Visualization and Manipulation of Plasma Membrane-Endoplasmic Reticulum Contact Sites Indicates the Presence of Additional Molecular Components within the STIM1-Orai1 Complex

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STIM1, a recently identified endoplasmic reticulum (ER) protein, rapidly translocates to a plasma membrane-adjacent ER compartment upon depletion of the ER Ca2+ stores. Here we use a novel means, namely a chemically inducible bridge formation between the plasma and ER membranes, to highlight the plasma membrane-adjacent ER compartment and show that this is the site where STIM1 and its Ca2+ channel partner, Orai1, form a productive interaction upon store depletion. By changing the length of the linkers connecting the plasma and ER membranes, we show that Orai1 requires a larger space than STIM1 between the two membranes. This finding suggests that Orai1 is part of a larger macromolecular cluster with an estimated 11–14-nm protrusion to the cytoplasm, whereas the cytoplasmic domain of STIM1 fits in a space calculated to be less than 6 nm. We finally show that agonist-induced translocation of STIM1 is rapidly reversible and only partially affects STIM1 in the juxtanuclear compartment. These studies are the first to detect juxtaposed areas between the ER and the plasma membrane in live cells, revealing novel details of STIM1-Orai1 interactions.

It has long been known that Ca2+-mobilizing agonists activate a Ca2+ entry pathway subsequent to their mobilization of intracellular Ca2+ stores by a mechanism that has eluded identification until most recently. In 1986, Putney (1) had postulated that enhanced Ca2+ entry is a consequence of the depletion of the intracellular Ca2+ stores, introducing the term capacitative Ca2+ entry or store-operated Ca2+ entry pathway (SOCE). Most recent developments began to shed light on the molecular details underlying the SOCE phenomenon. Screening with libraries of RNA interference, two groups have identified proteins, previously known as stromal-interacting molecule (STIM) 1 and -2 (2, 3), as essential components of SOCE (4–6). STIM1 and STIM2 are ER-resident proteins that contain a single membrane-spanning domain and an EF hand motif in the luminal side of the ER that serves as a Ca2+ sensor. Remarkably, STIM1 shows rapid translocation to a plasma membrane (PM)-adjacent region of the ER upon depletion of the ER luminal Ca2+ (4, 5, 7), but it does not have the structural hallmarks of an ion channel. In parallel studies, another protein necessary for SOCE and with a channel-like structure has been identified and named Orai1 (8) or CRACM1 (9). Although overexpression of STIM1 alone is a poor enhancer of SOCE, together with Orai1, it dramatically enhances store-operated Ca2+ entry consistent with the hypothesis that STIM and Orai form a functional complex, (10, 11). Finally, three groups have recently provided strong evidence that Orai1 indeed is the molecular entity forming the channel pore through which Ca2+ enters the cells upon store depletion (12–14). These studies have laid the groundwork for the molecular definition of the capacitative Ca2+ entry process.

Several questions have been raised concerning the movements of STIM1 within the ER and between the ER and the PM upon store depletion. Since STIM1 can be glycosylated and is also found in the PM, it was of great importance to determine whether the rapid appearance of STIM1 in the form of numerous puncta at the PM upon store depletion represents a translocation of the protein within the ER from the reticulo-tubular to a membrane-adjacent region or whether it also involves the incorporation of the molecule into the PM. Some studies suggested that ER depletion increases the amount of STIM1 in the PM (5) and that the increased Ca2+ entry could be blocked by STIM1 antibodies acting from the outside of the cells (7). In

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1 This article was selected as a Paper of the Week.
2 The on-line version of this article (available at http://www.jbc.org) contains three supplemental figures and three movies.
3 The abbreviations used are: SOCE, store-operated Ca2+ entry pathway; ER, endoplasmic reticulum; PM, plasma membrane; FRB, fragment of mTOR that binds FKBP12; mTOR, mammalian target of rapamycin; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; InsP3, inositol 1,4,5-trisphosphate; STIM, stromal interaction molecule; Tg, thapsigargin; YFP, yellow fluorescent protein; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; CFP, cyan fluorescent protein; TIRF, total internal reflection fluorescence microscope; TK, thymidine kinase; FKBP, FK506-binding protein.
suggests that Orai1 is part of a large macromolecular complex sizes of their respective predicted cytoplasmic sequences. This STIM1 between the PM and ER, a difference not justified by the spatial requirements of both STIM1 and Orai1, unexpected distance between the PM and the ER permitted determination of the region where STIM1 and Orai1 form a rapidly reversible entry process (18) or that it can cluster at the PM after store depletion (19).

The functional importance of the fraction of ER positioned closely to the PM has been recognized long before the STIM1-Orai1 movements were described. Co-purification of the InpF3 receptor located in the ER with PM has been described more than 20 years ago (20, 21), and the importance of the PM-associated fraction of the ER in phospholipid synthesis and transfer in both metazoan cells and yeast have been well documented (22, 23). The newly recognized significance of this compartment in Ca2+ signaling and its suspected role in phosphatidylinositol (PtdIns) synthesis and transfer to the PM prompted us to find new means to visualize and possibly functionally modulate this ER compartment. To this end, in the present study, we describe a novel chemically inducible heterodimerization approach to detect points of proximity between the ER and the PM and apply this technique to study the relationship between this compartment and the movements of the STIM1 and Orai1 proteins during Ca2+ depletion of the ER. Highlighting of this ER compartment in COS-7 cells allowed us to show that this is the region where STIM1 and Orai1 form a rapidly reversible complex upon Ca2+ store depletion. Manipulation of the distance between the PM and the ER permitted determination of the spatial requirements of both STIM1 and Orai1, unexpectedly revealing that Orai1 requires a significantly larger gap than STIM1 between the PM and ER, a difference not justified by the sizes of their respective predicted cytoplasmic sequences. This suggests that Orai1 is part of a large macromolecular complex with a sizeable (11–14-nm) protrusion into the cytoplasm.

