STIM1 and Orai1 are essential components of Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels (CRACs). After endoplasmic reticulum Ca\(^{2+}\) store depletion, STIM1 in the endoplasmic reticulum aggregates and migrates toward the cell periphery to co-localize with Orai1 on the opposing plasma membrane. Little is known about the roles of different domains of STIM1 and Orai1 in protein clustering, migration, interaction, and, ultimately, opening CRAC channels. Here we demonstrate that the coiled-coil domain in the C terminus of STIM1 is crucial for its aggregation. Amino acids 425–671 of STIM1, which contain a serine-proline-rich region, are important for the correct targeting of the STIM1 cluster to the cell periphery after calcium store depletion. The polycationic region in the C-terminal tail of STIM1 also helps STIM1 targeting but is not essential for CRAC channel activation. The cytoplasmic C terminus but not the N terminus of Orai1 is required for its interaction with STIM1. We further identify a highly conserved region in the N terminus of Orai1 (amino acids 74–90) that is necessary for CRAC channel opening. Finally, we show that the transmembrane domain of Orai1 participates in Orai1-Orai1 interactions.

Ca\(^{2+}\) has been long recognized as an important second messenger in regulating a variety of physiological processes (1). In both excitable and non-excitatory cells, it has been found that extracellular Ca\(^{2+}\) enters the cytosol in a typical pathway after depletion of the endoplasmic reticulum (ER) Ca\(^{2+}\) store (2), which is therefore termed either capacitative calcium entry (3) or store-operated calcium entry. Since the identification of a long polar region in the C terminus of STIM1 which is therefore termed capacitative calcium entry (3), it has been long recognized as an important second messenger (1). In both excitable and non-excitable cells, it has been found that extracellular Ca\(^{2+}\) enters the cytosol in a typical pathway after depletion of the endoplasmic reticulum (ER) Ca\(^{2+}\) store (2), which is therefore termed either capacitative calcium entry (3) or store-operated calcium entry (3) or store-operated calcium entry (3).

**EXPERIMENTAL PROCEDURES**

**Solutions and Chemicals**—For intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) measurements, we used standard extracellular Ringer’s solution containing the following (in mM): 145 NaCl, 4.5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 D-glucose, and 5 Hepes (pH 7.4, adjusted with NaOH). The CaCl\(_2\) was replaced by 1 mM EGTA.
and 2 mM MgCl₂ in Ca²⁺-free Ringer’s solution. In patch clamp experiments, the standard external solution contained the following (in mM): 145 NaCl, 4.5 KCl, 10 CaCl₂, 1 MgCl₂, 10 d-glucose, 10 mM tetraethylammonium and 5 Hepes (pH 7.4, adjusted with NaOH). The divalent-free solution contained (in mM): 145 NaCl, 4.5 KCl, 10 EDTA, 10 mM tetraethylammonium, and 5 Hepes (pH 7.4, adjusted with NaOH). The pipette solution contained (in mM): 140 cesium glutamate, 8 MgCl₂, 12 BaCl₂, 10 Hepes (pH 7.2, adjusted with CsOH).

Stock solutions of thapsigargin (TG) were prepared in Me₂SO at a concentration of 2 mM. External solutions were applied using a gravity-driven perfusion system, and the local solution was completely exchanged in less than 2 s. Fura-2/AM was purchased from Invitrogen. Unless otherwise specified all reagents and chemicals were from Sigma-Aldrich.

