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Biochemical properties and cellular localisation of STIM proteins

Marie A. Dziadek [a,*], Lorna S. Johnstone [b]

[a] *Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia*
[b] *School of Biological Sciences, University of Auckland, Auckland, New Zealand*

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Abstract

Human and murine STIM1 were originally discovered as candidate growth regulators in tumours and in the bone marrow stroma, and the structurally related vertebrate family members, *STIM2* and the *Drosophila* homologue *D-Stim*, were subsequently identified. STIM proteins are ubiquitously expressed type I single-pass transmembrane proteins which have a unique combination of structural motifs within their polypeptide sequences. The extracellular regions contain an N-terminal unpaired EF-hand Ca^{2+} binding motif adjacent to an unconventional glycosylated SAM domain, while the cytoplasmic regions contain alpha-helical coiled-coil domains within a region having homology to ERM domains adjacent to the transmembrane region, and phosphorylated proline-rich domains near the C-terminus. STIM1, STIM2 and D-Stim diverge significantly only in their structure C-terminal to the coiled-coil/ERM domains. The STIM structural domains were predicted to function in Ca^{2+} binding as well as in mediating interactions between STIM proteins and other proteins, and homotypic STIM1–STIM1 and heterotypic STIM1–STIM2 interactions were demonstrated biochemically. However, the functional significance of the cellular localisation of STIM1 and its domain structure only became evident after recent breakthrough research identified STIM1 as a key regulator of store-operated calcium (SOC) entry into cells. It is now clear that STIM1 is both a sensor of Ca^{2+} depletion in the endoplasmic reticulum (ER) lumen and an activator of Orai1-containing SOC channels in the plasma membrane. On the basis of recent functional studies a model can be proposed to explain how the biochemical properties of STIM1 contribute to its precise membrane localisation and its function in regulating SOC entry.

Keywords: STIM1; STIM2; SOC channels; Calcium binding; Conformational changes; Aggregation

1. Discovery of STIM gene products

Human *STIM1* (where STIM1 is stromal interaction molecule; initially named GOK) was identified by chromosome walking as a novel gene adjacent to *RRM1* on chromosome 11p15.5, in a search for novel tumour suppressor genes within the region of 11p15.5 implicated in several childhood and adult tumours as well as Beckwith–Wiedemann syndrome [1]. Transfection of full length *STIM1* cDNA into either rhabdoid tumour or rhabdomyosarcoma cell lines inhibited cell proliferation and induced cell death, consistent with a potential tumour suppressor role in myogenic cells [2]. Murine *Stim1* was identified independently at approximately the same time as a bone-marrow stromal gene product through signal sequence 'trap' screening [3]. The recombinantly expressed extracellular region of Stim1 was shown to bind to the cell surface of pre-B lymphoid cells and promote their clonal expansion *in vitro*, implicating Stim1 as a stromal cell surface protein that mediates interactions between stromal and haematopoietic cells within the bone marrow. Sequence comparisons showed 96% amino acid identity between murine Stim1 and human STIM1. The second vertebrate STIM family member, *STIM2*, and the *Drosophila* homologue, *D-Stim*, were identified using bioinformatic approaches to search gene databases for translated protein sequences related to the full-length STIM1 amino acid sequence [4]. Such searches have revealed that only two *STIM* genes are present in mammalian species, while a third family member has been more recently identified in zebrafish (Johnstone and Dziadek, unpublished). A sin-

* Corresponding author. Tel.: +61 2 9295 8195; fax: +61 2 9295 8101.
E-mail addresses: m.dziadek@garvan.org.au (M.A. Dziadek), ls.johnstone@auckland.ac.nz (L.S. Johnstone).

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gle *STIM* gene appears to exist in invertebrates (*Drosophila melanogaster*, *Caenorhabditis elegans*), while no *STIM*-like genes have been identified in prokaryotes or unicellular eukaryotes (e.g. yeast) [4]. The initial data indicating a function of STIM1 in growth regulation prompted a more extensive biochemical characterisation of STIM1, STIM2 and D-Stim protein structures. This information contributed to the recent elucidation of their function in regulation of Ca^{2+} entry after sensing depletion of Ca^{2+} stores in the endoplasmic reticulum (ER) [5,6]. STIM1 was identified in limited functional screens for genes involved in store-operated Ca^{2+} (SOC) entry using RNAi technology in *Drosophila* S2 cells (170 genes, [6]), and human HeLa cells (2304 genes, [5]). These screens targeted genes coding for proteins with transmembrane domains that also have a possible function as ion channels or in cell signalling. STIM1, STIM2 and D-Stim were the only proteins identified in these functional screens whose knockdown by RNAi resulted in reduced SOC entry.