**EXPERIMENTAL PROCEDURES**

*Materials*—Rapamycin and thapsigargin were purchased from Calbiochem. Apyrase and ATP were obtained from Sigma. All other chemicals were of the highest analytical grade.

*DNA Constructs*—YFP- and mRFP-STIM1 plasmids were made by inserting the fluorescent proteins after the signal sequence, starting at residue 23 with a linker (KLGAGAGAGAILNSRV) placed between the C terminus of the fluorescent protein and the rest of the human STIM1 sequence (obtained as an expressed sequence tag clone: C50DI607YJ06 from Invitrogen). The whole cDNA of this construct was inserted within the NheI and the KpnI restriction sites of the pEGFP-C1 (Clontech) vector.

The human Orai1 was obtained as an expressed sequence tag clone (id: 3914595, Open Biosystems) and was tagged with CFP, YFP, or mRFP at its C terminus with a linker (AGAN-SGAGAGAGAILSRGAAAGAAGPVAT) inserted between Orai1 and the fluorescent protein based on the pEGFP-N1 vector in which the starting Met of GFP was changed to Leu. The cytomegalovirus promoter in some of the STIM1 and Orai1 constructs was replaced by the thymidine kinase (TK) promoter amplified from the pRL-TK vector of Promega (nucleotides 7–1029) using the VspI and NheI restriction sites.

For PM targeting, the N-terminal palmitoylation/myristoylation signal of the Lyn protein (MGCIKSKGKDSAGA) was used (24), and it was fused to the N terminus of the human FKBP12 protein either through a short linker (DPTRSAAGAGAGAILSR) or through a longer helical linker (DPTRSAANS(EEAAR)6NSAGAGAGAILSR). This fusion protein was tagged with CFP or mRFP using the pEGFP-N1 plasmid backbone.

For targeting of the FRB protein to the cytoplasmic surface of the ER, the C-terminal localization sequence (residues 521–587) of the human SacI phosphatase (obtained as an expressed sequence tag clone: 3049075 from Open Biosystems) was added to the C terminus of the FRB fragment with a linker of SGAGAGAGAILNSRV between the two proteins. This sequence was placed behind CFP, mRFP, or GFP using the pEGFP-C1 plasmid backbone. A construct in which a longer helical linker of SGAGAGAGAILNS(EEAAR)6NSRV was inserted between the FRB and the ER targeting sequence was also created.

The plasmids designed for the rapamycin-induced PM recruitment of the type-IV 5-phosphatase domain have been described elsewhere (25). All constructs have been sequenced.

*Confocal Analysis, Cytoplasmic Ca2+, and TIRF Measurements of Single Cells*—COS-7 cells were cultured on glass coverslips (3 × 105 cells/35-mm dish) and transfected with the indicated constructs (2 μg of DNA total/dish) using Lipo-Fectamine 2000 for 24 h as described previously (25). Confocal measurements were performed at 35 °C in a modified Krebs-Ringer buffer containing (in mM) 120 NaCl, 4.7 KCl, 1.2 CaCl2, 0.7 MgSO4, 10 glucose, 10 sodium Hepes, pH 7.4, using a Zeiss LSM 510-META scanning confocal microscope and a ×63/1.4 objective. Data were acquired with the multitrack mode with scanning in frame mode using the 405, 488, and 543 nm laser excitation of CFP, YFP (or GFP), and mRFP, respectively. The three channels were recorded with the following emission filters (CFP, 420–490 when together with GFP or 420–505 when together with YFP; GFP or YFP, 505–545, mRFP, 560LP). Post-acquisition picture analysis was performed using the Photoshop (Adobe) software to expand to the full dynamic range, but only linear changes were allowed.

TIRF analysis was performed at room temperature (24 °C) in an Olympus through the lens TIRF microscope system equipped with a Hamamatsu EM-CCD camera and a PlanApo 60/1.45 objective. Excitation with 488 or 568 nm lasers was used for the YFP or Fluor4 and mRFP, respectively, and scans were performed at every 10 s. For data acquisition the Openlab Software (Improvision) was used, and the pictures were exported as TIFF files for processing with the Metamorph software (Molecular Devices). Quantitation of the membrane intensities was determined after defining the regions of individual cells and thresholding. Due to the large variations of the intensities of individual cells because of the different footprint size and translocation responses, these responses were normalized, taking their maximal thapsigargin (Tg)-induced translo-
cation as 100%. These recordings were then averaged, and their S.E. was calculated and plotted against time.