**Plasmid Construction—**pHluorin-STIM1, STIM1-mOrange, Orai1-EGFP, and Orai1-mOrange were constructed as previously described (21). The pH-sensitive fluorescent protein pHluorin was inserted immediately downstream of the predicted signal peptide region of human STIM1 (NM_003156) to produce pHluorin-STIM1. STIM1 tagged with different fluorescent proteins at either terminus exhibited identical spatial distribution, and either chimeric protein reconstituted SOC Ca²⁺ entry and large CRAC current when co-expressed with Orai1 (Figs. 3 and 5), indicating that they were functionally equivalent. To obtain the Orai1–3XFLAG construct, Orai1 cDNA (NM_032790) was amplified by PCR from human placenta cDNA library (Invitrogen), tagged with 3XFLAG, and ligated into a pcDNA3.1 Zeo(+) vector. STIM1-DN (Δ23–200), STIM1–ΔC1 (Δ233–685), ΔC2 (Δ425–685), and ΔC3 (Δ672–685) were amplified by PCR from pHluorin-STIM1 and re-ligated into a KpnI- and EcoRI-digested pHluorin-STIM1 vector. Orai1–ΔN (Δ1–90), Orai1–ΔN73 (Δ1–73), Orai1–ΔC (Δ267–301), and Orai1–ΔD (Δ1–90 and Δ267–301) were amplified by PCR and cloned into pEGFP-N1 plasmid, respectively. The N-terminus of Orai1 (1–90) and pHluorin tagged transmembrane domain (266–288) of Sytustin1A (Stx1A) were amplified from Orai1-EGFP and Stx1A-pHluorin, respectively (26), ligated together in-frame by PCR and inserted into an EcoRI- and XhoI-digested pcDNA3.1 Zeo(+) vector (Orai1-Nt-TM). Similarly, an EGFP fragment was amplified by PCR, fused in-frame with the transmembrane domain of Stx1A, and ligated in a pcDNA3.1 Zeo(+) vector (EGFP-TM). All nucleotide sequences of constructs were verified by sequencing.

**Cell Culture, Transfection, and Fluorescence Imaging—**HEK293 cells were cultured as described previously (21) and were transfected with Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instruction. One day prior to the experiment, cells were transfected onto round poly-L-lysine-coated coverslips. Expression levels of pHluorin-tagged STIM1 vectors or EGFP-tagged Orai1 vectors were quantified as the averaged fluorescence intensities obtained from transfected HEK293 cells under wide field illumination, as summarized in supplemental Fig. S1.

For imaging experiments, transfected cells were viewed either under a confocal laser scanning microscope FV500 (Olympus Optical Co., Tokyo, Japan) with 60x oil objective lenses (numerical aperture (NA) = 1.40) or under total internal reflection fluorescence microscope (TIRFM) with 100x oil lenses (NA = 1.45). Detail information regarding the construction and application of confocal and TIRFM are described elsewhere (21). For confocal microscopy experiments, we only showed the middle focal plane throughout this report unless otherwise specified. All images were processed and analyzed using ImageJ (National Institutes of Health).

**Single Cell [Ca²⁺], Measurement—**Cells were preincubated with 6 μM fura-2/AM at room temperature for 30 min in standard Ringer’s solution. [Ca²⁺], was measured by a dual-wavelength excitation (340/380 nm) photometry system on an inverted microscope (IX71, Olympus, Tokyo, Japan) equipped with polychromatic xenon light source (TILL photonics, Gräfeling, Germany). The emission was collected at 510 ± 5 nm with a photodiode controlled by the TILL photometry system and X-Chart extension of Pulse software (HEKA, Lambrecht, Germany). [Ca²⁺], was calculated from the background-corrected fluorescence ratio R. Calibration parameters of R_{min} (0.145), R_{max} (4.069), and K_{eff} (3.900 μM) were determined previously (28).

**Electrophysiology—**Patch clamp experiments were conducted at room temperature using standard whole cell recording configuration. High resolution current recordings were acquired using the EPC-10 (HEKA). After establishment of the whole cell configuration, voltage ramps of 50-ms duration, spanning a range of −100 to +100 mV, were delivered from a holding potential of 20 mV every 2 s over a period of 300–500 s. All voltages were corrected for a liquid-junction potential of 10 mV. Currents were filtered at 2.9 kHz and digitized at a rate of 20 kHz. Capacitive currents were determined and corrected before each voltage ramp. The amplitude of the current recorded at −80 mV was used to monitor the development of IC_{CRAC} current. The maximum CRAC currents at −100 mV were used for statistical analysis. For noise analysis, 300-ms sweeps were acquired at the rate of 20 kHz at a holding potential of −100 mV, followed by a 50-ms ramp from −100 to +100 mV. Data were filtered offline at 1 kHz. The mean current and variant were calculated from a 200-ms segment in each 300-ms sweep.