2. Structure of STIM proteins

STIM1, *STIM2* and *D-Stim* code for proteins of 685, 746 and 570 amino acids, respectively [4]. Northern blotting shows two transcripts for *STIM1*, a predominant 4.1 kb transcript expressed in most adult tissues and a larger 4.5 kb transcript expressed in adult and fetal brain (Fig. 1A). Both transcripts are expressed in other fetal tissues (Fig. 1A). Immunoprecipitation and Western blotting analyses of cell lysates from a variety of cell lines have shown the presence of a single major 90 kDa protein product in all cell types

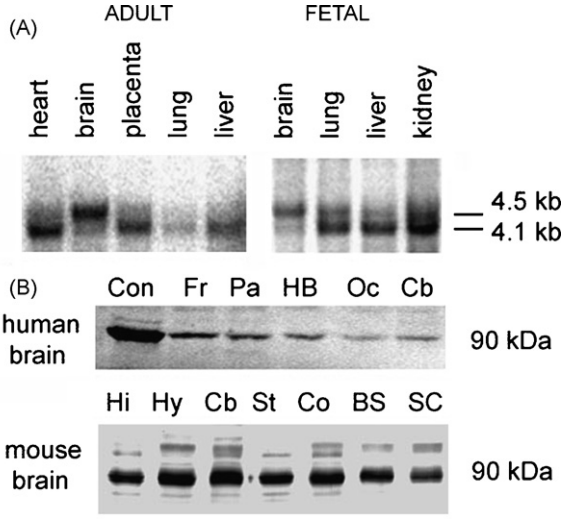

Fig. 1. (A) Northern blot analysis of *STIM1* transcripts in polyA RNA isolated from human adult and fetal tissues. Two STIM1 transcripts of 4.1 and 4.5 kb are evident. (B) Western blot analysis of protein lysates prepared from sections of normal adult human and mouse brain, and control K562 cells (Con) using antibodies specific to STIM1: frontal (Fr), parietal (Pa), hindbrain (HB), occipital (Oc), cerebellum (Cb), hippocampus (Hi), hypothalamus (Hy), striatum (St), cortex (Co), brainstem (BS) and spinal cord (SC). A 90 kDa STIM1 protein is evident in all samples.

[7]. A single 90 kDa STIM1 protein is also expressed in all regions of adult human and murine brain (Fig. 1B), indicating that the larger 4.5 kb STIM1 transcript does not code for a larger STIM1 protein isoform in the brain. In addition, RT-PCR analysis across the entire open reading frame of STIM1 transcripts in brain tissues did not reveal the presence of additional exons (unpublished data). Single polypeptide species have also been demonstrated for STIM2 (105 kDa) and D-Stim (64.5 kDa) which are subject to posttranslational modification [4]. All three STIM proteins are modified by N-linked glycosylation within the extracellular domain, STIM1 at two sites and STIM2 and D-Stim at one site (Fig. 2, [4,8]). Both STIM1 and STIM2 are phosphorylated predominantly on serine and threonine residues, and differences in the degree of phosphorylation is the major factor that causes the variation in electrophoretic mobility of STIM2 in cells [4].

