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The STIM1/Orai signaling machinery

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Ca^{2+} influx via store-operated Ca^{2+} release activated Ca^{2+} (CRAC) channels represents a main signaling pathway for T-cell activation as well as mast-cell degranulation. The ER-located Ca^{2+}-sensor, STIM1 and the Ca^{2+}-selective ion pore, Orai1 in the membrane are sufficient to fully reconstitute CRAC currents. Their identification, but even more the recent structural resolution of both proteins by X-ray crystallography has substantially advanced the understanding of the activation mechanism of CRAC channels. In this review, we provide a detailed description of the STIM1/Orai1 signaling pathway thereby focusing on the critical domains mediating both, intra – as well as intermolecular interactions and on the ion permeation pathway. Based on the results of functional studies as well as the recently published crystal structures, we portray a mechanistic view of the steps in the CRAC channel signaling cascade ranging from STIM1 oligomerization over STIM1-Orai1 coupling to the ultimate Orai1 channel activation and permeation.

Introduction to CRAC channels

The Ca^{2+} ion represents a major intracellular messenger in eukaryotic cells. Changes in cytosolic Ca^{2+} concentrations are required for many physiological processes such as proliferation, contraction, secretion.[1,2] Among the pathways that allow Ca^{2+} entry from the extracellular matrix, Ca^{2+} release-activated Ca^{2+} (CRAC) channels belong to the group of store-operated channels (SOC) which have been extensively studied during the past 2 decades in T-lymphocytes and mast cells.[3] In 2005 and 2006, STIM1 and Orai1, the key components fully reconstituting CRAC currents have been identified.[4-7] One form of hereditary severe combined immune deficiency (SCID) syndrome which is linked to a defect in CRAC channel function has opened the way to the identification of the Orai1 channel protein and its mutated form (Orai1 R91W) in SCID patients.[5] Moreover, here Feske et al.[5] have combined a modified linkage analysis with single-nucleotide polymorphism arrays and a *Drosophila* RNA interference screen that has conclusively led to the identification of Orai1 (also initially termed CRACM1[6]) as the CRAC channel pore forming unit. Sequence database research has revealed a family consisting of three homologous proteins in higher vertebrates, i.e., Orai1, Orai2, and Orai3. Bioinformatic analysis predicts each of the Orai family members as a transmembrane (TM) protein with 4 TM spanning segments and 3 cytosolic strands including the N-terminus, the second loop connecting TM2 and TM3 and the C-terminus. Electrophysiological characterization by several groups has revealed that Orai proteins represent unequivocally the pore forming entity in the CRAC channel complex, providing the high Ca^{2+}-selectivity and low single channel conductance.[8-11] Further biophysical experiments have shed light on the Orai isoforms' distinct properties like inactivation profiles and 2-aminoethyldiphenyl borate (2-APB) sensitivity.[11-23]

In 2005, Liou et al.[7] as well as Roos et al.[4] have characterized the CRAC channel activating protein, i.e., stromal interaction molecule 1 (STIM1). By knocking down 2300 signaling proteins in HeLa and Drosophila S2 insect cells using an RNA interference-based screen, they have identified 2 proteins essential for endoplasmic reticulum (ER) store depletion mediated Ca^{2+} influx, STIM1 and STIM2. These ER-located Ca^{2+} sensors are responsible for activating CRAC channels following Ca^{2+} depletion of the ER. STIM2 shares approximately 61% sequence identity with STIM1.[24] Both proteins contain an ER luminal N-terminus including the Ca^{2+} sensing EF-hand, 1 TM spanning segment and a long cytosolic strand which couples to and activates Orai.[4,7,25] At resting cell conditions, STIM1 exhibits a tubular distribution throughout the cytosolic ER compartment[26,27] and has also been detected to a small extent in the plasma membrane (PM).[28-31] Upon store depletion, the luminal STIM1 EF hand loses Ca^{2+} which triggers homomerization and translocation of STIM1 to the cell periphery near the PM, i.e., ER-PM junctions. In these microdomains the STIM1 oligomers form punctuate clusters which interact with and activate Orai channels.[32-36] The small portion of the STIM1 pool that integrates into the plasma membrane is not essentially required for CRAC channel activation. It has been shown that STIM1 has an additional role in TRP channel regulation[19,31,37-39] and leukotriene C4-[40,41] as well as arachidonate-[42,43] stimulated Ca^{2+} channels where Orai1 and Orai3 are essential components.

In 2012, the crystal structures of both cytosolic fragments of STIM1[42] and full-length Orai[43] have been reported, representing a milestone toward detailed elucidation of intra- and intermolecular interactions of these proteins and their conformational changes. In the following, mechanistic aspects of the STIM/Orai signaling machinery are presented and discussed in the context of these structures.

STIM1

STIM1 oligomerization and translocation

Overexpressed fluorescence-tagged STIM1 proteins exhibit a dramatic change in localization in response to store depletion. In resting cell with full ER Ca^{2+} stores, STIM1 is

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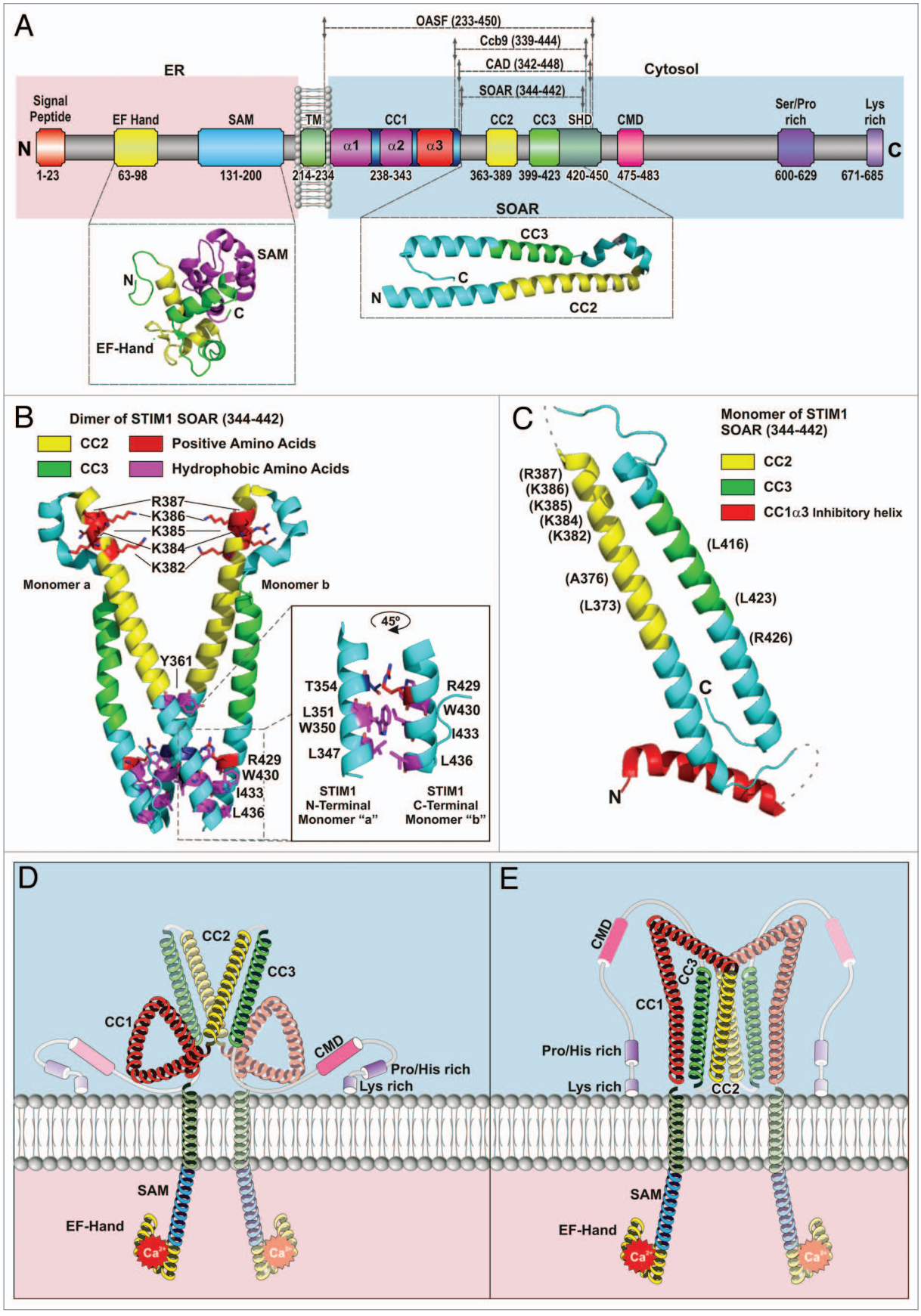