**Co-immunoprecipitation and Western Blotting—**For transfection-based co-immunoprecipitation assays, HEK293 cells were transfected with the indicated plasmids, lysed in 500 μl of lysis buffer (50 mM Tris at pH 8.0, 500 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor tablets from Sigma-Aldrich), and immunoprecipitated with anti-FLAG-agarose beads (Sigma-Aldrich) overnight at 4 °C. The beads were washed four times with the lysis buffer and eluted in SDS sample buffer. Proteins in the cell lysates or immunoprecipitated samples were separated by SDS-PAGE, followed by Western blotting with anti-FLAG or anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA) antibody according to the standard procedures.

**Data Analysis—**Unless noted otherwise, all current traces were corrected for leak currents. Data analysis was conducted using IGOR Pro 5.01 (Wavemetrics, Portland, OR). Averaged results were presented as the mean value ± S.E. with the number of experiments indicated. Statistical significance was evaluated using Student’s t test. Asterisks denote statistical signifi-
Interacting Domains of STIM1 and Orai1

RESULTS

Interrogations of Functions of Different Domains in STIM1—To study the function of different domains of STIM1 in Ca\(^{2+}\) store depletion-induced STIM1 aggregation and interaction with Orai1, we constructed different truncated forms of STIM1 and co-expressed them with wild-type (WT) Orai1. As shown in Fig. 1A, STIM1-ΔN was devoid of the EF hand and SAM domains in the N terminus but retained the signal peptide; STIM1-ΔC1 was lacking the entire cytosolic C terminus, whereas STIM1-ΔC2 lacked the serine-proline-rich region and the polycationic residue region. The STIM1-ΔC3 mutant was truncated such that it lacked most of the polycationic residues in the C terminus.

In accord with our previous data (21), WT pHluorin-STIM1 displayed diffused ER distribution in the resting state; it migrated toward cell periphery and co-localized with Orai1-mOrange on the plasma membrane after store depletion (supplemental Fig. S2). Deletion of the EF-hand and SAM domain in the N terminus of STIM1 led to a spontaneously targeting of STIM1 to the plasma membrane that was independent of store depletion, as shown in the confocal images obtained from the same cell before and after TG (1 μM) pretreatment for at least 5 min (Fig. 2A). Moreover, the fluorescence intensity profile of STIM1-ΔN and Orai1-mOrange along the line that was imposed across the cell overlapped, indicative of spatial co-localization of these proteins. Consistent with the optical experiment, cells co-expressing WT Orai1 and STIM1-ΔN exhibited a elevated basal [Ca\(^{2+}\)](supplemental Fig. S3A) and a spontaneously active inward current. The channel was blocked by 10 μM La\(^{3+}\) (supplemental Fig. S3C) and had a current voltage relationship similar to native CRAC channel (supplemental Fig. S3D), indicating that it represented a bona fide CRAC channel. Expressing the pHluorin-tagged ΔC1 mutant in HEK293 cells resulted in a diffuse ER distribution, which failed to aggregate into clusters upon ER Ca\(^{2+}\) store depletion (Fig. 2B). In contrast, STIM1-ΔC2 clusters were found in non-stimulated HEK293 cells, but many were not targeted to the cell periphery, as manifested by the fact that fluorescence intensity profile of STIM1-ΔC2 peaked in the cell interior in Fig. 2C. After store depletion, STIM1-ΔC2 clusters did not migrate to the surface membrane, neither did they co-localize with the WT Orai1 on the surface membrane. In parallel, SOC Ca\(^{2+}\) influx was inhibited in cells co-expressing Orai1-mOrange with either STIM1 mutant (supplemental Fig. S3, A and B), and no CRAC current was detected in these cells, either (supplemental Fig. S8).

On the other hand, STIM1-ΔC3 exhibited normal ER distribution in rest condition; upon store depletion, they aggregated and migrated to the cell periphery and co-localized with Orai1-mOrange on the surface membrane, as shown by the overlapped fluorescence intensity profiles of the STIM1-ΔC3 and Orai1-mOrange along the lines that ran across the cell (Fig. 2D). Moreover, the STIM1-ΔC3 mutant was fully capable of reconstituting SOC influx when co-expressed with Orai1 in HEK293 cells (supplemental Fig. S3, A and B). Co-expressed STIM1-ΔC3 with Orai1 also produced large CRAC current (Fig. 3A). The I-V relationship of IC\(_{\text{CRAC}}\) current was not altered by expression of the STIM1-ΔC3 mutant (Fig. 3B), and the averaged current density was also similar in cells co-expressing STIM1-ΔC3 and Orai1-mOrange (30 ± 6 pA/picofarad, n = 8), as compared with cells co-expressing pHluorin-STIM1 and Orai1-mOrange (34 ± 5 pA/picofarad, n = 8). However, intracellular perfusion of BAPTA induced slower initiation of IC\(_{\text{CRAC}}\) current in cells co-expressing STIM1-ΔC3 and Orai1 (delay = 62 ± 11 s), as compared with cells co-expressing WT STIM1 and Orai1 (delay = 104 ± 23 s, p < 0.05) (Fig. 3D). This effect was not due to a difference in the exogenous fusion protein expressed, because overexpressed STIM1-ΔC3 and WT STIM1 were at a similar level (supplemental Fig. S1A). Taken together, these experiments indicate that different regions in STIM1 play different roles in clustering and migration of STIM1 and its functional interaction with Orai1 upon store depletion.

