Cooperative Binding of Stromal Interaction Molecule 1 (STIM1) to the N and C Termini of Calcium Release-activated Calcium Modulator 1 (Orai1)*

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Calcium flux through store-operated calcium entry is a central regulator of intracellular calcium signaling. The two key components of the store-operated calcium release-activated calcium channel are the Ca2+-sensing protein stromal interaction molecule 1 (STIM1) and the channel pore-forming protein Orai1. During store-operated calcium entry activation, calcium depletion from the endoplasmic reticulum triggers a series of conformational changes in STIM1 that unmask a minimal Orai1-activating domain (CRAC activation region (CAD)). To gate Orai1 channels, the exposed STIM1-activating domain binds to two sites in Orai1, one in the N terminus and one in the C terminus. Whether the two sites operate as distinct binding domains or cooperate in CAD binding is unknown. In this study, we show that the N and C-terminal domains of Orai1 synergistically contribute to the interaction with STIM1 and couple STIM1 binding with channel gating and modulation of ion selectivity.

Store-operated calcium entry represents a key mechanism by which cells generate Ca2+ signals and maintain Ca2+ homeostasis by replacing Ca2+ lost from the endoplasmic reticulum (ER)2 with Ca2+ that enters the cytoplasm through plasma membrane channels. The Ca2+-release-activated Ca2+ (CRAC) channel is a prototypical store-operated calcium entry channel whose essential components are STIM1, the Ca2+-sensor of the ER, and Orai1, the CRAC channel pore-forming subunit. STIM1 is a single-pass ER membrane protein with several functional domains, including three coiled-coil domains (CC1, CC2, and CC3) facing the cytosol, with CC2 and CC3 forming part of a minimal CRAC channel activation domain called CAD (1) or STIM-Orai activating region (SOAR) (2) (spanning residues 339–448 in hSTIM1, referred to hereafter only as CAD). Each Orai1 subunit has four transmembrane segments, with the N and C termini of the protein facing the cytosol, and Orai1 channels are assembled from hexamers of Orai1 subunits. The activation of CRAC channels starts when depletion of calcium from the ER causes rearrangement of STIM1 and unmasking of CAD (3–8). When exposed, CAD binds to a site in the Orai1 N terminus (N-terminal binding domain (NBD), residues 74–87) and to a second site in the Orai1 C terminus (C-terminal binding domain (CBD), residues 267–292) (1, 9). This interaction results in clustering of STIM-Orai at ER-PM (plasma membrane) junctions and in pore opening of Orai1 channels, which mediate calcium influx into cells (10–17). We and others have shown recently that STIM1 binding to both the Orai1 NBD and CBD is critical for channel activation and that binding of STIM1 at these N- and C-terminal domains of Orai1 likely induces rearrangements in proximal membrane segments to open the channel (18–20). Whether the Orai1 NBD and CBD contribute to STIM1 binding independently or through a more complex manner is unknown.

In this study, we determine the relative contribution of the Orai1 NBD and CBD to interaction with STIM1 by studying the effect of cycling mutations between the NBD and CBD on STIM1-Orai1 physical interaction and on channel gating. Our results reveal that STIM1 binding to the NBD and CBD of Orai1 occurs in a cooperative manner to control the gating and ion selectivity of CRAC channels.

Materials and Methods

Cell Culture and Transfection—HEK293 cells were cultured in DMEM as described previously (21). Plasmid transfection of cells was performed using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. For electrophysiological experiments, 6–8 h after plasmid transfection and 12–15 h before the start of experiments, cells were plated onto 18-mm coverglass coated with L-polylysine. For Ca2+ imaging, co-localization, and FRET experiments, cells were plated onto 18-mm coverglass coated with L-polylysine, and, 6–8 h after plasmid transfection, culture medium was replaced to wash off the transfection reagent. To avoid a constitutive calcium rise in cells expressing all forms of Orai1-SS or V102A mutants, cells were cultured in high-glucose, Ca2+-free DMEM supplemented with 50 μM La3+.