Figure 1. For figure legend, see page 332.

Figure 1 (See previous page). STIM1: (**A**) Schematic representation of human, full-length STIM1 depicting regions essential to the STIM1/Orai1 signaling cascade. Moreover, the structure of the EF-SAM domain as well as the STIM1 SOAR (344–442) fragment is shown. (**B**) Cartoon representation of a STIM1 SOAR (344–442) – dimer including coiled-coil domain 2 (CC2) and 3 (CC3) exhibiting a V-shaped structure. Furthermore, residues mediating dimer interaction and those involved in the coupling to Orai1 (positively charged residues) are highlighted. Inset depicts magnified view of interacting residues between Monomer "a" N-terminal and Monomer "b" C-terminal segments. (**C**) Cartoon representation of a STIM1 SOAR (344–442) monomer together with the inhibitory helix (aa 310–337), depicting critical residues the mutation of which modifies STIM1 function (aa numbering refers to human STIM1). (**D and E**) Two hypothetical models of STIM1 in the resting state.

homogenously distributed and moves rapidly along microtubules. Following store depletion STIM1 oligomerizes, consequently slowing down its movement along the microtubules.[44] In a former study, Baba et al.[32] have demonstrated a dynamic, constitutive movement of STIM1 in resting cells, while store depletion leads to redistribution of STIM1 into discrete puncta located at ER-PM junctions. Examination of several mutants with deletion in the cytosolic strand of STIM1 has revealed coiled-coil (CC) regions and the serine/proline rich domain of STIM1 (**Fig. 1A**) as essential for the constitutive movement of STIM1, while the puncta formation involves both luminal as well as cytoplasmic STIM1 domains.[32] As derived from FRET experiments, store depletion consequently leads to the formation of stable STIM1 oligomers.[36,45,46] Elegant experiments performed by Luik et al.[35] utilizing artificial luminal cross-linking of STIM1 demonstrates luminal dimerization as trigger signal for STIM1 oligomerization and accumulation at ER-PM junctions where it interacts with and activates Orai1 channels. In summary, these results point to the fact that initial di – and/or oligomerization on the luminal side is the first step in the activation process of STIM1 induced by store depletion.[4,7,47-49]

STIM1 essential domains in the ER luminal strand

STIM1 contains an ER luminal N-terminus, a single TM spanning domain and a cytosolic C-terminus (**Fig. 1A**). The ER luminal part includes a canonical and a hidden EF-hand followed by a sterile-α motif (SAM).[50-52] The EF-hand consists of a helix-loop-helix motif where negatively charged aspartates and glutamates bind Ca^{2+} as long as the stores are full. Ca^{2+} store depletion is the initial signal for STIM1 oligomerization, with Ca^{2+} dissociating from the EF hand (K_d ~200–600μM) thereby destabilizing the entire EF-SAM entity.[51] The low Ca^{2+} binding affinity perfectly matches the Ca^{2+} concentration range (~400–800 μM) at which an ER Ca^{2+} sensor protein is able to accurately respond to changing ER Ca^{2+} concentrations. Stathopulos et al.[52] have examined in detail the EF-SAM complex (**Fig. 1A**). They have demonstrated that holo EF-SAM loaded with Ca^{2+} contains high α-helicity whereas in the absence of Ca^{2+} (apo EF-SAM) it is less compact. The holoform has proven to be a monomer whereas the apoform in contrast forms at least a dimer.[51,52] The calculated Hill coefficient for the luminal domains of STIM1 under low $[Ca^{2+}]_{ER}$ concentrations is ~4 which is rather high and in line with a probable multimeric state.[35] Accordingly, Covington et al.[53] have shown that a STIM1 deletion mutant lacking the whole, cytosolic C-terminus is also able to di-/oligomerize via the luminal strands in response to store depletion as derived from FRET increases. Both STIM1 and STIM2 contain EF-SAM domains in their ER luminal part, however, these domains behave differently.[25] As STIM2 activates CRAC currents upon smaller decreases in $[Ca^{2+}]_{ER}$, STIM2 has been proposed to be part of a feedback system to keep $[Ca^{2+}]_{ER}$ in tight limits.[25] The reason for the distinct behavior of EF-SAM of STIM1 and STIM2 has been explained by a difference in the luminal, structural stability of STIM1 and STIM2.[54,55] Next to the SAM domain a TM segment spans the ER membrane (**Fig. 1A**). Whether the TM domain acts as a passive entity or affects STIM1 oligomerization actively remains to be seen.

STIM1 essential domains in the cytosolic strand

The long, cytosolic strand includes three putative coiled-coil (CC1, CC2, CC3) regions, the CRAC modulatory domain CMD, a serine/proline – and a lysine-rich region (**Fig. 1A**). As demonstrated by Huang et al.[56] and Muik et al.,[57] the STIM1 C-terminus is sufficient to activate CRAC channels. Based on this finding, several groups have analyzed shortened cytosolic STIM1 fragments to identify the key domains that are sufficient to activate Orai channels. Finally and nearly at the same time, OASF (233–450), CAD (342–448), SOAR (344–442), and Ccb9 (339–444) have been identified[57-60] (**Fig. 1A**). All these fragments have the CC2 (363–389) and CC3 (399–423) regions with additional 19 residues (424–442) in common (**Fig. 1A**), integrating an Orai coupling and activating domain as well as a STIM1 homomerization domain (SHD). The latter domain has been assigned to the segment ~421–450 and it is involved in cytosolic STIM1 homomerization.[57] Deletion of the SHD in OASF results in substantially reduced FRET values in homomerization experiments and abolishes activation of Orai1 channels in patch clamp recordings.[57]

Crystal structure of the Orai1 activating entity of STIM1

In 2012 the first crystal structure of a cytosolic portion of STIM1 has been reported revealing intra– and intermolecular interactions of a dimeric assembly.[42] The crystallized hSOAR protein (345–444 $_{L374M, V419A, C437T}$) forms a dimer (**Fig. 1B**) which possibly corresponds to human STIM1 in the activated state. The structure of the monomeric SOAR molecule resembles that of the capital letter "R." It consists of 2 long α-helices, i.e., CC2 and CC3, arranged in antiparallel manner that are linked by 2 short α-helices. The dimer`s interface is generated by CC interactions with the C – and N-termini, respectively, from the other monomer. C-terminal residues (R429, W430, I433, L436) from 1 monomer interact with N-terminal amino acids (T354, L351, W350, L347) of the other monomer resulting in an overall V-shape structure of the SOAR dimer (**Fig. 1B**). The tyrosines 361 from each monomer form a stacking interaction at the crossing point of both CC2. Amino acid mutations (of L347A-W350A-L351A or W430A-I433A-L436A) within the dimer interface region in SOAR as well as full-length STIM1 disrupt co-localization with and activation of Orai1 channels.[42] A cluster of positively charged residues, i.e., K382, K384, K385, K386, R387, is located on either tip of the V-shape structure of the hSOAR dimer (**Fig. 1B**).