For calcium measurements, cells were loaded with fura2/AM or (Fluo4/AM in the TIRF analysis) (3 μM, 45 min, room temperature). Calcium measurements with Fura2 were also performed at room temperature in the same solution supplemented with 200 μM sulfin-pyrazone. An Olympus IX70 inverted microscope equipped with a Lamba-DG4 illuminator and a MicroMAX-1024BFT digital camera and the appropriate filter sets was used. The MetaFluor (Molecular Devices) software was used for data acquisition.

RESULTS

Characterization of STIM1 Movements in COS-7 Cells—COS-7 cells can provide a better spatial resolution of the ER-PM compartment, yet they have not been used in the published literature for STIM studies. When using these cells, we noted that overexpression of STIM1 has grossly altered the ER architecture and produced large sheets of ER contacting the PM (supplemental Fig. 1). This important observation may have significant relevance to the role of STIM1 in shaping the ER and, therefore, is currently under further investigation. In the meantime, to make STIM1 studies possible in COS-7 cells, we generated STIM constructs designed for low expression levels. For this purpose, we replaced the cytomegalovirus promoter in the STIM1 (and in some other constructs) with the herpes simplex virus TK promoter. Expression of this construct yielded moderate expression levels with features of STIM1 distribution observed in other cells. Unless otherwise noted, all STIM1 constructs referred to in this study are driven by the TK promoter.

The movements of STIM1 were followed in live cells after the expression of a YFP-STIM1 (or mRFP-STIM1) fusion construct containing the fluorescent protein in the luminal side of the ER between the signal sequence and the EF hand essentially as described in Ref. 4. As shown in Fig. 1 and supplemental Movie 1, STIM1 appears mostly in the tubular ER and in the nuclear envelope as observed by previous studies. Activation of endogenously expressed Ca^{2+}-mobilizing P2Y receptors of the cells with ATP caused the rapid appearance of distinct STIM1 puncta in the periphery of the cells, but the localization of STIM1 in the nuclear envelope was only partially affected in most cells. However, the addition of Tg, the sarcoendoplasmic reticulum Ca^{2+}-ATPase blocker, led to the disappearance of STIM1 from the deeper ER structures with a simultaneous enhancement of the peripheral puncta (Fig. 1 and supplemental Movie 1). These data suggested that the depletion of the ER Ca^{2+} is not uniform after agonist stimulation and that it affects the peripheral ER pools before reaching the juxtanuclear compartments. However, the difference in response to ATP and Tg may just reflect the fact that activation of endogenous P2Y receptors generates a relatively low level of InsP_3 in COS-7 cells, thus limiting the Ca^{2+}-mobilizing action of this messenger.

To determine the reversibility of the STIM1 translocation process, two approaches were used. First, the agonist action of
ATP was terminated by the addition of the enzyme apyrase, an ecto-ATPase that rapidly degrades extracellular ATP. Second, the agonist-induced response was terminated by the rapid removal of the PM pool of PtdIns(4,5)P2 by the use of our recently developed chemically induced translocation of the type-IV phosphoinositide 5-phosphatase enzyme to this compartment (25). The process of STIM1 translocation was followed by TIRF analysis where the translocation of STIM1 to the membrane compartment attached to the coverslips can be monitored and quantified (16, 17). As shown in Fig. 1B, the addition of apyrase to cells in which STIM1 had been translocated to the peripheral puncta by ATP application rapidly reversed the process, and STIM1 quickly relocated into the tubular ER compartment. The subsequent addition of Tg to such cells still could evoke a massive translocation of STIM1.

Similar results were obtained when the agonist effect was terminated by eliminating PtdIns(4,5)P2 by the rapamycin-induced PM recruitment of the 5-phosphatase (25). Here the localization response of YFP-STIM1 was simultaneously monitored with the recruitment of the mRFP-FKBP-5-phosphatase by TIRF analysis. ATP-induced localization of STIM1 was rapidly reversed as the 5-phosphatase was recruited to the membrane after rapamycin addition, but Tg was still able to evoke STIM1 translocation even without PtdIns(4,5)P2 in the PM (Fig. 2A). The relocation of STIM1 clearly required the refilling of the Ca2+ stores as neither apyrase (not shown) nor the termination of InsP3 generation by the membrane-translocated 5-phosphatase failed to remove STIM1 from the cell periphery unless Ca2+ was present in the medium (Fig. 2B). These data clearly demonstrated the usefulness of COS-7 cells for these studies and that STIM1 not only translocates to the peripheral ER compartments quickly upon store depletion but that this process is readily reversible upon refilling of the intracellular Ca2+ stores.

The Effects of Tg on Ca2+ Influx and STIM1 Translocation Can Be Significantly Delayed, Showing Considerable Variations between Individual Cells—Next we examined the effect of simultaneous expression of Orai1 and STIM1 on the Ca2+ response of COS-7 cells stimulated either by ATP or by Tg. It has been shown recently that simultaneous expression of these two proteins causes a large capacitative Ca2+ influx response upon store depletion in other cell types (10, 11). Here we compared the Ca2+ responses to application of an agonist or Tg. The addition of Tg alone led to a relatively rapid and small increase in [Ca2+]i, reflecting the rapid Ca2+ release from the ER, followed by a significantly delayed large Ca2+ increase indicating the opening of the capacitative Ca2+ entry pathway (Fig. 3A, blue traces). This delay showed large cell-to-cell variations but was not observed if Tg was added after the agonist, ATP (Fig. 3B). The addition of ATP to cells expressing both STIM1 and Orai1 evoked an immediate, large Ca2+ response that exceeded the increases observed in control cells and showed a significantly enhanced plateau phase. These Ca2+ responses showed two kinds of patterns; in cells that showed moderately enhanced Ca2+ peaks and a steady sustained Ca2+ plateau, the subsequent Tg-induced increase was also moderately enhanced (Fig. 3B, red traces). In contrast, many cells showed large oscillatory Ca2+ waves, and in these cells, the subsequent Tg response was also greatly enhanced (Fig. 3B, blue traces).