Electrophysiological Recordings—Membrane currents were recorded under voltage clamp conditions using the whole-cell patch clamp configuration on an Axopatch 200B amplifier (Axon Instruments). Patch pipettes were fabricated from boro-
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Co-localization Analysis—Cells co-expressing mCherry-Orai1 or various mCherry-Orai1 mutants together with EGFP-S1C (an hSTIM1 fragment corresponding to residues 343–465) were plated and cultured as described above. Midplane sections of transfected cells were captured using an LSM 780 confocal microscope (Zeiss) controlled by Zen imaging software (Zeiss). The imaging system consisted of a Nikon Diaphot inverted microscope equipped with a ×20 objective and charge-coupled device camera (SenSys, Photometrics). Axon Imaging Workbench 4.0 (Axon Instruments) controlled both filters and collection of data.

Statistical Analysis—Statistical significance of data was calculated using one-way analysis of variance with Bonferroni correction (Kaleidagraph, Synergy Software) when comparing three or more dataset groups and unpaired two-tailed Student’s t test when comparing two dataset groups (Microsoft Excel 2010). Predicted parameters for linear additive effects of double mutants were calculated by first scaling the measured parameters of each single mutant to the corresponding control. The resultant single mutation parameters were then multiplied pairwise to generate values for the relevant combinations of single mutations. To calculate the standard deviation of these predicted parameters, we employed standard propagation of error analysis using the standard deviation values derived from each single mutant dataset. The MSD (Mean, Size, Standard...
Deviation) format of the Student's t test analysis (SigmaPlot 11, Systat) was used to evaluate significance between measured and predicted parameters for double mutants. All data are reported as mean ± S.E.

**Results**

**Cooperativeness of the N- and C-terminal Domains of Orai1 Determines Channel Gating by S1C—In vitro binding assays have shown that C-terminal fragments of STIM1, harboring the CAD domain, can bind to synthetic fragments of either the NBD or CBD of Orai1 (1, 9, 23–25). Cell-based binding assays, however, showed that, although the NBD contributes to CAD-Orai1 interaction, it does not interact with CAD when key residues in the CBD are deleted (19). This has been interpreted to indicate that the NBD-CAD interaction is of such low affinity that it cannot be detected. We wondered whether this observation could, instead, reflect a more complex interaction of CAD with the NBD and CBD in which it is more than a sum of NBD and CBD interactions.

To investigate the functional interaction between the NBD and CBD, we introduced point mutations into the NBD, the CBD, or both and analyzed the effect on channel activation. Because several mutations in the Orai1 CBD abolish STIM1 binding, we forced a high local concentration of CAD near the channel by employing an earlier approach in which dimers of STIM1 C-terminal fragments (residues 340–485, S domain) are directly linked to Orai1 (Fig. 1A and Refs. 18, 19, 27, 28).

Recent studies have suggested that pairs of Orai1 CBD from neighboring subunits interact in a conformation that is optimal for STIM1 binding. This raises the concern that the Orai1 CBD may be distorted simply by the covalent attachment of the STIM1 fragments. To verify that the Orai1-SS constructs represent a reliable framework to study the nature of interactions between STIM1 and Orai1, we asked whether tethering the STIM1 fragments to the Orai1 C terminus would prevent binding of untethered STIM1 fragments. Fig. 1 shows that tethering of the STIM1 fragment to the Orai1 C terminus does not prevent functional interaction between Orai1 and untethered STIM1 fragments. Despite its poor membrane expression, Orai1-SS R429C, which carries the loss-of-function mutation R429C in both of the tethered STIM1 fragments, is activated when co-expressed with a soluble STIM1 C-terminal fragment that is similar to CAD (S1C, residues 342–465 in hSTIM1; Fig.

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**FIGURE 1.** Untethered STIM1 fragments interact with Orai1-S or with the R429C Orai1-SS mutant. A, schematic of Orai1, the SOAR/CAD-like S1C fragment, and the Orai1-SS channels used in this work. B, summary of current densities (n = 5–8 cells, left panel) and representative plots of the current-voltage relationship (right panel) of currents recorded from cells expressing Orai1-S alone or from cells co-expressing S1C together with Orai1 or Orai1-S. pF, picofarad. C, summary of current densities (n = 5 cells, left panel) and representative plots of the current-voltage relationship (right panel) recorded from cells expressing Orai1-SS with the loss-of-function mutation R429C alone or together with WT S1C (left panel). D and E, representative fluorescence images (D) and summary of fluorescence densities (E) measured from cells expressing the WT or the R429C Orai1-SS-EGFP mutant (n = 6 regions in each). f.u., relative fluorescence units. Scale bar = 10 μm.
Similarly, the Orai1 S channel, which displays sub-maximal channel activation when expressed alone, is maximally activated when co-expressed with the soluble S1C fragment (Fig. 1B). These results suggest that the CBD of Orai1-SS retains the ability to bind to and be activated by STIM1.