The role of CC1 in controlling the activation state of STIM1

In addition to the Orai1 activating entity of STIM1, i.e., SOAR, the CC1 domain (aa 238–343, **Fig. 1A**) plays an important role in

transducing luminal di-/oligo-merization to the cytosolic strand of STIM1 resulting in SOAR exposure and homomerization.[42,61-64] Covington et al.[53] have analyzed the impact of the respective cytosolic CC domains on homomerization by the STIM1 C-terminal deletion mutants STIM1-CC1 (aa 1–344) and STIM1-CC1-CAD (aa 1–448) concluding that CC1 leads to store independent, yet unstable oligomerization, while CC3+SHD (see **Fig. 1A**) within CAD enables store dependent oligomerization. Although STIM1-CC1 demonstrates weak oligomerization potential per se, the role of CC1 has to be evaluated in the context of CC1-SOAR/CAD which mimics the inactive state as long as stores are full.[62,65,66] Current models of STIM1 activation show CC1 oligomerization subsequent to store depletion.[42,65,66] The detailed role of CC1 still remains puzzling, as its mechanistic function in controlling SOAR exposure (see below) and oligomerization has not been sufficiently resolved. Bioinformatic secondary structure predictions suggest the presence of 3 α helices (α1, α2, and α3) within the CC1 structure (**Fig. 1A**).[63] Helix α1 comprises aa 238–271, helix α2 aa 278–304, and helix α3 aa 308–337 (also known as inhibitory helix IH[42]). Several hypothetical models on the mechanism of CC1 in the control of STIM1 activation status have been proposed (**Fig. 1D or E**). Korzeniowski et al.[67] suggest that STIM1 C-terminus is kept in a quiescent state due to an autoinhibitory, intramolecular electrostatic interaction between an inhibitory acidic segment within α3 of CC1 and a short basic region (aa382–387) within SOAR. However, as evident from the crystal structure of *C.elegans* of CC1+SOAR (**Fig. 1C**), the so called inhibitory helix ($\alpha3_{CC1}$) is less likely in the position to form the suggested electrostatic clamp-[42]with the basic region at the tip (see **Fig. 1C**). Yang et al.[42] propose that $\alpha3_{CC1}$ forms intramolecular interactions with residues at the beginning of CC2 and at the end of CC3 (**Fig. 1C**). Their activation model describes a STIM1 dimer in the resting state where the SOAR domain is responsible for dimerization and is occluded by the inhibitory helix ($\alpha3_{CC1}$). Another work by Machaca's group[64] presents an intramolecular shielding model of STIM1 that keeps it in a quiescent state. They show that the acidic region within $\alpha3_{CC1}$ is neither involved in electrostatic interactions nor in CC formation as deletion or substitutions of this segment reveals no effect. However, multiple mutations within $\alpha3_{CC1}$ affecting the amphipathic character of the helix seem to have an impact. Therefore, they conclude that the amphipathic nature of the $\alpha3_{CC1}$ regulates the STIM1 activation status.[64]

In an attempt to monitor conformational re-arrangements, we have developed a double-labeled YFP-OASF-CFP conformational sensor to show that STIM1 aa233–474 (OASF), comprising both CC1 and SOAR, folds into a rather closed conformation which may represent the quiescent state of STIM1 when Ca^{2+} stores are full. Consistently, Zhou et al.[68] reported recently, based on Tb^{3+}-acceptor energy transfer measurements, a similarly tight conformation of STIM1 C-terminus (233–685) where the polybasic segment at the end is near to the residue 233 at the beginning. In attempt to reveal the molecular steps that guide the cytosolic strand of STIM1 from the quiescent into the active form, either mutations or artificial crosslinking have been performed. By introducing point mutations in $\alpha1_{CC1}$ (L251S) or CC3 (L416S L423S) (see **Fig. 1C**) the OASF sensor then adopts an extended conformation as measured by FRET.[62] When introducing these CC1 or CC3 point mutations in full length STIM1, constitutive CRAC currents have been observed despite the stores are not depleted. Hence, we have suggested that intramolecular CC interactions exist within the quiescent STIM1 that are released by these mutations or physiologically upon store depletion and/or interaction with Orai1.[62] In an alternative approach by Hogan`s group, such an extended conformation exposing the polybasic cluster at the very end of STIM1-CT has been induced by artificial crosslinking of the CC1 domains from 2 STIM1 C-termini that stabilizes the α-helical portion and promotes dimerization of CC1.[68] Introducing the "activating" mutation L251S into STIM1-CT also results in conformational extension of STIM1-CT[68] which is in line with Muik et al.[62] and emphasizes the impact of $\alpha1_{CC1}$ on the transition of STIM1 from a quiescent to an active state. Furthermore, Zhou et al.[68] demonstrate that only monomeric CC1 is able to interact with SOAR implying that CC1 dimerization as a consequence of STIM1 activation yields the extended conformation due to release of the interaction with SOAR. Based on these data, we present in **Figure 1D and E** two hypothetical models that depict quiescent STIM1 conformations in the resting cell. In either model, besides the luminal EF hand and SAM domains, the CC1 domain plays a dominant role in controlling the inactive state of STIM1 with both $\alpha1_{cc1}$ and $\alpha3_{cc1}$ helices contributing to that function, via intramolecular interactions with the SOAR domain.[62,66,68] The difference between these models can be seen in the arrangement of the SOAR domain where the V-like structure is directed away from (**Fig. 1D**) or toward (**Fig. 1E**) the ER membrane. In the latter case the polybasic segment aa382–387 directs toward the ER membrane. In addition these models differ in the arrangement of the CC1 domain. Crystallization of human STIM1 will be essential to elucidate the CC1-SOAR interaction, as the CC1 domain of *C.elegans* is shorter and the residues involved in the intramolecular interaction of $\alpha3_{cc1}$ with SOAR are not well conserved. Moreover, the cytosolic N-terminal portion of *C.elegans* STIM1 corresponding to human 233–306 has not been resolved in the crystal structure. Upon store depletion CC1 dimerization is suggested to release the inhibitory clamp leading to exposure of SOAR and oligomerization, key to the interaction with Orai1 cytosolic strands (see section below and **Fig. 3**).[66,68]

Several questions still need to be resolved in more detail: Which STIM1-CT domains interact intramolecularly keeping STIM1 in a tight, quiescent state, particularly regarding the α1 of CC1? What are the specific roles of α1, α2, and α3 of CC1 in controlling STIM1-CT re-arrangement? What is the interplay of the different STIM1-CT domains in the course of activation following store depletion? Which domains are involved in and how do they control the formation of higher order STIM1 oligomers?