To determine whether STIM1 translocation also shows a comparable delay after Tg treatment, cells expressing STIM1
were examined by TIRF analysis. As shown in Fig. 4A, STIM1 translocation in response to Tg showed similar cell-to-cell variations and was significantly delayed when compared with the rapid effects of ATP (shown in Figs. 1 and 2). When the Fluo4 (to monitor Ca^{2+}) and TIRF analysis were done simultaneously, there was a good correlation between the delays of the two responses (Fig. 4B). These results suggested that the Tg-induced store depletion has to reach a certain level before it results in the movement of STIM1 to the cell periphery to activate Ca^{2+} influx, and the variability of the delay is probably due to the different ER Ca^{2+} leak activity of the individual cells.

**Visualization of the ER Compartment Making Contacts with the Plasma-Membrane**—After establishing the COS-7 cell system as a model, we wanted to determine whether there are pre-existing ER compartments beneath the PM that would be preferential sites of STIM1 translocation. To visualize such a compartment, we used a chemically inducible molecular bridge formation strategy. The FRB fragment of the mTOR protein was targeted to the cytoplasmic surface of ER in the form of a CFP fusion protein, whereas the FKBP-12 protein fused to mRFP was targeted to the inner surface of the PM as described under “Experimental Procedures.” The addition of rapamycin causes heterodimerization of the FKBP and FRB modules (24), and we reasoned that a molecular bridge can only be formed at sites where the ER membrane and the PM are at close proximity since both of these proteins are retained in their respective membranes. It was also expected that once such a contact zone had been established, it would be extended as more molecules are recruited to this region. Fig. 5A shows that the two respective constructs show a characteristic PM and ER localization when co-expressed in COS-7 cells. After the addition of rapamycin, both constructs rapidly started to concentrate in small flat areas at the cell surface where they showed tight co-localization. This process could be detected as early as 15–30 s after rapamycin addition. The adjoining areas developed into a relatively stable steady state within 5 min, after which the structures were stable and showed only very slow expansion (see also supplemental Movie 2).

These results indicated that the FRB and FKBP fusion proteins with high lateral mobility within the PM and ER membranes became trapped within their respective membranes at the areas of juxtaposition upon heterodimerization. Although this process also extends the area beyond the initial contact points, it will still pinpoint the sites where the ER membrane and the PM are at close proximity. To determine the extent to which the ER itself is reorganized during the development of these membrane contacts, several markers that visualize the ER membrane were used. These included the type-I InsP_{3} receptor in the form of a YFP fusion protein, a GFP construct targeted to the ER-lumen, and the GFP-fused C-terminal targeting signal of the inositol phosphatase, SacI. In all of these cases, we could observe the flattening of a fraction of the peripheral tubular ER compartment at the sites of FRB-FKBP interaction. Fig. 5B shows an example with the GFP-ER(SacI) protein. However, this manipulation hardly affected the deeper juxtanuclear ER compartments (Fig. 5B), indicating that only the peripheral portion of the ER is subject to the changes induced by the rapamycin-induced PM attachment.

**FIGURE 3. Cytoplasmic Ca^{2+} responses of COS-7 cells transfected with TK-YFP-STIM1 and untagged Orai1.** COS-7 cells were transfected with TK-YFP-STIM1 and an untagged Orai1 construct. After 24 h, cells were loaded with 3 μM Fura2/AM for 45 min at room temperature. Cells were then washed twice, and their single cell Ca^{2+} responses were monitored and their YFP signal was also determined, indicating the expression of the proteins (see “Experimental Procedures” for further details). A, [Ca^{2+}], responses of the individual YFP-STIM1-expressing cells (blue traces) to 200 nM Tg are plotted against the averaged responses of non-transfected cells (n = 28) recorded in the same field (black trace). Note the large variations in the onset of the huge Ca^{2+} influx response relative to the relatively uniform onset of Ca^{2+} release. B, [Ca^{2+}], responses of cells stimulated with ATP (50 μM). The ATP responses of cells expressing YFP-STIM1 fell into two relatively well distinguishable groups. Some cells showed an increased peak and a sustained and elevated steady plateau increase of [Ca^{2+}], after ATP stimulation, and these had a moderate Ca^{2+} response to a subsequent Tg challenge (red traces). In contrast, many cells displayed large oscillatory Ca^{2+} waves, and these also showed a large response to subsequent Tg stimulation (blue traces). These differences probably reflect the different extent of STIM1-Orai1 expression. The black trace shows the averaged responses of non-transfected cells (n = 20) recorded in the same field.
The Site of STIM1 Translocation Corresponds to the PM-ER Contact Sites—Next we examined whether STIM1 translocation to the peripheral ER sites upon store depletion corresponds to the sites that can be established by chemical cross-bridging. COS-7 cells were co-transfected with the YFP-fused form of STIM1 in addition to the PM-targeted mRFP-FKBP and the ER-targeted CFP-FRB fusion proteins (in some cases, both constructs were tagged with CFP). Such cells were then treated with Tg to translocate STIM1 into the peripheral ER and form the subplasmamembranal puncta (Fig. 6A). Tg treatment did not alter the localization of either the PM (Fig. 6A, panel a) or the ER-targeted (not shown) fusion proteins. The addition of rapamycin to Tg-treated cells rapidly established the PM-ER contact patches, and importantly, these were always initiated from the sites of STIM1 localization (Fig. 6A, panels d–i). These results suggested that the appearance and expansion of these membrane contacts upon chemical cross-bridging indeed originate from the contact points between the ER and the PM and that STIM1 defines this compartment in a store-depleted state.