Having seen that STIM1 binding and activation are retained in the STIM1-tethered Orai1, we proceeded to ask how mutations in Orai1 that compromise activation by STIM1 interact in combination. We analyzed three mutants of Orai1 that are not activated by soluble STIM1 but retain activation by tethered STIM1, indicating that they weaken interaction with STIM1: K85E in the NBD and L276D and L273S in the CBD (data not shown and Refs. 18, 19, 27). We reasoned that if each interaction contributed separately to STIM1 binding, then the effect of NBD/CBD double mutants would reflect the sum of the effects of the single mutations (Fig. 2B, dashed red columns) but that a cooperative process would result in a deviation from linearity.

We found that even though the single mutants had normal or almost normal channel activation in Orai1-SS, as assessed either by the density of membrane current or by intracellular Ca²⁺ levels, channel activation was abolished in both double NBD-CBD mutants (K85E/L273S or K85E/L276D) (Figs. 2B).

Expression profiles of the single and double mutants were comparable with that of WT channels (Fig. 2C and D), and the conductance of the double mutant channels could be restored by the constitutively activating V102A mutation (Fig. 4), indicating that the effect of the mutations was on their ability to be activated by CAD and not on their expression levels or their...
ability to conduct large currents when the gate is constitutively open. These supralinear effects therefore suggest that the Orai1 NBD and CBD operate synergistically during channel activation by CAD.

Cooperativeness of the N- and C-terminal Domains of Orai1 Determines Channel Gating by Full-length STIM1—Having observed non-linearity in the effect of mutations on the activation of Orai1 by CADs that are directly fused to the channel, we next searched for mutations that would enable us to ask whether normal coupling to full-length STIM1 has the same properties. Previous studies (18, 19, 24, 29) have shown that NBD or CBD point or deletion mutations at positions Trp-76 or Leu-276, respectively, introduce mild to strong effects on channel activation and physical interaction between Orai1 and full-length unlinked STIM1. We therefore examined the effect of combining mutations in these positions on activation of Orai1 by STIM1 or by tethered STIM1 fragment (Orai1-S). We recorded similar current densities from cells that co-expressed STIM1 together with WT Orai1 or with the individual Orai1 mutants W76C or L276C (Fig. 3, A and B). Similarly, cells that expressed WT Orai1-S or Orai1-S carrying the W76C or L276C mutations displayed similar current densities. Although all WT and mutant Orai1 or Orai1-S displayed similar expression levels and plasma membrane localization (Figs. 3, C, D, G, and H, and 4A), a significant decrease in current density was measured for the double NBD/CBD mutant Orai1-S W76C/L276C or when STIM1 was co-expressed with the Orai1 W76C/L276C (Fig. 3, A and B). Therefore, non-linear interaction with the Orai1 NBD and CBD is not only a characteristic of activation by tethered CAD fragments but also of normal activation by full-length STIM1.

Cooperativeness of the N- and C-terminal Domains of Orai1 Determines the Regulation of Ion Selectivity in V102A Orai1-SS Channels—Activation of Orai1 channels by STIM1 has been shown earlier to occur in a stepwise manner that involves a non-linear dependence on the number of STIM1s that bind to the channel (27, 30), leading to the proposal that Orai1 subunits operate cooperatively (30). Having observed a non-linear interaction in channel activation by STIM1 or its C-terminal fragment S1C with the NBD and CBD of Orai1, we wondered whether cooperativity was restricted to the activation process. We therefore asked whether the regulation of ion selectivity in constitutively active channels follows the same rules. We analyzed the ion selectivity of V102A Orai1-SS mutants that contain the NBD point mutation K85E, the CBD point mutations L273S or L276D, or the double NBD/CBD mutations K85E/L273S or K85E/L276D by quantifying the reversal potentials of mutant channel currents in a calcium-containing solution. Reversal potentials recorded from cells expressing V102A Orai1-SS channels with wild-type NBD/CBD or with a single mutation in either the NBD (K85E) or CBD (L273S or L276D) were of similar values of about 40–50 mV (Fig. 4, A and B). In
contrast, even when directly linked to CAD, the double NBD/CBD mutations (K85/L273S and K85/L276D) displayed non-selective currents with a reversal potential of 19.5 ± 4 mV for K85E/L273S and 6.2 ± 1.2 mV for K85E/L276D. These findings indicate that, as shown above for gating, the regulation of ion selectivity of constitutively active Orai1 channels by STIM1 also involves non-linear interaction with the NBD and CBD of Orai1.