Orai

Assembly of Orai subunits

Individual Orai subunits are composed of 4 transmembrane domains, the N- and C-terminal cytosolic strands, 1 intracellular and 2 extracellular loops (**Fig. 2A**). The CRAC channel complex is composed of an oligomer of Orai subunits, potentially forming homo- as well as heteromeric Orai channels.[12,69-71] As

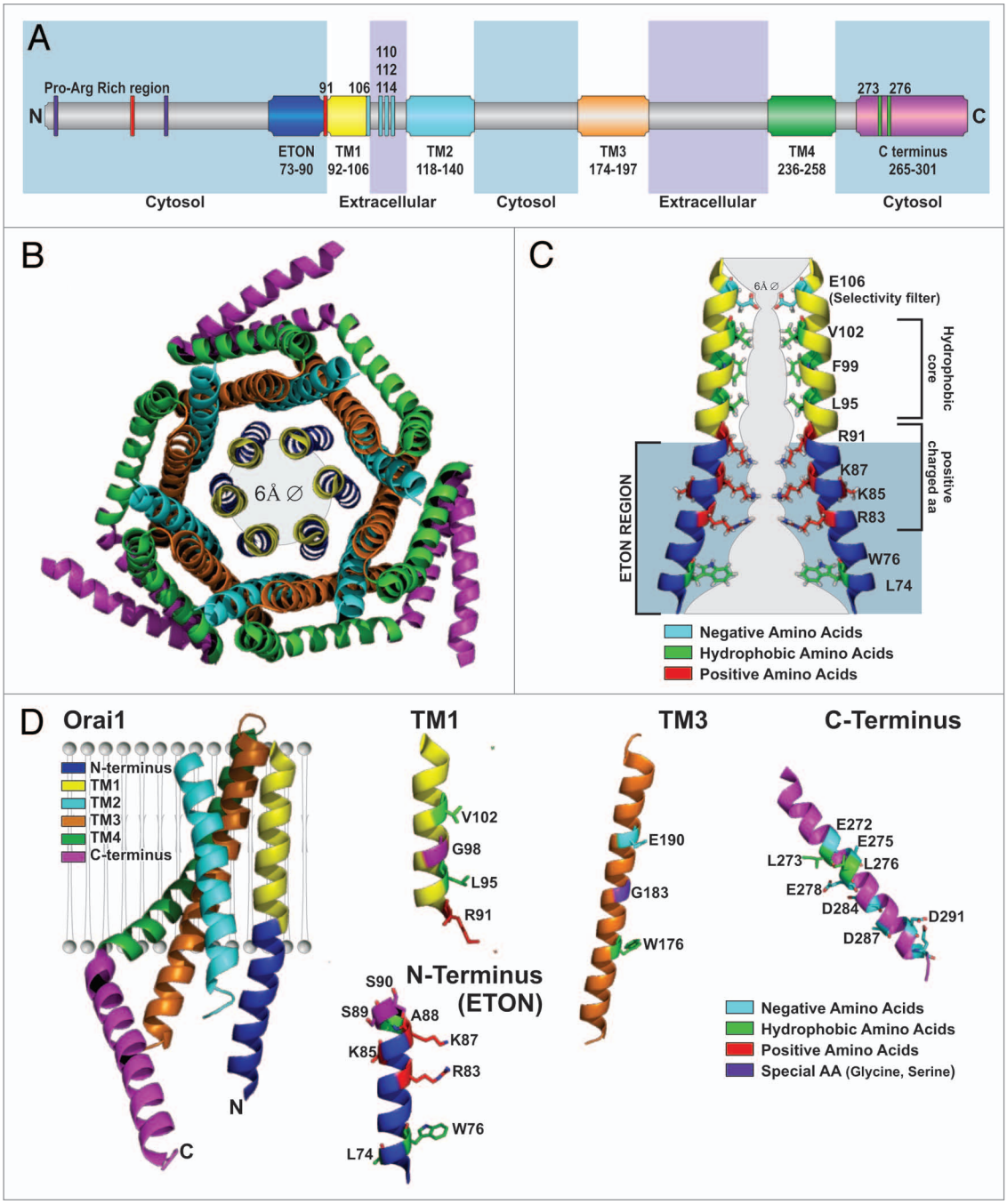

Figure 2. Orai1: (**A**) Schematic representation of human, full-length Orai1 depicting the overall structure and residues essential to Orai1 function. (**B**) Cartoon showing the assembly of Orai subunits in a hexamer based on the X-ray crystal structure of Drosophila Orai. Transmembrane domains of the six subunits are arranged as concentric rings around the ion pore, with TM1 forming the inner ring surrounding the ion pore. (**C**) Cartoon representing the human Orai1 pore by 2 TM1 strands together with the cytosolic, helical extensions including the conserved ETON region. Relevant amino acids contributing to the pore, especially the selectivity filter, the hydrophobic core as well as residues within the ETON region are highlighted. (**D**) Cartoon representing a single Orai1 subunit with the 4 transmembrane (TM) regions, the N-terminal as well as the C-terminal elongated helices, shown in different colors used throughout (**A–D**). TM1, TM3, the C-terminus and the N-terminus are additionally shown in separate with residues relevant to Orai1 function highlighted (aa numbering refers to human Orai1).

the truncation of the cytosolic strands does not generally affect aggregation of Orai subunits, their multimerization is assumed to be mainly established by their transmembrane regions.[34,36] Hitherto, a detailed mapping of crucial regions especially within the transmembrane segments but also in the cytosolic portions is still missing. The recently reported hexameric drosphila Orai crystal structure (see below **Fig. 2B**)[43] has revealed dimerization of C-termini within each of the 3 Orai dimers and may provide further clues for experiments on key residues or regions holding the Orai subunits together.