FIGURE 4. STIM1 recruitment to the subplasmamembranal ER shows variable delays after thapsigargin treatment. COS-7 cells were co-transfected with TK-mRFP-STIM1 and untagged Orai1 constructs for 1 day, and in some cases, loaded with the Ca\(^{2+}\) probe Fluo4/AM (3 \(\mu\)M) for 45 min. After washes, TIRF recordings were made using a detection of STIM1 in the red channel, and when applicable, a simultaneous monitoring of Fluo4 fluorescence in the green channel. A, the appearance of STIM1 close to the PM shows a delay with large variations (compare these responses with those observed after ATP in Fig. 3). B, simultaneous Ca\(^{2+}\) recordings from selected cells show that the initial Ca\(^{2+}\) release does not differ between cells, but the secondary Ca\(^{2+}\) rise due to Ca\(^{2+}\) influx through the Orai1 channels shows variations and correlates with the STIM1 translocation process. Note that the relative magnitude of the initial rise of Ca\(^{2+}\) (due to release) appears bigger in these recordings when compared with those obtained with Fura-2 due to the higher Ca\(^{2+}\) affinity of Fluo4 and that the Ca\(^{2+}\) changes appear transient, but this is due to the photobleaching of the probe as it is not used in a ratiometric mode. The continuous lines, the dotted lines, and the dashed lines identify corresponding calcium and translocation responses of individual cells.

FIGURE 5. Visualization of PM-ER contact regions by chemical cross-bridging of subplasmamembranal ER with the PM. A, COS-7 cells were transfected with a PM-targeted FKBP-mRFP construct and a CFP-FRB construct targeted to the cytoplasmic face of the ER. These constructs show their respective membrane localization (panels a and b). Due to the recording close to the cell surface, the ER architecture is somewhat fuzzy (panel b). The addition of rapamycin (rapa, 100 nM) results in the concentration of both proteins in small patchy areas where they show tight co-localization (panels d–i, see also supplemental Movie 2). B, cells were transfected with the PM-targeted FKBP-mRFP and CFP-FRB-ER constructs plus a GFP construct targeted to the ER by the same targeting sequence as in panel A but without FRB. This latter construct is not expected to participate in the cross-bridging and is used to monitor the overall changes in ER morphology during the cross-bridging process. The CFP channel is not shown for clarity. Although the tubular ER network is largely preserved after rapamycin addition, there are flattened ER areas (panel e) that correspond to the patchy regions formed by the ER and PM contacts (panel d). Bars shown are 10 \(\mu\)m.
**STIM1 Clustering**

**A**

PM-FKBP-mRFP | TK-YFP-STIM1 | merge
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Panel a: after Tg | Panel b: TK-YFP-STIM1 | Panel c: merge

**B**

PM-FKBP-mRFP | TK-YFP-STIM1 | merge
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Panel a: after rapamycin | Panel b: TK-YFP-STIM1 | Panel c: merge

**FIGURE 6.** PM-ER contact regions correlate with the location of STIM1 in thapsigargin-treated cells. A, COS-7 cells were transfected with the PM-targeted FKBP-mRFP and CFP-FRB-ER constructs together with TK-YFP-STIM1. Cells were treated with Tg (200 nM) until STIM1 appeared in the juxtamembrane ER compartment (panel b). At this point, the PM-FKBP-mRFP is found at the PM (panel d), whereas the CFP-FRB-ER protein is in the ER (not shown in the figure). The addition of rapamycin (100 nM) results in the rapid formation of ER-PM junctions (indicated by the PM-FKBP-mRFP signal shown in panel d), and these develop at sites that correspond to the localization of STIM1 (panels e–i). Remarkably, during this process, the YFP-STIM1 reorganizes into small puncta that are always located at the periphery but attached to the cross-bridged ER-PM areas (panels g–i) but occasionally were trapped within those (two examples are indicated in panels g and i by the arrows). In this latter case, their negative imprint is clearly visible in the red channel (panel g). B, cells were transfected with the same three constructs as in panel A, but here rapamycin was added first before Tg. The addition of rapamycin leads to the development of ER-PM junctions (panel d), and STIM1 is also located in these flattened ER areas (panel b). The addition of Tg results in the movement of STIM1 from these areas to their periphery, where it forms intense puncta attached to the areas of PM-ER junctions (panels e and h) (see also supplemental Movie 2). Bars shown are 10 μm.