Orai1 Interacts with STIM1 at Its Cytoplasmic C Terminus—Orai1 is reported to functionally and physically interact with STIM1 and Orai1 (24, 25, 29). To locate sites in Orai1 that were responsible for this interaction, we truncated Orai1-EGFP of cytoplasmic C terminus (Orai1-ΔC), N terminus (Orai1-ΔN), and both

FIGURE 1. A schematic representation of STIM1 (A) and Orai1 (B) mutants used in this study. WT STIM1 and STIM1 mutants were fused with either pHluorin at the N terminus or mOrange at the C terminus. Functional domains of STIM1 include EF-hand, SAM, the transmembrane domain, coiled-coil (CC), the Ser/Pro-rich domain (SP), and the polylysine residues region (K). Orai1 includes four transmembrane domains with its N and C termini in the cytosol. Fluorescence tags (EGFP or mOrange) were fused to the C terminus of Orai1.
cytoplasmic C and N terminus (Orai1-D/H9004). We also made a cytoplasmic N terminus of Orai1 fused to the trans-membrane domain of Syx1A tagged by pHluorin (Orai1-Nt-TM) (Fig. 1B). As a control, we have previously shown that overexpressed STIM1-mOrange in HEK293 cells aggregated into clusters that co-localized with Orai1-EGFP on the surface membrane after store depletion (21, 27). In contrast, as observed using TIRFM in Fig. 4A, Orai1-ΔC on the surface membrane failed to aggregate and co-localize with overexpressed STIM1-mOrange clusters after Ca$^{2+}$ store depletion by applying another sarcoplasmic-endoplasmic reticulum calcium ATPase pump blocker tert-butyldihydroquinone (100 μM). Moreover, neither significant store-operated Ca$^{2+}$ entry (supplemental Fig. S4, A and B) nor CRAC current (supplemental Fig. S9) was observed in cells co-expressing Orai1-ΔC and STIM1-EGFP. In contrast, truncation of the cytoplasmic N terminus of Orai1 did not affect its cellular distribution, because Orai1-ΔN exhibited normal periphery distribution in rest conditions and co-localized with STIM1 clusters upon store depletion (Fig. 4B). Orai1-D, where the double cytoplasmic region was removed, behaved similar to Orai1-ΔC when overexpressed with STIM1 (data not shown), implying that the cytoplasmic C terminus of Orai1 is crucial for its aggregation and co-localization with STIM1. This is further supported by the fact that overexpressed chimeric protein Orai1-Nt-TM, containing the cytoplasmic N terminus of Orai1, was targeted to the plasma membrane but failed to co-localize with STIM1 clusters after store depletion (Fig. 4C).

Amino Acids 74–90 in the Cytoplasmic N Terminus of Orai1 Are Required for CRAC Channel Activation—By comparing the amino acid sequence of the human, mouse, Drosophila, and C. elegans Orai families (supplemental Fig. S5), we found that the
amino acids from 74 to 90 in the cytoplasmic N terminus of Orai1 were highly conserved among different species. Therefore, we constructed a truncation sparing the conserved cytoplasmic N terminus (Orai1-ΔN73) (Fig. 1B). The amplitude of store-operated Ca\(^{2+}\) entry was increased by 2-fold in HEK293 cells co-expressing Orai1-ΔN73 and STIM1-mOrange (1.1 ± 0.12 μM, n = 20), compared with non-transfected cells (0.36 ± 0.05 μM, n = 27, p < 0.001), as shown in Fig. 5 (A and B).