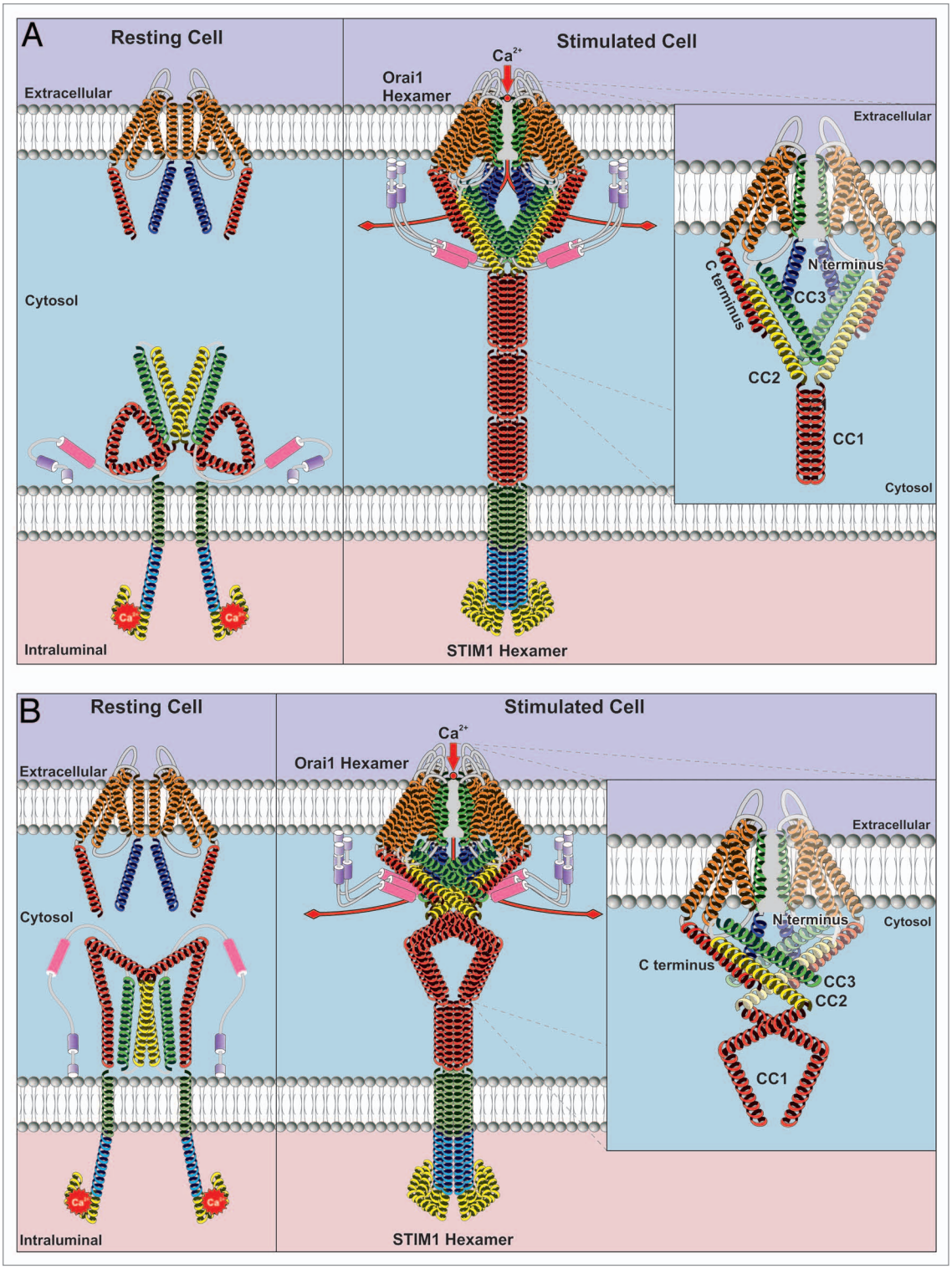

Figure 3. Cartoons representing 2 hypothetical models (**A and B**) for STIM1-Orai1 coupling based on distinct STIM1 conformations in the resting state as displayed in **Fig. 1D and E**. Upon store depletion, STIM1 proteins adopt an extended conformation and oligomerize, processes that are controlled by the CC1 domain. Insets depict activated Orai1 channel with CC2 and CC3 involved in the coupling to Orai1 C – and N-termini. The models emphasize distinct CC1 arrangements in the STIM1 activated state rather than focusing on the stoichiometry of Orai1/STIM1 interaction.

Orai stoichiometry

Orai channel structure displays no clear homology to other calcium channels. Hence, speculations concerning their function and stoichiometry have soon arisen.[72,73] Biochemical and fluorescence studies have mainly revealed a tetrameric Orai structure,[74-79] in contradiction to the recently published

hexameric crystal structure of the Orai channel of *Drosophila melanogaster*.[43]

In the early stage of research on Orai proteins, at least stable dimers have been discovered by biochemical approaches, while chemical cross-linking has enabled to observe tetramers.[70] Tetrameric or higher order Orai1 aggregates have been confirmed by disulfide cross-linking.[80] A tetrameric stoichiometry has also been visualized by electron microscopy studies.[81] This oligomeric state of 4 Orai subunits forming an active and conducting Ca^{2+} channel is further underlined by functional assays. Store-operated Ca^{2+} currents mediated by expressed, tetrameric Orai1 concatamers remain unaffected by co-expression with a dominant-negative Orai1 mutant.[75,81] Furthermore concatenated Orai1 tetramers with an increasing number of non-functional Orai1 R91W subunits have displayed a gradual loss in Ca^{2+} currents.[76] This current reduction is independent on the position or order of Orai1 R91W, but directly dependent on the number of mutant subunits in the tetramer.[76]

Employing single molecule fluorescence microscopy, which allows counting the Orai1 subunits within the channel complex, several groups[74,77,82] have investigated the stoichiometry of Orai1 channels in the activated and resting state. For this, stepwise photobleaching has been applied to immobile Orai1 aggregates in fixed cells,[74] confirming that STIM1-activated, conducting CRAC channels form tetramers. Under resting cell conditions, Ji et al.[77] have detected Orai1 tetramers in fixed HEK 293 cells. In contrast, Penna et al.[74] have observed a dimeric state for immobile Orai aggregates in *Xenopus levis* oocytes and they have suggested that dimerization of Orai1 dimers is induced by STIM1, leading to the tetrameric, activated CRAC channel.[74] These controversial results regarding the stoichiometry of Orai1 in the resting cell state may result, as suggested by Cahalan's group,[79] from fixation of cells which artificially causes store depletion and thus leads to tetramer formation of Orai subunits.

Alternative to stepwise photobleaching, direct single molecule brightness analysis[72,73,78] has also been utilized to analyze the stoichiometry of resting state Orai1 proteins in living HEK 293 cells, where the majority of Orai1 is mobile. Upon exclusively measuring the stoichiometry of mobile GFP-Orai1 proteins, the largest Orai1 fraction emerged to be purely tetrameric.[78] Furthermore, the robust FRET between 2 differentially-labeled dimeric Orai1 concatemers does not change upon store depletion, suggesting that Orai1 has already accomplished its final oligomeric state which is at least tetrameric or higher.[78] Demuro et al.[79] have also studied the stoichiometry of Orai3 channels by single-molecule photobleaching reporting that at resting state Orai3 remains a dimer, while it forms a tetramer in the presence of STIM1 C-terminus. In contrast, 2-APB stimulation leaves Orai3 in its dimeric state leading to the hypothesis of a functional dimeric channel assembly gated by 2-APB. Orai3 single molecule brightness analysis measurements as well as FRET experiments of differently labeled Orai3-dimers have not yet been reported.