STIM1, STIM2 and D-Stim share several structural features within both the extracellular and the intracellular regions of the polypeptide chain (Fig. 2). While the extracellular region of all three proteins are very similar in their domain structure, D-Stim contains an additional 70 amino acid region near the N-terminus that is not present in STIM1 and STIM2 and has no obvious homology with any known functional or structural sequence motifs (Fig. 2, [4]). All three STIM proteins have signal peptides at the N-terminus and contain two cysteine residues spaced eight amino acids apart that are situated N-terminal of a single helix-loop-helix region which conforms to the consensus motif for an EF-hand calcium binding domain [4]. A conserved five helical bundle single sterile alpha motif (SAM) domain is situated between the EF hand and the transmembrane region in each STIM protein (Fig. 2). An N-linked glycosylation site at the N-terminal limit of the SAM domain is conserved in all three proteins, while STIM1 contains an additional N-linked glycosylation site within the SAM domain itself (Fig. 2, [4]). These glycosylation sites are endoglycosidase H sensitive in both STIM1 and STIM2 [4,8] indicating that they are not fully processed to an endoglycosidase resistant mature form within the ER. Further terminal processing to an endoglycosidase H resistant oligosaccharide chain is often, but not

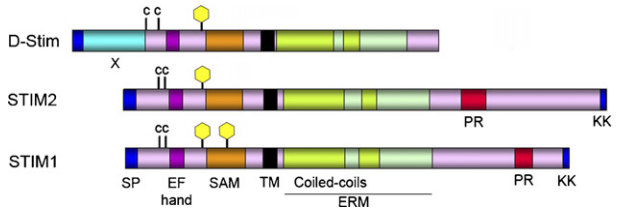

Fig. 2. Diagrammatic representation of the structural domains in vertebrate STIM1 and STIM2 proteins, and *Drosophila* D-Stim: signal peptide at the N-terminus (SP), EF hand, SAM domain, transmembrane region (TM), coiled-coils within an ERM domain, proline-rich region (PR) and lysine-rich tail at the C-terminus (KK). A pair of cysteine residues is present near the N-terminus (cc), and a conserved N-linked glycosylation sites resides N-terminal to the SAM domain (hexagon) and also within the STIM1 SAM domain. D-Stim contains an extra protein sequence adjacent to the signal peptide (X) that is not present in vertebrate STIMs.

always, associated with translocation of glycoproteins from the ER to the plasma membrane [9,10]. To our knowledge the SAM domain in STIM proteins still remains the only SAM domain identified to date that has an extracellular localisation. SAM domains are common protein–protein interaction motifs, having now been identified in over 1300 proteins, and show a high degree of versatility in their functional properties [11]. SAM domains can interact with SAM domains in other proteins in a homotypic or heterotypic fashion to form dimers or oligomers, or can bind to non-SAM containing proteins, and these protein–protein interactions mediate a wide range of biological processes including cell signalling and transcriptional regulation [11].

The cytoplasmic regions of STIM1, STIM2 and D-Stim contain two adjacent alpha-helical structures near the transmembrane segment that are predicted to form coiled-coils [4]. These coiled-coils reside within a larger region recently identified as having homology to ERM (ezrin/radixin/moesin) domains (Fig. 2, [12,13]). ERM proteins were originally identified as a family of proteins that mediate linkage of the cytoskeleton to the plasma membrane [14] and have a structural and regulatory role in the function of the cortical cytoskeleton [15]. ERM homology domains have been found in several proteins localised at the plasma membrane, such as talin, focal adhesion kinase, and protein tyrosine phosphatases, that are thought to mediate protein–protein and protein–membrane interactions to anchor protein complexes at specific membrane sites [15]. An interesting feature of ERM proteins is that they exist in a conformationally 'inactive' form in which the binding sites for other proteins are masked by intramolecular head-to-tail electrostatic interactions within the ERM domain, and an 'active' form that requires a conformational change to unmask binding sites that can associate with plasma membrane and cytoskeletal proteins [16–18]. This conformation can be regulated and stabilised by phosphorylation of specific sites within the C-terminal tail of the ERM proteins [17,19,20]. Given the structural and functional characteristics of ERM domains the coiled-coil/ERM domain is predicted to play an important role in mediating the functional properties of STIM proteins.