Intracellular perfusion of high concentrations of BAPTA induced pronounced whole cell currents in cells co-expressing Orai1-ΔN73 and STIM1-mOrange (Fig. 5C), which displayed similar CRAC channel characteristics such as an inwardly rectifying current-voltage relationship (Fig. 5D), an increase in divalent-free current in Ca\(^{2+}\)-free bath solution, and first potentiation then inhibition of the current by 50% of maximum activation of the CRAC channel, respectively.

The amplitude of SOC Ca\(^{2+}\) entry, and 36% of I\(_{\text{CRAC}}\) current compared with cells co-expressing STIM1-mOrange and Orai1-ΔN73, current-voltage relationship (Fig. 5). This potentiation was increased by 2-fold in HEK293 cells co-expressing Orai1-mOrange plus pHluorin-STIM1 (n = 24), compared with non-transfected cells (n = 22) (Fig. 6A), presumably reflecting a direct interaction of endogenous Orai1 with endogenous STIM1 or Orai1 that led to a functional interaction between different Orai1—Endogenous store-operated Ca\(^{2+}\) entry was severely inhibited in HEK293 cells overexpressing WT Orai1 (70 ± 18 nm, n = 21) compared with non-transfected control cells (360 ± 53 nm, n = 24) (Fig. 6A), presumably reflecting a direct interaction of endogenous Orai1 with endogenous STIM1 or Orai1 that led to a functional interaction between different Orai1.

Because Orai1-ΔΔ was incapable of forming clusters co-localized with STIM1, we wondered if Orai1-ΔΔ inhibited SOC influx through interacting with endogenous Orai1. To test that possibility, 3× FLAG-tagged WT Orai1 was co-expressed with either EGFP-tagged Orai1-ΔΔ or EGFP-tagged transmembrane domain of Syx1A (EGFP-TM). The two forms of Orai1
C. The cytoplasmic C terminus of Orai1 is essential for its co-localization with STIM1. TIRFM was used to examine distributions of EGFP-tagged Orai1 mutants and mOrange-tagged STIM1 mutants before or after store depletion. A, Orai1-ΔC was distributed throughout the surface membrane at rest (upper left panel); Depletion of the ER Ca^{2+} store induced Orai1-mOrange aggregation into puncta underneath the plasma membrane, whereas Orai1-ΔC failed to co-localize with STIM1. As shown in the merged image (right), there is little overlap of Orai1 and STIM1 either before or after store depletion. B, Orai1-ΔN and STIM1-mOrange exhibited normal distribution at rest. After store depletion, Orai1-ΔN (left) and STIM1 (right) aggregated into co-localized clusters. C, Orai1-Nt-TM distributed homogeneously throughout the surface membrane before and after store depletion and was incapable of forming clusters that co-localized with STIM1-mOrange. Each set of images represents at least five similar experiments.

interacted with one another, as shown by immunoblotting Orai1-ΔC-EGFP, which was co-immunoprecipitated with Orai1-3×FLAG (Fig. 6C). As a control, anti-FLAG beads failed to co-immunoprecipitate EGFP in control cells transfected with 3×FLAG-tagged Orai1 and EGFP-TM. These results indicate that the transmembrane domain of Orai1 is involved in the Orai1-Orai1 interaction.

**DISCUSSION**

It is firmly established that STIM1 forms aggregates after ER Ca^{2+} store depletion (6, 7, 21), which depends on the EF-hand and SAM domains in the N terminus of STIM1 (11, 16). In the present study, we further confirm this theory, because deletion of EF-hand and SAM domains leads to spontaneous plasma membrane targeting of STIM1 to the cell periphery and constitutive opening of the CRAC channel (Fig. 2 and supplemental Fig. S3). We also found that STIM1-ΔC1 was unable to form aggregates before or after store depletion, whereas STIM1-ΔC2 spontaneously aggregated into clusters in resting condition. The difference is likely to be the presence of the coiled-coil domain, which is present in STIM1-ΔC2 but absent in the ΔC1 mutant, therefore strongly supporting an essential role for the coiled-coil domain in STIM1 aggregation (31). Huang et al. (13) reported that the ERM domain of STIM1 is essential for its binding with the TRPC1 channel, therefore suggesting a similar interaction between STIM1 and Orai1. In our study, Ca^{2+} store depletion failed to induce STIM1-ΔC2 migration and Orai1 aggregation in cells co-expressing these two vectors, and this phenomenon was rescued by replacing STIM1-ΔC2 with STIM1-ΔC3. Because STIM1-ΔC3 vector contains a serine-proline-rich region and part of the ERM domain compared with the ΔC2 mutant (13), we further delineate these regions as possible STIM1-Orai1 interaction sites.