In contrast to a tetrameric Orai assembly, the recent crystallographic study[43] reported – against all expectations—a hexameric structure of Orai from *Drosophila melanogaster* (**Fig. 2B**). The 4 transmembrane helices are arranged in 3 concentric rings, where TM1 forms the inner ring surrounding the ion pore, TM2 and TM3 are arranged in a second, and TM4 forms the third ring.[43] Compatible with the hexameric crystal structure of Orai1, quantification of the particle size of purified Orai1 alone or together with CAD in negative stain electron microscopy has revealed higher order oligomers with more than 4 Orai subunits in a complex together with CAD.[58] One fraction has shown complexes of maximal 6 particles matching with the hexameric crystal structure. It is of note that both the atypical structure of Orai1 Ca^{2+} channel subunits and the hexameric stoichiometry are distinctly different to features of other Ca^{2+}-selective channels such as TRPV6 or L-type voltage-gated channels.[72,73]

Besides the tetrameric and hexameric assemblies of Orai subunits, Shuttleworth's group has reported that Orai subunits may also form pentameric complexes to generate the arachidonate-regulated Ca^{2+} (ARC) channel. These channels contain 3 Orai1 and 2 Orai3 subunits, thus forming a heteromeric assembly.[75,76,83] Therein, the Orai3 N-terminus represents the critical determinant which switches a store-operated channel into an arachidonate-regulated one.[82]

The Orai channel permeability

Orai channels conduct Ca^{2+} ions with a selectivity 1000 times higher than that of Na^{+}.[84] Hence, strongly inward rectifying Orai Ca^{2+} currents display a reversal potential of more than + 60 mV.[12,15,85] Monovalent ions are also able to permeate through Orai channels, as long as the solution is free of divalent ions. These monovalent Orai currents are blocked by addition of Ca^{2+} at μM concentrations. Furthermore, Orai1 channels possess a very low single channel conductance of about 20 fS in the presence of 110 mM Ca^{2+}, which further decreases at physiological Ca^{2+} concentrations.[86,87]

The Orai1 channels are regulated via intracellular Ca^{2+}, which induces Orai1 channel inactivation, displaying a fast and a slow phase. The members of the Orai family differ in their inactivation profiles, based on differences in the sequence of the N-terminus, the cytosolic loop2 between TM2 and TM3 as well as the C-terminus.[12,88]

In contrast to other Ca^{2+} channels, such as TRPV6[89] and L-type channels,[90] Cs^{+} is unable to pass through Orai channels. Permeation studies with methylated derivatives of ammonium[91] have revealed that the narrowest region of the Orai1 pore possesses a diameter of 3.8 to 3.9 Å.[11,22,92] Novel findings derived from the crystal structure (see **Fig. 2B and C**) suggest for the narrowest area in the ion pore a diameter of ~6 Å,[43] which is at variance with the experimentally obtained diameter with methylated ammonium derivatives. A recent study by Thompson and Shuttleworth[93] has focused on the selectivity of tetrameric vs. hexameric concatemeric channel assemblies. Interestingly, the hexameric concatemer shows reduced Ca^{2+} selectivity compared with the tetrameric form, compatible with the fact of ~6 Å diameter observed for less Ca^{2+} selective Orai1 pore mutants. Thus, the discrepancy between the experimentally and structurally resolved pore diameter is so far unclear. The presence of STIM1 may affect Ca^{2+} selectivity[94] and pore diameter when co-crystallized. Nonetheless, the hexameric Orai1 concatemers exhibiting the reduced Ca^{2+} selectivity have been activated via STIM1.[93]

The Orai1 pore and the TM1

The distinct amino acid sequence and permeation properties of Orai channels compared with other Ca^{2+} ion channels has led to the proposal of a unique selectivity filter for the CRAC channel.[74,75,77] The permeation pathway of Orai channels is mainly formed by TM1 surrounding the ion pore together with at least a part of the ETON (see below) region (**Fig. 2C**). A negatively charged glutamate E106 has been determined to exclusively contribute to the selectivity filter; hence based on the hexameric Orai crystal structure, the selectivity filter is composed of a ring of 6 glutamates from the six TM1 regions surrounding the ion pore. A single point mutation E106D[9,10,12] in TM1 reduces already the Ca^{2+} selectivity together with an enlargement in the minimum pore size to 5.4 Å. This increase in the pore diameter is accompanied by a further relief of the steric hindrance for Cs^{+} permeation[22] leading to reduced Ca^{2+} selectivity.

Besides the E106 in TM1, the first extracellular loop of Orai1 containing 3 negatively charged residues (D110/112/114, see **Fig. 2A**) also contributes to the attraction of Ca^{2+} ions and the selectivity. Mutation of all 3 negatively charged residues to alanines drastically decreases Ca^{2+} selectivity.[10,86] Analogous sites in the other Orai isoforms are composed of a mixture of glutamates, glutamines and aspartates. While homomeric channels, independent of the isoform, display inward rectifying Ca^{2+} selective currents, heteromeric channel assemblies, as evaluated for Orai1/Orai3, display an asymmetric combination of glutamates and aspartates and less Ca^{2+} selectivity with an increased Cs^{+} permeation.[69] Thus, the acidic Ca^{2+} coordination site in the first loop may additionally regulate Ca^{2+} selectivity of Orai channels, besides E106, and prevent monovalent outward currents.[10,22] Several cysteine loop1 mutants form disulfide bonds and dimerize, which suggests a close proximity of 2 adjacent first loops within an Orai1 channel complex. Small MTS reagents as well as larger 6 to > 8 Å MTS probes coupled to the cysteine mutants in the first loop result in decreased currents, suggesting these loops flank a vestibule large enough to accommodate bulky compounds.[86] These results may further indicate that the first loop is a flexible segment that can undergo conformational changes.[86] Hence, Ca^{2+} permeation is optimized by the first extracellular loop, which attracts Ca^{2+} ions via its negative residues, thereby functioning as a Ca^{2+} sink to enhance the local Ca^{2+} concentration at the pore entrance close to the selectivity filter.[9,95] Consequently the Ca^{2+} ions are guided from the extracellular loop via E106 through the pore maintaining the high Ca^{2+} selectivity[8-11,22] of the Orai channels.

Toward the cytoplasmic side the pore opens to a wider cavity including hydrophobic side chains like valine 102, phenylalanine 99 and leucine 95 (see **Fig. 2C**). These residues point directly into the pore as visible in the Orai crystal structure which is, except for F99, in accordance with their ability to dimerize upon cysteine substitution in cysteine crosslinking experiments.[80,86]

Mutation of hydrophobic (V102) as well as polar (G98) residues in TM1 (**Fig. 2D**) has shown that the channel can be locked in an open conformation.[94,96] The constitutively active Orai1 V102C/A channel additionally displays changes in the selectivity filter leading to a reduced Ca^{2+} selectivity due to an increased pore size.[94,96] Interestingly, Orai1 V102C/A currents regain Ca^{2+} selectivity in the presence of STIM1.[94] Upon introduction of L273S in the Orai1 C-terminal coiled-coil domain (see **Fig. 2D**) impairing STIM1 interaction,[36] the V102C mutant remained non-selective even in the presence of STIM1. Thus, STIM1 binding regulates the ion selectivity and pore architecture of Orai1 channels. Prakriya and coworkers[94] have speculated that the close proximity of the gating residue V102 to the selectivity filter at E106 contributes to the tight coupling of permeation and gating during channel activation. Furthermore, since STIM1 interaction regulates selectivity of Orai1 V102A/C, these mutants have been utilized as tool to monitor alterations in STIM1 binding, which has allowed for determination of STIM1 key interaction sites on Orai1 cytsosolic strands[94,97] (see below).

Subsequent to the selectivity filter and within the hydrophobic cavity a flexible glycine hinge (G98) has been identified[96] (**Fig. 2D**) that assumedly enables flexion of the upstream pore-lining region to reduce the impedance of Ca^{2+} flow after passing the selectivity filter.[98] Its mutation to G98D results in non-selective, constitutive currents,[96] which, in contrast to Orai1 V102A, do not re-gain Ca^{2+} selectivity in the presence of STIM1. Moreover, the G98D mutation even restores function of the non-functional R91W SCID mutant.[94,96] Hence, the constitutive G98D mutant seems to extend the channel gate more effectively than the V102A/C mutant, preventing STIM1 N-terminal interaction and hydrophobic packing associated with the R91W mutation.