The sequences of the three STIM proteins diverge significantly C-terminal to the coiled-coil/ERM domain [4]. STIM1 and STIM2 contain unique proline-rich regions near the C-terminus of each protein, while the truncated D-Stim protein lacks such a region (Fig. 2). In STIM1 the proline-rich region includes multiple serine residues (SPSAPPGGSPHLDSSRRHSPSSPDPDTPSP) while the STIM2 proline-rich region contains multiple histidine residues in addition to several serines (PSSPQPQRAQLAPHAPHPSHPRHPHHPQHTPHSLPSPDP). Proline-rich sequences are very common in proteins in all organisms, and are important motifs for mediating protein–protein interactions that regulate many cellular processes [21]. A variety of specific consensus motifs exist that bind different classes of proteins that contain proline-recognition domains, such as the SH3 domain [21]. Both STIM1 and STIM2 contain several PXXP core motifs recognized by SH3 binding proteins. The C-terminal tails of STIM1 and STIM2 are lysine-rich sequences that contain di-lysine residues, which are also absent in D-Stim (Fig. 2). Such domains frequently function as ER retrieval and retention signals, dependent on the length and structure of the cytoplasmic tail [22].

3. Cellular localisation

STIM proteins are ubiquitously expressed [4,7], consistent with their critical roles as sensors of Ca^{2+} within the ER lumen and activators of SOC channels. A large proportion of STIM1 appears to be localised intracellularly within the ER, while in at least some cells a proportion is also localised at the cell surface. While the question of plasma membrane localisation remains controversial, and has influenced concepts of how STIM1 regulates SOC channel activity [5,6,13,23,24], there is now sufficient evidence from a number of laboratories which clearly demonstrates cell surface localisation in some cell types. In our own studies we used specific antibodies against an N-terminal sequence of STIM1 in immunofluorescence of unfixed live cells [7] and FACS analysis [8,25] to detect cell surface expression of endogenous and transfected STIM1, respectively. Cell surface biotinylation studies which have adequately controlled for absence of access of the biotin to intracellular proteins have demonstrated that some 15–25% of STIM1 protein can be detected on the cell surface [7,12,25,26]. More direct evidence of a plasma membrane role of STIM1 comes from studies showing inhibition of SOC activity when cells are incubated with STIM1 N-terminal antibodies, suggesting a functional role for cell surface STIM1 in regulating SOC activity [24,27]. While one study has shown that store depletion stimulates rapid cell surface expression of STIM1 [27], other studies have shown that the amount of STIM1 on the cell surface is not increased after store depletion [25,28]. There is, however, clear evidence that store-depletion causes aggregation of STIM1 beneath the PM but not incorporation into the PM [28,29]. The role of cell surface STIM1 in regulation of SOC activity at the plasma membrane remains controversial.

A recent study has identified a role for STIM1 in regulation of arachidonic acid-regulated Ca^{2+}-selective (ARC) channels in the plasma membrane, the activity of which are independent of store depletion [26]. This study is the first to report a potential function of STIM1 as a more universal regulator of Ca^{2+} entry into cells, and identifies a function of cell-surface localised STIM1 that is independent of SOC channels. Other studies using epitope-tagged STIM1 constructs have failed to detect cell surface STIM1 [5,28–30], which may be due to the epitope tag influencing localisation despite the ability of these constructs to express STIM1 protein that is functional in SOC activation, or may represent differences in either cells utilised in these studies or particular experimental protocols. In summary though, the evidence strongly implies that STIM1 can be translocated to the plasma

membrane where it may have a number of different functions that remain to be defined. The cellular localisation of STIM2 has been much less studied, but a single investigation indicates that, unlike STIM1, STIM2 cannot be detected on the cell surface by either FACS analysis or by surface biotinylation, even when levels of STIM2 expression are similar to that of STIM1 [25]. No cellular localisation studies have so far been reported for D-Stim.

4. Tissue expression

STIM1 transcripts are expressed in both fetal and adult tissues (Fig. 1), and the limited data available indicates developmental regulation in many tissues, with relatively low, uniform levels of expression in many cells and higher expression in differentiated cell types (e.g. differentiated skeletal muscle). Immunostaining for STIM1 in tissue sections reveals low expression throughout many tissues, but abundant localisation in certain cell types and cellular regions (Fig. 3). STIM1 is readily detected in both the fetal and adult central and peripheral nervous systems, particularly in axonal projections within cortical regions of the brain, ganglia and in dermal tissue in the fetus (Fig. 3). Purkinje cells in the cerebellum are highly stained (Fig. 3). This localisation pattern suggests a potential role for STIM1 in regulation of store-operated calcium entry in the nervous system [31]. High levels of STIM1 are also present throughout all layers of the developing stratified epidermis (Fig. 3). The specific role of STIM1 in epidermal cells has not yet been investigated. A particularly high accumulation of STIM1 is seen in the apical, microvillus surface of epithelia in several tissues, including the gut, palate, kidney, oviduct, epididymis, uterus, and choroid plexus (some examples shown in Fig. 3). Ca^{2+} signalling complexes have been shown to be selectively concentrated at the apical surface of polarised secretory epithelia [32], and our observations suggest that STIM1 may participate in the assembly and/or the function of these complexes in highly polarised Ca^{2+} signalling in epithelial tissues *in vivo*.