A remarkable finding of these experiments was that the STIM1 puncta were almost always pushed to the periphery of the PM-ER junctional zones. Only in a few cases were there STIM1 puncta trapped within these contact zones (Fig. 6A, panel i, arrows). These data raised the possibility that STIM1 might be part of a larger molecular complex that is excluded from the narrow gap between the PM and ER membranes as enforced by the chemical cross-bridge.

**STIM1 Forms Clusters within the Subplasmalemmal ER Compartment upon Store Depletion—**To further investigate the movements of STIM1 during ER Ca\(^{2+}\) depletion in relationship to the rapamycin-induced PM-ER junctions, similar experiments were performed as described above, but the order of addition was reversed. First, the PM-ER contacts were established by the addition of rapamycin followed by Ca\(^{2+}\) depletion by Tg. In quiescent cells, the three proteins (the YFP-fused form of STIM1, the PM-targeted mRFP(or CFP)-FKBP, and the ER-targeted CFP-FRB) showed their characteristic localization. The addition of rapamycin caused the formation of the PM-ER contact sites, and STIM1 (found in the ER close to the periphery) was also present in this flattened ER compartment (Fig. 6B). These changes were consistent with the rapamycin-induced recruitment and juxtapositioning of the peripheral tubular ER in which STIM1 is already localized. When Tg was added to such cells to deplete the ER Ca\(^{2+}\) stores, a striking movement of STIM1 from the patchy areas into discrete puncta anchored to the perimeter of the patches occurred (Fig. 6B and supplemental Movie 3). Similar changes were observed when ATP was used instead of Tg (supplemental Fig. 2). This very prominent ATP-induced clustering was rapidly reversible upon termination of the response by apyrase addition (supplemental Fig. 2).

This clustering together with the moving of STIM1 out to the periphery of the PM-ER junctions strongly suggested that although the cytosolic segment of the STIM1 molecule fits within the tight space formed by the cross-bridged PM-ER junctions in control cells, upon store depletion, STIM1 interacts with a protein that is larger than the space between the chemically cross-bridged membranes, and this interaction occurs in discrete contact sites.

**Orai1 in the PM Becomes Part of the STIM1 Clusters upon Store Depletion—**Next we investigated the distribution of Orai1 during these manipulations. For this, Orai1 was tagged at its C terminus with YFP, CFP, or mRFP with different linkers between the two proteins. Expression of these fusion proteins showed mostly PM localization (Fig. 7A), but in a small percentage of cells, they were also seen in the Golgi and even in tubular ER structures, probably reflecting the different stages as the protein made its way to the PM (not shown). The Orai1 protein fused to the fluorophores with a short linker was found to be active based on Ca\(^{2+}\) measurements and elicited large Ca\(^{2+}\) influx responses in the cells when expressed together with TK-YFP-STIM1 (Fig. 7B). In cells co-expressing YFP-STIM1 and Orai1-mRFP (or -CFP), the two constructs showed their respective localizations, Orai1 in the PM and STIM1 in the ER (Fig. 7C). However, in some cells, STIM1 was already localized to membrane-adjacent ER areas, and Orai1 was also enriched in the same locations of the PM (not shown). The addition of Tg (together with ATP to speed up the ER Ca\(^{2+}\) depletion process) caused the rapid concentration of PM Orai1 in the same areas where the STIM1 puncta appeared (Fig. 7C). Importantly, in cells transfected with Orai1-mRFP (or -CFP or -YFP) with-
To determine the localization of Orai1 in cells where the PM-ER junctions were transfected with the ER-targeted FRB and the PM-targeted FKBP together with Orai1-YFP. The addition of rapamycin to such cells induced the formation of PM-ER junctions (Fig. 8B). To examine the movements of both STIM1 and Orai1 simultaneously and to determine their relative positions to the PM-ER junctions, the ER-targeted FRB and the PM-targeted FKBP (both tagged with CFP in this case) were expressed together with TK-YFP-STIM1 and Orai1-mRFP. The addition of rapamycin to such cells induced the formation of the PM-ER junctions with the peripheral STIM1 localized in them, but Orai1 was prominently excluded from these areas (shown only after the Tg addition in Fig. 8C). Application of Tg induced the movement of both of these proteins into prominent clusters always at the periphery of the membrane patches where they showed almost perfect co-localization (Fig. 8C). This involved the movement of STIM1 out of the membrane patches as described above. These data suggested that the two proteins formed a complex at the border of the PM-ER zones. For this to happen, STIM1 retained in the ER had to move to the periphery of the PM-ER zones to meet Orai1 of the PM that was excluded from these areas.