The extended TM1 Orai1 N-terminal (ETON) region

The Orai crystal structure[43] has revealed that the helical structure of the TM1 domain extends even further into the cytosol by about 20 Å forming the so called extended TM1 Orai1 N-terminal (ETON) region.[98] At the cytosolic side the TM1 helix together with the ETON region contains three positively charged residues R91, K87, and R83 which directly line the pore thereby creating an unusual environment for a cation channel. These positively charged residues have been suggested to provide both barrier as well as electrostatic stabilization to the elongated pore controlled via interaction with STIM1.[98] The mutation of arginine R91 to hydrophobic residues inhibits STIM1-dependent Orai1 currents, associated with the SCID disease.[5,99] In order to let Ca^{2+} pass into the cell cytosol, this barrier of the 3 positive charges possibly linked to anions[43] is likely released, accomplished by a coupling of STIM1 to the conserved ETON region.[98]

Further, Orai1 gating is additionally controlled by another positively charged amino acid, i.e., K85, in the ETON-region (**Fig. 2C and D**). As the Orai1 K85E mutant completely lacks activation via STIM1,[100] despite an only partially reduced binding between an N-terminal Orai1 fragment and CAD, this loss of function most likely results from a defect in gating. Mechanistically, the latter defect may be linked to an impairment of intramolecular interactions of K85 stabilizing the Orai1 channel structure as essential for STIM1-dependent gating.

In addition the ETON region includes 2 serines (S89 S90) which are located between the 2 basic residues R91 and K87

(see **Fig. 2D**) and may contribute to the flexibility of the adjacent N-terminal and TM1 segments.[99] The exchange of the 2 serines by 2 glycines, promoting helical flexibility, has enhanced Orai1 currents, while increased rigidity or helical break introduced by 2 prolines has resulted in loss of function.[99] The crystal structure displays in this area a slight bend (**Fig. 2D**), suggesting a potential flexibility or hinge for S89 S90 in the gating of Orai1 by STIM1. Additionally, the inner portion of TM1 residues (aa 91–98) seems to possess more flexibility than the outer half region (aa99–104) that forms a rather rigid conformation.[80]

The TM3 of Orai1

The third TM domain does not line the pore but modulates both selectivity and gating of the Orai1 channel (see **Fig. 2D**). The tryptophan at position 176 in TM3 regulates gating as a cysteine substitution at this position switches the channel into a constitutively active less Ca^{2+}-selective form.[95] Single point mutation of E190 in TM3 has drastically affected the permeation properties thereby increasing the pore diameter to 7 Å.[8,9,11,22] As Orai1 E190C has failed to form crosslinked dimers, it has been suggested that this position in TM3 rather allosterically affects the pore properties probably by altering intramolecular TM interactions.[80,86] Accordingly, the glycine residue G183 located between W176 and E190 (see **Fig. 2D**) might play such a role. An alanine (G183A) substitution abolishes store-operated activation, but renders the mutant Orai1 channel sensitive to 2-APB stimulation.[95] The analog mutation in Orai3 to cysteine (Orai3 G158C)[101] has resulted in altered kinetics upon 2-APB activation and prevention of full channel closure following 2-ABP washout. These effects have been deduced to formation of a cysteine bridge between G158C and a native cysteine in TM2, suggesting this TM2-TM3 interaction may control the open or closed state of Orai channels.[101] Regarding indirect, allosteric interference/coupling of TM2/TM3 with the pore, detailed analysis of the crystal structure may provide further clues into the interplay of the TM domains and the pore structure.

In summary, Ca^{2+} ions possibly attracted by the negatively charged residues in the first extracellular loop enter the flexible outer pore vestibule. Subsequently they move forward into the pore formed by the helical TM1 segments[86] toward the selectivity filter with 6 glutamate residues (E106). Here, in this transient interaction, Ca^{2+} ions are supposed to lose at least partially water molecules, but get rehydrated when entering the subsequent, larger hydrophobic cavity. Finally, they reach the elongated pore formed by the ETON regions that likely provides electrostatic barrier by the three positively charged residues R91, K87, and R83. These residues are supposed to inhibit Ca^{2+} influx in the closed channel state either due to bound anions or simply by electrostatic repulsion in this region. Ca^{2+} passage is inhibited by a single point mutation of R91 to a hydrophobic residue associated with SCID, most likely by artificially generating a robust hydrophobic packing[43] that prevents Ca^{2+} flow into the elongated pore. As additional function, the ETON regions provide the binding interface for Orai interaction with STIM1 that culminates in Ca^{2+} exit into the cytosol, which is described in more detail in the following section.

STIM1/Orai1 Coupling Domains

STIM1 coupling to the C-terminus

The coupling of STIM1 oligomers and the Orai1 channel involves several domains in the cytosolic strands of STIM1 and Orai1 (**Fig. 1A**; **Fig. 2A and D**). Truncation of the Orai1 C-terminus, as performed in 3 independent studies[34,36,58], has provided the first insight into the STIM1/Orai1 coupling process. Co-localization and FRET experiments have clearly revealed that Orai1 C-terminus is indispensable for the coupling with STIM1. In accordance, Orai1 C-terminus forms the cytosolic extension of TM4,[43] thus easily accessible to STIM1 for cytosolic binding. Partial deletion of the C-terminal strand (Orai1 Δ283–301) has still enabled co-clustering with STIM1 indicating that the second half of Orai1 C-terminus is not essential for coupling to STIM1.[19] Bioinformatic analysis has predicted a weak coiled-coil probability of Orai1 C-terminus, while Orai2 and Orai3 display a 15–17 fold higher coiled-coil probability.[102] Consistently, a single point mutation in the Orai1 coiled-coil domain (L273S or L276D, see **Fig. 2D**) is sufficient to abrogate the coupling with STIM1 C-terminus,[36,102,103] whereas a double point mutation has been required in Orai2 and Orai3. In accordance, Zhang et al.[40] have shown constitutive co-localization of STIM1 with Orai3 before store depletion, in contrast to Orai1, very likely due to the increased coiled-coil probability of Orai3 compared with Orai1. These findings point to the affinity of STIM1 increasing with the higher probability of Orai C-terminal CC domains. In line, a reduction of the probability of the CC2 domain of STIM1 C-terminus by a single mutation (L373S) still allows partial activation of Orai2 and Orai3 channels, but not of Orai1.[102] Communication to all 3 Orai channels is disrupted by a double mutation in CC2 domain of STIM1. Thus, these CC domains are essential for the coupling of STIM1 and Orai.[102] Nonetheless, it remains unclear why both Orai1 and Orai3 activate with comparable kinetics upon store depletion, as constitutive STIM1 coupling to Orai3 may imply faster activation. Hence, further domains besides STIM1-Orai-C-terminus coupling control the kinetics of Orai activation.

It is of note that the Orai1 C-terminal hydrophobic residues L273 and L276, the mutation of which disrupt Orai1 coupling to STIM1,[36,103] play an additional role in their involvement of C-terminal dimerization of each Orai dimer within the hexameric Orai crystal structure.[43] Hence it is tempting to speculate that their mutation has impaired either the geometry of the dimeric arrangement or/and the interacting sites, both essential for the coupling to STIM1.