5. Function of STIM structural domains

The last 2 years have seen remarkable progress in defining the critical roles of STIM1 and Orai1 in the regulation of SOC entry into cells [13,28,33,34], and this information is presented in detail in other articles in this edition of *Cell Calcium*. The molecular characterisation of these proteins supports the 'conformation coupling' hypothesis that was put forward prior to identification of the key players, whereby SOC activation was proposed to be regulated by interactions between proteins in the ER and protein components of the Ca^{2+} channel in the plasma membrane [35,36]. These close interactions necessitate reorganisation of the cortical actin cytoskeletal network to enable close, but reversible, physical communication between microdomains of the ER and

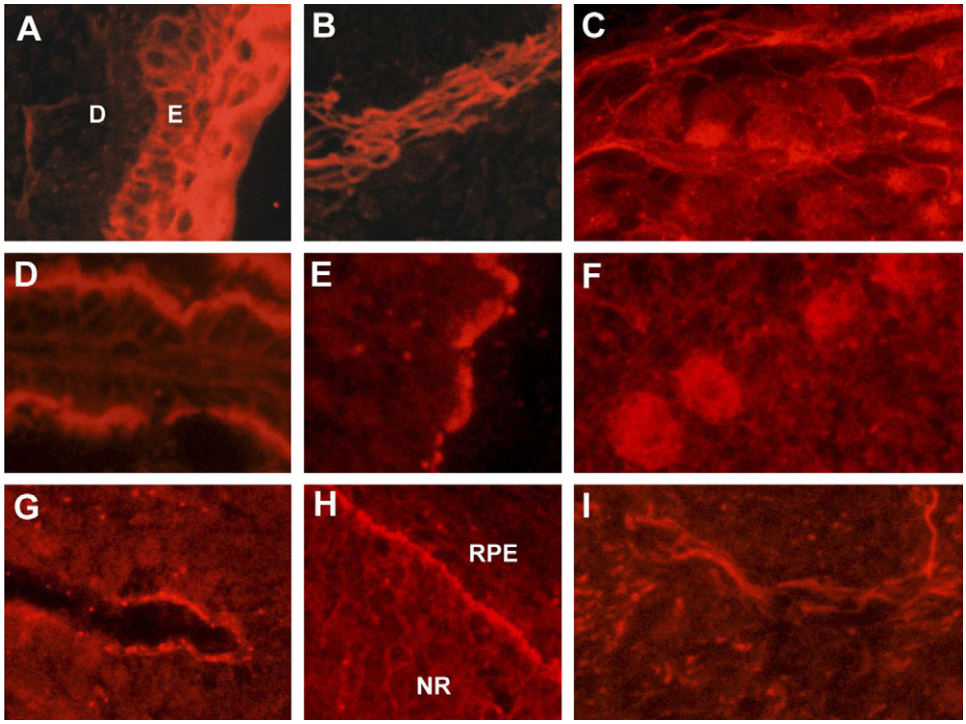

Fig. 3. Immunofluorescent staining of STIM1 in fixed frozen sections of fetal and adult mouse tissues: (A) day 16.5 fetal skin showing epidermis (E) and dermis (D); (B) day 16.5 dermis showing peripheral nerve tracts; (C) day 16.5 dorsal root ganglion; (D) adult oviduct; (E) day 16.5 fetal palate; (F) adult cerebellum showing Purkinje cell layer; (G) day 16.5 fetal gut, showing crypt of villus; (H) day 16.5 retina showing retinal pigmented epithelial layer (RPE) and neural retina (NR); (I) cortical region of adult cerebellum.