Human STIM1 has a polycationic tail, which is found to be essential for the gating of the TRPC1 channel (13). Many membrane-residing small GTPase proteins share a similar structure and use this polycationic tail to guide their targeting to the phosphatidylinositol 4,5-bisphosphate-enriched region in the plasma membrane (32). Therefore, it is tempting to speculate that this polycationic region determines the targeting of STIM1 to the cell surface. In agreement with this hypothesis, both TG-induced migration of STIM1-ΔC3 puncta to the cell periphery (data not shown) and development of I_{CRAC} current induced by intracellular perfusion of BAPTA were slowed down, implying a reduced efficiency in the trafficking of STIM1 to the cell periphery. However, the amplitude of averaged I_{CRAC} current reconstituted by STIM1-ΔC3 and Orai1 was not significantly different from that reconstituted by STIM1 and Orai1, indicating that the tail region is not required for CRAC activation. It is possible that the presence of the single basic amino acids in the tail region (amino acid 671) in our constructs, which was absent in previous studies (13), provides enough guiding signal for STIM1 to the cell surface. In agreement with this hypothesis, both TG-induced migration of STIM1-ΔC3 puncta to the cell periphery (data not shown) and development of I_{CRAC} current induced by intracellular perfusion of BAPTA were slowed down, implying a reduced efficiency in the trafficking of STIM1 to the cell periphery. However, the amplitude of averaged I_{CRAC} current reconstituted by STIM1-ΔC3 and Orai1 was not significantly different from that reconstituted by STIM1 and Orai1, indicating that the tail region is not required for CRAC activation. It is possible that the presence of the single basic amino acids in the tail region (amino acid 671) in our constructs, which was absent in previous studies (13), provides enough guiding signal for STIM1 to the cell surface. 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FIGURE 5. Orai1 is capable of forming functional CRAC channels without amino acids 1–73 at the cytoplasmic N terminus. A, HEK293 cells were co-transfected with Orai1-EGFP and STIM1-mOrange, Orai1-ΔN73, and STIM1-mOrange. Non-transfected cells were used as controls. Cells were pre-loaded with fura-2/AM and stimulated with TG (1 μM) in the Ca^{2+}-free solution to deplete the ER Ca^{2+} store. Averaged time course of store depletion induced Ca^{2+} entry in control HEK293 cells (circle, n = 27), cells expressed Orai1-ΔN73 and STIM1-mOrange (right-facing triangle, n = 20) and cells expressed Orai1-EGFP and STIM1-mOrange (square, n = 22). B, averaged peak Ca^{2+} elevation in the indicated cells by re-addition of 2 mM Ca^{2+} after store depletion. Significances of the t test are labeled on the histogram. C, representative time course of CRAC channel activation in cells as in A. No definitive CRAC current was detected in control cells, whereas substantial CRAC currents were observed in cells expressing fusion proteins. D, I-V curves at the time of maximal activation of the CRAC channel in C. E, summary of peak current densities at −100 mV in the indicated cell categories (n = 8 for each category); p values are labeled on the histogram. F and H, mean value and variance of CRAC current in cells co-expressing Orai1 plus STIM1 or Orai1-ΔN73 plus STIM1 after passive store depletion induced by dialysis of 12 mM BAPTA through the pipette. Each point represents the values calculated from a 200-ms segment of current data. G and I, the variances of CRAC current from F and H were plotted against mean currents and fitted with a linear function. The slope of the fit revealed the unitary single-channel CRAC currents at −100 mV in 10 mM extracellular Ca^{2+} solution (see “Experimental Procedures”).
We identified a highly conserved region of CRAC channel. We believe that the N terminus of Orai1 is essential for the opening of the CRAC channel. Finally, we propose that the transmembrane domain of Orai1 participates in Orai1-Orai1 interactions.