Besides these hydrophobic amino acids, the Orai1 C-terminus includes a series of acidic residues (**Fig. 2D**). These negatively charged amino acids have been proposed to interplay with a highly conserved cluster of basic residues in STIM1 (KIKKKR – aa 382–387 of human STIM1), to mediate STIM1/Orai1 coupling.[104-106] STIM1 mutants lacking this positively charged segment fail to associate with Orai upon store depletion. However, coupling occurs between this STIM1 mutant or wild-type STIM1 with the mutant Orai1 that has all 6 negative charges neutralized in its C-terminus, indicating that other structural

components dominate in the coupling process.[106] In aggregate, heteromeric coiled-coil interactions between CC2 of STIM1 and Orai1 C-terminus are mainly mediated by hydrophobic residues and to a weaker extent stabilized by salt bridges.[107,108]

In our hypothetical STIM1/Orai coupling models (**Fig. 3A and B**), STIM1 gets fully activated via oligomerization both of the ER luminal part as well as the cytosolic coiled-coil domains accompanied with SOAR exposure. The proposed models display the 2 conformations for quiescent STIM1 as previously depicted in **Figure 1D and E**. The main difference in the activated form of STIM1 that interacts with the Orai1 channel can be seen in the arrangement of the CC1 domain that tightly controls STIM1 activation status. SOAR exposure as well as oligomerization is induced either by a parallel (**Fig. 3A**) or at least in part antiparallel (**Fig. 3B**) arrangement of CC1 triggered via store depletion. In either model, the CC2 domain interacts with Orai1 C-terminus, while the Orai1 N-terminal interaction domain of STIM1 is still unknown.

STIM1 coupling to Orai1 N-terminus

In addition to Orai1 C-terminus, its N-terminus functions as another binding partner for STIM1 (or CAD), though to a weaker extent[34,36,58] as revealed by co-immunoprecipitation studies.[58] Here, in all Orai proteins, the conserved ETON region (aa73–90) forming the elongated extension of TM1 into the cytosol is essential for the interaction with STIM1. While a complete deletion of Orai1 N-terminus or a deletion of aa74–90 results in loss of Orai1 function, a partial truncation up to residue 74/75 maintained Orai1 channel activity.[34,97] Consistently, Orai1 current activation is completely abolished upon deletion of the first 76 N-terminal residues or beyond. Additionally, a mutagenesis approach has revealed the double point mutation of L74 and W76 (see **Fig. 2D**) resulting in loss of function in accordance with substantially reduced STIM1 interaction, suggesting their involvement in the Orai1 N-terminal STIM1 binding interface.[97] Further downstream the ETON region, the positively charged R83 and K87 residues (see **Fig. 2D**) also contribute to the interaction with STIM1, while additionally providing electrostatic barrier as well as stabilization to the elongated pore.[97] In summary, almost the whole ETON region functions as binding interface for Orai1 interaction with STIM1 and additionally provides electrostatic gating elements to fine-tune the shape of the elongated pore.

Intriguingly, despite the ETON region is fully conserved between Orai1 and Orai3, STIM1-dependent activation of the latter is still retained upon extensive truncations that already abolish Orai1 function.[14,97] Hence, Orai3 activation via STIM1 seems to involve additional structures that compensate for the extensive N-terminal deletions, the location of which still remains to be elucidated.[58]

In summary, STIM1 coupling to and activation of Orai1 channels involves both Orai1 N – as well as C-terminus, the bridging of which is most likely accomplished by the SOAR domain of STIM1. Thereby, a force is generated to induce a conformational re-arrangement of the elongated TM1 helices surrounding the Orai1 pore which probably removes the electrostatic barrier provided by the ETON region and culminates in Ca^{2+} entry into the cell cytosol. Whether the conformational re-arrangement is mediated only via Orai1 cytosolic strands or additionally involves alterations in TM helical interactions is so far unclear.

Stoichiometry within the STIM1/Orai1 Complex

After ER store-depletion, STIM1 oligomerizes and redistributes to puncta in ER-PM junctions. This process is accompanied by a conformational change leading to SOAR exposure, interaction with and activation of Orai channels.[4-7,32,33,45,47,48,109-111] The STIM1/Orai complex formation leads to a reduction in the molecular mobility of Orai1 in comparison to Orai proteins at resting state[112]. The STIM1/Orai complex is formed by oligomeric aggregates of STIM1 and Orai1, while the exact stoichiometry of interacting subunits still remains unclear. Orai1 proteins have been detected in biochemical and fluorescence studies mainly as a tetramer, while the recent crystal structure has revealed that Orai1 occurs as a hexameric complex. Regarding STIM1, expression studies with varying STIM1:Orai1 ratios as well as patch-clamp measurement on Orai1-STIM1 C-term fusion proteins have revealed that eight STIM1 molecules are required for full CRAC current activation and inactivation[113,114], even if only 1 to 2 STIM1 molecules are sufficient to trap Orai1 channels at ER-PM junctions.[113] The extent of inactivation is related to the number of STIM1 molecules which bind to the Orai1 channel complex in as that the more STIM1 associated, the more Orai1 currents inactivate.[115] Furthermore, an increase in the Ca^{2+} selectivity of Orai1 has additionally been observed the more STIM1 molecules are interacting with Orai1.[86,94] Hence, CRAC channel activation occurs not in an "all-or-none" fashion but develops via a graded process involving up to 8 STIM1 molecules.[114] Regarding a potential tetrameric Orai complex, the STIM1:Orai1 stoichiometry has been suggested as 2:1. However, in light of the crystallized hexameric Orai complex, it remains unclear as to how 8 STIM1 molecules may easily couple to six Orai subunits. In that case, 12 STIM1 molecules may be required to fully activate the Orai hexamer. Alternatively, one could also envisage a 1:1 stoichiometry of STIM1:Orai1 proteins involving 2 STIM1 molecules coupling to each of the 3 Orai1 dimer entities in the hexameric assembly.

Perspective

Despite substantial progress, particularly with access to 3D atomic structures, has been obtained in the STIM/Orai field in the past 8 y, our understanding of the CRAC channel signaling machinery is far from complete. Regarding STIM1, the intra-/intermolecular interactions which keep STIM1 in the quiescent state and the activation steps linking store depletion to exposure of SOAR and oligomerization need to be more precisely defined. The gating of Orai channels by STIM1 is only partially understood, particularly as the STIM1 segment interacting with Orai1 N-terminal region is unknown. Moreover, it remains to be clarified whether additional Orai domains beside the N- and C-terminal strands play a role in transducing the coupling of STIM1 into Orai1 gating. Crystallization of STIM1 active fragments with Orai

or of Orai mutants locked in the open state will certainly help to understand this process of gating together with a clarification on the Orai:STIM1 stoichiometry. In addition to these 2 key proteins of the CRAC signaling machinery, more modulatory proteins such as CRACR2A,[116] SARAF[117]or Septin[118] are and will be emerging, the knowledge of which will enhance our understanding of CRAC current regulation and also widen the repertoire for interference with novel drugs. Finally, an impressive increase is currently seen in studies characterizing STIM/Orai involvement in many processes of various cell types, beside the immune system, that additionally will reveal differences in terms of activation, regulation, subunit composition etc. useful for specific therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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