plasma membrane [37]. The role of STIM1 in the sequence of events leading to activation of plasma membrane SOC channels in response to emptying of Ca^{2+} stores in the ER can be briefly summarised as follows (see also Fig. 5): (1) STIM1 in the ER membranes acts as a Ca^{2+} sensor to detect Ca^{2+} depletion in the ER lumen; (2) upon Ca^{2+} depletion STIM1 is redistributed from a uniform ER pattern to discrete lateral aggregates, or punctae, in the ER membrane juxtaposed to the plasma membrane; (3) STIM1 induces clustering of Orai1 within the plasma membrane resulting in activation of functional SOC channels and entry of Ca^{2+} into the cytoplasm. While the precise molecular mechanisms underlying the regulation of SOC activation remain to be elucidated, experimental studies using mutated STIM cDNA constructs have illuminated how the extracellular and intracellular structural domains of STIM1 are involved in Ca^{2+} sensing, in aggregation and translocation of STIM1 complexes in the ER, and in SOC activation at the plasma membrane.

5.1. The extracellular domain contains the ER Ca^{2+} sensor

The single unpaired EF hand motif in STIM1 is, as expected, responsible for Ca^{2+} sensing in the ER lumen, with low affinity Ca^{2+} binding ($K_d \sim 0.2$–0.6 mM) that compares to that of other ER Ca^{2+}-binding proteins such as calreticulin [38]. Ca^{2+} sensors are proteins that undergo a conformational change in response to binding and dissociation of Ca^{2+} that has downstream effects to ultimately regulate a Ca^{2+}-dependent cellular function [39]. Mutations of critical amino acids in the STIM1 EF hand prevent Ca^{2+} binding and render STIM1 in a constitutively active form that behaves as if ER stores were constantly depleted. Thus, in cells which are store-replete, EF hand-mutated STIM1 nevertheless aggregates and translocates to form punctae in the ER adjacent to the plasma membrane, which couple with and activate SOC channels in the plasma membrane [5,24,34]. These studies demonstrate that Ca^{2+} binding to this domain in wild type STIM1 is sufficient to prevent the entire sequence of events leading to SOC activation. In recent studies, a sequence containing only the EF and SAM domain-containing region has been recombinantly expressed in *Escherichia coli* and the effects of Ca^{2+} binding on its conformation studied *in vitro* [38]. These biophysical analyses show that in the presence of Ca^{2+} the EF hand/SAM domain exists as a monomer that has a compact alpha-helical structure. In contrast, when Ca^{2+} is depleted, the EF hand/SAM domain changes to a less alpha-helical, less compact conformation that promotes aggregation to dimers and oligomers. Repletion of Ca^{2+} results in disaggregation of this domain back to monomers, demonstrating complete reversibility of this Ca^{2+}-dependent conformational change. The conformational change is not restricted to the EF-hand domain, but also encompasses the SAM domain, suggesting that conformational changes of the SAM domain may mediate the initial homotypic STIM1 interactions observed upon Ca^{2+}-depletion in the ER lumen [38]. Since the EF-hand/SAM region was expressed in *E. coli*, the role of N-linked glycosylation within the SAM domain cannot be ascertained. The only known function of N-linked glycosylation demonstrated so far is in localisation of STIM1 to the plasma membrane [8], such that when glycosylation is inhibited by tunicamycin, FACS analysis shows a reduction in cell surface STIM1 levels [8].

Deletion of the SAM domain alone renders STIM1 incapable of store depletion-mediated punctae formation and does not result in constitutive STIM1 aggregation into punctae nor constitutive activation of SOC channels [30]. These data support a critical role of the SAM domain in determining the Ca^{2+}-dependent conformational transition of the extracellular region. Since expression of protein containing only the cytoplasmic region of STIM1 appears to elicit the same effects as an EF hand mutant in constitutive activation of SOC channels in store-replete cells [12], it can be concluded that the extracellular domain of STIM1 plays a critical role in *preventing* the lateral aggregation of STIM1 oligomers into punctae and subsequent SOC channel activation in store-replete cells, rather than having a necessary and sufficient role in actively promoting these events after store depletion. However, a role of this region in enhancing STIM1–STIM1 interactions in normal cells, when STIM1 is not so abundant, cannot be ruled out. Ca^{2+} binding to the EF hand may thus be the single critical endogenous regulatory mechanism that prevents the SAM-dependent conformational change in the extracellular region of STIM1 required for aggregation and translocation of STIM1 into punctae in the ER. It is not known whether this conformational change involves alterations in the interaction of the SAM domain with other proteins (including STIM1), or whether the SAM domain in STIM1 only mediates intramolecular interactions within the extracellular region.