**An Ensemble of Orai1 NBD and CBD Forms a Distinct Interaction Site for S1C**—Having studied functional coupling between S1C and the N and C termini of Orai1, we turned to investigate their molecular association. We expressed WT Orai1 or Orai1 with mutations in either the NBD (W76C or K85E), TM4-CBD linker region (S263P), CBD (L276C), or with combination NBD/linker/CBD mutations (W76C/S263P, K85E/S263P, W76C/L276C, or K85E/L276C) and performed co-localization and FRET analyses. We found that two of the single mutants, W76C (NBD) and L276C (CBD), did not significantly change the interaction with S1C, whereas the K85E (NBD) mutation and S263P (TM4-CBD linker) moderately weakened this interaction (Fig. 5, A–C). Combining either NBD mutation W76C or K85E or CBD mutation L276C with the linker mutation S263P yielded an additive reduction in S1C-Orai1 association in each double mutant. In contrast, the double mutant Orai1 W76C/L276C, which combined an NBD mutant with a CBD mutant that individually had no significant effect, exhibited a substantially reduced S1C-Orai1 association in each double mutant. Similarly, interaction was nullified in the Orai1 K85E/L276C double mutant (Fig. 5B–C). The results were also compared with the co-localization and FRET values predicted for the additive effects of these combined mutations (Fig. 5, B and C, dashed red columns). Therefore, just as we observed supra-
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linear effects on gating and on regulation of ion selectivity of combining NBD and CBD mutations, as above, so too did combining NBD and CBD mutations have a supralinear effect on association with S1C.

Discussion

Our finding that STIM1-Orai1 protein association, channel activation, as well as modulation of ion selectivity are governed by cooperative interactions between STIM1 and the Orai1 N- and C-terminal regions sheds new light on important aspects of CRAC channel gating and permeation mechanisms. First, it implies that cooperativity in Orai1 channel activation by STIM1 begins in the binding step and is imparted to the subsequent gating and permeation processes. Second, it provides new evidence to support direct roles for both the Orai1 N- and C-terminal domains in channel gating. Importantly, they also suggest that CRAC channel activation involves simultaneous binding of STIM1 to both the Orai1 N- and C-terminal domains. Third, although alternative scenarios can be considered, as detailed below, taken together with earlier reports (1, 13, 18, 19, 22), the findings from this work support the idea that the Orai1 NBD and CBD assemble to form a distinct binding site for STIM1 and that STIM1 binding to this site controls channel gating and modulation of ion selectivity.

A recent NMR structure of the STIM1 CC2 and the Orai1 CBD regions added structural insights on the interacting residues within the CAD-CBD complex (25). Notably, several of these residues have been shown to play important roles in channel activation. The exact regions in the Orai1 NBD and the corresponding residues in CAD that contribute to NBD-CAD interactions remain, however, poorly defined. Although our results suggest that CAD directly interacts with the Orai1 NBD through interactions with Trp-76 and Lys-85, we cannot rule out other interpretations. First, through allosteric coupling between the Orai1 NBD and CBD, a conformational change in the NBD, induced by the mutations employed in this work, may affect the affinity of the CBD for CAD, and second, the Orai1 NBD may indirectly contribute to communication with CAD through interactions with additional regulatory components, like calmodulin (31) or CRACR2A (26), that have been shown previously to involve the implicated NBD residues Trp-76 and Lys-85, respectively. Ultimately, further structural work is needed to shed light on and resolve this fundamental issue.

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