**Size Estimates of the Orai1 Complex**—To determine the minimal size of the gap between the two membranes that would accommodate the Orai1 complex, the constructs used for chemical cross-linking were modified. A long helical linker constructed with a (EAAAR)₉ sequence was placed between the localization signals and the FKBP (or FRB) molecule (see Fig. 10). First, we expressed either one of the partners containing the extended linker with the original “short” binding partner (which would extend the intermembrane space to ~9 nm). The formation of rapamycin-induced ER-PM junctions in this case still excluded Orai1-YFP (or -mRFP) from the junctions (not shown). However, when the extended versions of both the PM-targeted and the ER-targeted constructs were used (allowing a calculated intermembrane space up to ~14 nm), Orai1-YFP (or -mRFP) was no more forced out from the rapamycin-induced PM-ER junctions (Fig. 9A). When the localization of mRFP-STIM1 and Orai1-YFP was simultaneously followed in such cells after establishing ER-PM contact zones, STIM1 was found within these zones, whereas Orai1 showed its characteristic even PM localization (Fig. 9B, upper row). The addition of ATP/Tg rapidly induced the movement of Orai1 into the same contact zones within the PM, indicating its interaction with STIM1 (Fig. 9B, lower row). The co-localization of STIM1 and Orai1 at these PM-ER sites is clearly observed even upon prolonged incubations, and unlike in the case of the short linkers, it did not yield clusters at the periphery of the contact zones but rather an even co-localization with them (Fig. 9C). These data suggested that the molecular complex containing Orai1 can be accommodated into a space that has to be larger than ~9 but smaller than ~14 nm, and in such contact zones, Orai1 can form a complex with STIM1.
DISCUSSION

The present study was designed to investigate the features of the ER compartment that is closely apposed to the PM. Although the functional significance of such an ER compartment has been postulated before, its importance became highlighted by the recent discovery of the STIM1 protein and its association with the PM-localized Orai1 Ca^{2+} channel in response to depletion of the ER Ca^{2+} stores (4–12). To localize and visualize this compartment of the ER, we developed a new approach based on the rapamycin-induced dimerization of the FRB fragment of mTOR and FKBP-12 (26). By targeting the FKBP and FRB proteins to the cytoplasmic surface of the PM and ER, respectively, rapamycin-induced binding of the two proteins can take place, but only where the two membranes are within a certain distance. One can assume that the PM-ER contact sites are very dynamic in intact cells, only providing cells with transient juxtapositions that are formed and depart rapidly. However, by adding the dimerizer, these contact zones are stabilized, and the two membranes are tightly held together with little gap between them, mimicking the process that takes place when the Orai1 and STIM1 proteins form a complex during ER Ca^{2+} store depletion. By altering the length of the linkers, the size of the gap between the two membranes can be changed, and we observed that the shortest linkers yielded larger ER-PM areas relatively quickly, developing after dimerization. However, when using the longer linkers, the ER-PM areas were more reminiscent of those developing with STIM1 after pool depletion (i.e. they did not expand to large sizes quickly). Interestingly, the formation of the artificial contacts (whether with the short or long linkers) had no obvious functional consequence for the capacitative Ca^{2+} influx pathway, as we did not observe a cytosolic Ca^{2+} increase after cross-bridging or any obvious difference between the lag times or magnitudes of the Tg-induced cytoplasmic Ca^{2+} signals in such cells when compared with controls (supplemental Fig. 3). This was surprising as these contact sites do contain a fraction of the expressed STIM1 protein. In this context, it is important to emphasize that STIM1 can appear in the ER compartment that is juxtaposed to the PM without being active, i.e. leading to the opening of the Orai1 channel. This patchy distribution that can be almost indistinguishable from the STIM1 clusters evoked by store depletion is observed in quiescent cells expressing slightly higher levels of STIM1 (see supplemental Fig. 1) or in cells where the peripheral ER is brought to the PM with the chemical cross-bridging. None of these situations yielded increased Orai1 activity (as judged by cytosolic Ca^{2+} increases), suggesting that without the conformational change imposed by store depletion and probably the consequential oligomerization (27), simple juxtaposition of STIM1 with the PM is not sufficient to induce Ca^{2+} influx.

This new approach has revealed several novel details of the STIM1-Orai1 interaction process at the PM-ER junctions. First, the artificially generated contact sites tightly coincided with those generated by store depletion regardless of whether...
they were formed before or after store depletion. The tightest artificial PM-ER junctions also flatten the peripheral portion of the ER and contain a significant fraction of the expressed STIM1 protein, but surprisingly, exclude the expressed Orai1 protein. This suggests that the gap between the membranes in this case is narrower than the natural contacts formed by STIM1-Orai1 interaction. This feature, however, allowed the monitoring of the reversible interaction of the STIM1 and Orai1 proteins regulated by store depletion in the plane of the membrane and clearly showed again that a simple juxtaposition of these proteins is not sufficient for their interaction. This interaction requires depletion of ER Ca\textsuperscript{2+} stores, causing prominent clustering of the two proteins but only at the perimeter of tight PM-ER junctions. The fact that these contacts were formed in discrete puncta suggests the existence of preferential sites where this interaction takes place; otherwise, these proteins would be expected to assemble evenly around the contact zones. The finding that STIM1 must travel to the periphery of the PM-ER junctions to form puncta even when Orai1 is not expressed indicates that endogenous Orai1 is also excluded from these junctions and that exclusion of the fluorescently tagged Orai1 protein is not simply caused by the added fluorescent protein tag.

