gating model to elucidate the unique gating mechanism of CRAC channels upon the STIM1-Orai1 interaction (Fig. 10).

In Step 1, STIM1 interacts with the C terminus of Orai1 following Ca\(^{2+}\) release from the ER. The importance of this first step, the initial binding between STIM1 and the C terminus of Orai1, is supported by two lines of evidence. First, truncations (ΔC1 or ΔC2) or a point mutation (L273S) in the C terminus of Orai1 prevents Orai1 gating upon TG-triggered store depletion (Fig. 2, B and D), presumably by disrupting its interaction with the SOAR/CAD region of STIM1 (Fig. 2A), and second, these mutants are able to form functional channels with an additional gain-of-function mutation at the putative gating hinge (Fig. 7). The fact that the currents through Orai1-ΔC2-G98M channels (Fig. 7D) are indistinguishable from the currents mediated by full-length Orai1-G98M channels (Fig. 5D) indicates that removing the C terminus of Orai1 does not significantly influence the overall structure of the channels. We speculate that the C terminus of Orai1 serves as a molecular post to tie the accessible STIM1 molecules to the Orai1 channels upon store depletion and to position STIM1 proximal to the pore region of the Orai1 channels.
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In Step 2, after binding to the Orai1 C terminus, STIM1 is in proximity to the N terminus of Orai1, allowing for its subsequent interaction. Initially, the functional data from Orai1 mutants carrying N-terminal truncations indicated that the deletion of this region leads to diminished channel function (32, 35–37). Subsequently, the results from in vitro co-immunoprecipitation and pulldown assays between the C terminus of STIM1 and Orai1 N-terminal fragments suggested their physical interaction (30, 31). However, the functional role of this interaction remained unclear. In the present study, our patch clamp and Ca²⁺ imaging data revealed that the Orai1 and Orai1-SS-eGFP mutants that lacked the putative interface for the physical contact between the Orai1 N terminus and STIM1 were defective (Fig. 2, C and D and Fig. 3), similar to the Orai1 mutants with disrupted C termini. In contrast, the Orai1-L273S-SS-eGFP and Orai1-ΔC1-SS-eGFP mutants retained their channel functions (Fig. 3). Together, these results indicate that the interaction between STIM1 and the N terminus of Orai1 is functionally vital for channel gating after the initial binding between STIM1 and the C terminus of Orai1. It appears that this second binding event has a very low affinity (30, 31). Thus, the first binding event may stabilize the STIM1-Orai1 complex and provide an opportunity for STIM1 to make contact with the N terminus of Orai1. This finding explains the critical role of the C terminus of Orai1 in channel activation. Steps 1 and 2 may occur simultaneously, but the functional integrity of the Orai1 N terminus does not rely on the presence of an intact C terminus.

In Step 3, the binding of STIM1 to the Orai1 N terminus triggers conformational changes of the pore-lining TM1 of Orai1, subsequently converting the channels from the closed state to the open state. We previously suggested the presence of a glycine gating hinge in Orai1 channels: the Gly-98 site lying in the middle of the TM1 helix (40). Note that the Gly-98 site of Orai1 is located directly above its N terminus. Mutants with point mutations at this putative gating hinge (e.g. G98D and G98M) are constitutively active (Figs. 4 and 5). Although C- and N-terminal Orai1 truncation mutants are deficient with respect to normal channel activities (Fig. 2), the additional gain-of-function mutation at the 98 site reopens the channels and bypasses the requirement for either terminus (Fig. 7). This set of data demonstrates that the channel-gating defects of the Orai1 truncation mutants are not due to the lack of a functional pore but result from the inability to form a proper interaction with STIM1. This evidence supports the dual-interaction model between STIM1 and Orai1 proposed above and also indicates that the assembly of Orai1 channels does not necessarily require the cytosolic domains of Orai1.

Collectively, based on the findings from our laboratory and others (30–32, 35, 36, 40), we suggest that the interaction of STIM1 and Orai1 is initiated between STIM1 and the C terminus of Orai1 (Step 1) followed by a second low affinity contact between STIM1 and the N terminus of Orai1 (Step 2), leading to channel activation (Step 3). Our data also indicate that both termini of Orai1 are necessary for STIM1-initiated gating but are not critical for channel assembly and opening. Because STIM1 has been suggested to modulate the ion selectivity of CRAC channels, it is possible that after STIM1 binds to the N terminus of Orai1, it pulls TM1 from the cytosolic side and reduces the “diameter” of the glutamate ring formed at the Glu106 site to confer Ca²⁺ selectivity (43).

A very recent study has described the crystal structure of modified Drosophila Orai proteins (residues 132–341 of Orai, termed Orai_cry) as hexamers in the absence of Stim molecules (26). The observed structure, which is apparently in the closed conformation, shows that the C termini of Orai form dimerized helices and are not in contact with the N termini, which is consistent with a bridging role for STIM1 in linking the N and C termini of Orai for channel gating. Our Drosophila Orai-G170M mutant (Fig. 6) may serve as a useful model for the open conformation. In parallel, the Orai1-ΔN2-SS-eGFP and Orai1-ΔC1-SS-eGFP mutants (Fig. 3) may serve as templates to mimic the closed and open states of wild-type Orai1 channels in association with STIM1, respectively.

Acknowledgments—We thank Dr. M. D. Cahalan for providing laboratory facilities for the initial screen of Orai1-G98X mutants; T. Xu, P. Xu, and M. Prakriya for the gift of the full-length Orai1-SS-eGFP construct; and A. Webb for technical support with confocal imaging. Confocal microscopy was performed at the Integrated Microscopy and Imaging Laboratory, Texas A&M Health Science Center.

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