**Interacting Domains of STIM1 and Orai1**

We also looked at functions of different domains of Orai1 in CRAC activation. It has recently been reported that the N terminus of murine Orai1 is essential for the store-operated Ca\(^{2+}\) entry (23). In agreement with that report, we also found that truncation of the cytoplasmic N terminus of Orai1 failed to reconstitute SOC Ca\(^{2+}\) entry when co-expressed with STIM1. Because there are five positively charged arginine residues in the N terminus of Orai1, it was postulated previously that these residues might bind to the ERM domain of STIM1 (23). However, truncation of the whole cytoplasmic N terminus did not affect store depletion-induced aggregation of Orai1 and its co-localization with STIM1, whereas deletion of the cytoplasmic C terminus diminished such responses. Conversely, overexpressing either Orai1-\(\Delta N\) or Orai1-\(\Delta C\) in HEK293 cells inhibited endogenous SOC Ca\(^{2+}\) entry. This inhibition was reduced in cells co-expressing Orai1-\(\Delta N\) and STIM1 but was not altered in cells co-expressing Orai1-\(\Delta C\) and STIM1 (supplemental Fig. S7). Moreover, the plasma membrane-tethered N terminus of Orai1 did not co-localize with STIM1 clusters formed after store depletion (Fig. 4C). These three lines of evidence suggest that it is the cytoplasmic C terminus and not the N terminus of Orai1 that is crucial for the STIM1-Orai1 interaction. Rather, we believe that the N terminus of Orai1 is essential for the opening of CRAC channel. We identified a highly conserved region (amino acids 74–90) in the N terminus that is absolutely required for \(I_{\text{CRAC}}\) activation. Because this region is conserved in human Orai1, Orai2, and Orai3, it may well explain the phenomenon wherein different Orai genes generate or augment store-operated Ca\(^{2+}\) entry and reconstitute \(I_{\text{CRAC}}\) current (25, 35–37). The reconstitute whole cell \(I_{\text{CRAC}}\) current was smaller in cells co-expressing STIM1 and Orai1-\(\Delta N73\), compared with cells co-transfected with STIM1 and Orai1. Because the unitary single CRAC channel current revealed by the variance analysis of mean whole cell CRAC current was not altered (Fig. 5), the reduction in current could be due to a decreased open probability of single CRAC channel or an increase in the number of silent CRAC channels on the surface membrane (30). Whether any one of these hypotheses is true requires further investigation.

Orai1 forms dimers or oligomers in the non-stimulated resting state and store-depleted state (24, 25). Functionally overexpressing Orai1 in HEK293 and HeLa cells is found to inhibit endogenous SOC Ca\(^{2+}\) entry (18, 35), implying that overexpressed Orai1 may interact with endogenous Orai1 and become insensitive to the store-depletion signal. In the present study, TG-induced endogenous SOC Ca\(^{2+}\) entry was significantly inhibited in cells overexpressing Orai1-\(\Delta C\) or Orai1-\(\Delta \Delta\). Because Orai1-\(\Delta C\) and \(\Delta \Delta\) are deficient in their interaction with STIM1, the inhibition could only be due to interaction of endogenous Orai1 with these truncations. Moreover, co-immunoprecipitation experiments directly showed that Orai1-\(\Delta \Delta\) physically interacted with full-length Orai1. On the other hand, endogenous SOC Ca\(^{2+}\) entry was inhibited by overexpressing Orai1-\(\Delta \Delta\) (Fig. 6, A and B), implying that the cytoplasmic N terminus may also participate in Orai1 oligomerization. Taking these findings together, we conclude that the transmembrane domain of Orai1 participates in Orai1 dimerization and oligomerization but does not exclude roles of other domains in the same processes.

In conclusion, after Ca\(^{2+}\) store depletion, STIM1 aggregation requires the coiled-coil domain in the C terminus of STIM1. STIM1 migrates toward the surface membrane under guidance of both the serine-proline-rich region and the polycationic region, interacts with the cytoplasmic C terminus of Orai1, and leads to clustering of the latter on the opposing plasma membrane. Although the N terminus of the Orai1 is not required for its interaction with STIM1, amino acids 74–90 are essential for the opening of the CRAC channel. Finally, we propose that the transmembrane domain of Orai1 participates in Orai1-Orai1 interactions.
Interacting Domains of STIM1 and Orai1

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