It is noteworthy that STIM1 was not excluded from even the tightest contact zones; in fact, it seemed to move freely within them. This discrepancy between the space requirements of Orai1 and STIM1 is not easily explained by the length of their putative cytoplasmic portions. Therefore, we assume that Orai1 is associated with a larger protein complex. The existence of additional molecular participants in the capacitative entry process is also indicated by several observations. First, the stoichiometry between these components has important bearings on the process. Expression of Orai1 alone is inhibitory rather than stimulatory on the Ca\textsuperscript{2+} entry process, suggesting that it sequesters a component important for the SOCE. However, together with STIM1, it causes large Ca\textsuperscript{2+} influx, indicating that under these conditions, STIM1 is the limiting factor. In contrast, STIM1 expression alone only marginally enhances Ca\textsuperscript{2+} influx upon store depletion (28, 29), suggesting that now the amount of Orai1 is limited. However, the ER morphology is extremely sensitive to STIM1 overexpression, causing juxtapositioning of large ER sheets with the PM (see supplemental Fig. 1). This may be indicative of STIM1 interacting with an abundant protein in the PM that cannot be Orai1 as this morphological change is not asso-

![STIM1 Clustering](image)
Recent cross-linking experiments have also indicated that Orai1 is recovered in a large molecular weight complex regardless of store depletion and that STIM1 is not part of this complex under any of the various detergent conditions used in that study (30). Immunoprecipitation of STIM1 with Orai1 is also controversial, being found independent of store depletion (12), increased after store depletion (14), or not demonstrable (30), perhaps indicating an indirect interaction and its dependence on the stoichiometry and state of putative additional components.

While characterizing the movements of Orai1 and STIM1 and the resulting Ca\textsuperscript{2+} responses in the larger COS-7 cells, we made several important observations. First, a rapid reversal of the STIM1 translocation process to the juxtamembrane ER was found when the agonist effect was terminated. Although it has been known that the capacitative Ca\textsuperscript{2+} entry pathway rapidly shuts down upon refilling of the ER Ca\textsuperscript{2+} pools, it was not clear whether this was due to a change in the conductivity of the Orai1 channel still complexed with STIM1 or the dissociation of the STIM1-Orai complex and relocalization of STIM1 away from the PM. Although the current results do not rule out that the termination process involves Orai1 channel closure before the relocalization of STIM1, they certainly show that the two processes follow very similar time scales.

The rapid change of STIM1 localization upon store depletion could simply be caused by the association of the STIM1 protein with Orai1 at the PM in the Ca\textsuperscript{2+} depleted state. However, the
rapid return of STIM1 to the deeper ER also suggests that STIM1 associates with a yet to be identified ER-resident protein in the Ca\(^{2+}\) replete state. The PM recruitment process is very reminiscent of the movement of the ER-localized FRB protein to the PM contact points after the addition of the dimerizer to interact with the PM-targeted FKBP (especially with the longer linkers in place), but this latter process cannot be rapidly reversed with our current methods.

Another notable finding of the present study was the clear dissociation of the translocation of STIM1 and activation of the Ca\(^{2+}\) influx pathway from the initial Ca\(^{2+}\) release after Tg addition. The delay was caused at the level of STIM1 translocation as the translocation and Ca\(^{2+}\) rise showed good correlation in agreement with findings of a recent report (15). This delay showed big cell-to-cell variations, raising the possibility that cells are different in their leak currents, taking more time to achieve the same decrease in the intralumenal Ca\(^{2+}\) concentration in some cells. Alternatively, cells are different in the Ca\(^{2+}\) threshold, at which point STIM1 changes conformation. A similar delay in SOCE relative to Ca\(^{2+}\) release in Tg-treated rat basophilic leukemia cells has already been described, and it was attributed to the release of Ca\(^{2+}\) first from an ER store related to protein synthesis but irrelevant to STIM1 movements (31). This could also be the case in the COS-7 cells. However, the finding that agonist addition, leading to Ins\(_P_3\) formation, eliminates this delay shows that once ER depletion is rapidly achieved, the time difference between these events becomes minimal. The difference between the STIM1 response to agonists and Tg was also displayed in experiments showing that agonist affected only the peripheral ER pool of STIM1, whereas Tg also mobilized STIM1 from the deeper juxtanuclear compartments. Similar findings were recently reported in human pancreatic acinar duct cells with a low concentration of Tg (32). A more prominent difference was the appearance of large oscillatory Ca\(^{2+}\) responses to ATP stimulation observed in some cells overexpressing Orai1 and STIM1 that was not observed with Tg. Because these large Ca\(^{2+}\) changes are due to Ca\(^{2+}\) influx, we assume that the Orai1 channel might undergo some rapid feedback regulation by Ca\(^{2+}\) or a Ca\(^{2+}\)-induced depolarization of cells. Although these observations need to be followed up with more detailed studies on the channels, it is worth noting that recent studies showed roles of STIM1 and Orai1 in agonist-stimulated oscillatory Ca\(^{2+}\) responses (33).

In summary, the present studies show that juxtamembrane compartments of the ER can be visualized in living cells and that such sites are formed and get stabilized by STIM1 and Orai1 when the luminal ER Ca\(^{2+}\) concentration is decreased. The formation of such contacts per se does not appreciably affect Ca\(^{2+}\) signaling. Using a chemically controlled juxtapositioning of the ER and PM membranes with variable size gaps, we demonstrate that Orai1 requires a larger space (11–14 nm) than STIM1 (4–6 nm) between the two membranes. This finding suggests that Orai1 is part of a larger molecular complex. Our data also highlight important spatial and kinetic differences between the effects of Tg and agonists in the process of activating Ca\(^{2+}\) entry.

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