5.2. The cytoplasmic coiled-coil/ERM domain mediates STIM1 aggregation and SOC activation

While the data presented above indicate that the extracellular region of STIM1 only dimerises in the absence of Ca^{2+}, STIM1 does not appear to exist in a monomeric form in the resting, Ca^{2+} replete state of cells *in vivo*, since both endogenous and overexpressed STIM1–STIM1 and STIM1–STIM2 complexes can be readily co-immunoprecipitated from store-replete cell lysates [8]. In fact, the formation of these complexes has been shown not to be dependent on the extracellular region, since STIM1–STIM1 interactions occur when the extracellular region of STIM1 is replaced with the non-STIM protein GCSF, but rather involves the cytoplasmic coiled-coil/ERM domain [8]. We propose that STIM1–STIM1 and STIM1–STIM2 interactions in store-replete cells represent homodimers and heterodimers, respectively, which subsequently aggregate into higher order oligomers within punctae upon Ca^{2+} depletion. These observations suggest that the cytoplasmic region, including the coiled-coil/ERM domains, exists in different

conformation states that either permit or prevent higher order oligomerisation.

When overexpressed in cells, the intracellular region of STIM1 protein is alone sufficient in mediating constitutive SOC entry [12], indicating that this region must assume a default active conformation that, like the EF hand mutant, is able to assemble into punctae in the ER and activate SOC channels in the PM. The role of the coiled-coil/ERM domain in this process has been investigated by deletion of this domain in full-length wild type STIM1 protein, in the constitutively active EF-hand mutant and in the constitutively active intracellular region of STIM1. In each case, deletion of the coiled-coil/ERM domain decreased translocation of STIM1 into punctae and inhibited activation of SOC channels [12,30]. In addition, deletion of this domain in the constitutively active EF-hand mutant inhibited store-depletion-dependent clustering of wild type STIM1 in a dominant-negative manner, indicating the critical role of this domain in mediating the response of STIM1 to absence of Ca^{2+} binding in the ER lumen [12]. The importance of the coiled-coil/ERM region in the translocation and function of STIM1 within the ER suggests that this domain mediates aggregation of STIM1 dimers into punctae below the plasma membrane, and also interactions between STIM1 in the ER and integral PM proteins, including protein components of SOC and other Ca^{2+} entry channels.

The demonstrated role for the coiled-coil/ERM domain in mediating quite different processes in store replete and store-deplete cells supports a model in which the coiled-coil/ERM domain undergoes a conformational change that determines the nature of the protein–protein interactions mediated through this region. Very few interacting partners have so far been identified for STIM1. STIM1 can be co-precipitated with plasma membrane TRPC proteins, and these interactions are mediated by the coiled-coil domain [12]. However, interactions between STIM1 and TRPC proteins are not regulated by store depletion. In contrast, interactions between STIM1 and Orai1 are significantly enhanced after store depletion [40], but it is not clear whether this is due to conformational changes in these proteins after store-depletion or simply due to localisation of STIM1 to ER sites where close association is physically possible. A role for the coiled-coil domain in mediating Orai1–STIM1 interactions has been suggested [41] but not demonstrated biochemically. However, since this is the only intracellular domain present in D-Stim, which does interact with *Drosophila* Orai1 to mediate SOC entry [34], the coiled-coil/ERM domain of D-Stim is almost certainly involved in their interaction.

As discussed above, ERM proteins are known to undergo conformational changes that cause them to be 'active' or 'inactive' in mediating interactions with other proteins. It will be of major importance to determine whether such conformational changes in the STIM1 coiled-coil/ERM domain are responsible for mediating different STIM1 associations in store-replete and store-deplete states, and whether conformational changes are influenced by the Ca^{2+}-binding dependent conformational status of the extracellular region. We suggest a model in which the coiled-coil/ERM domain can exchange

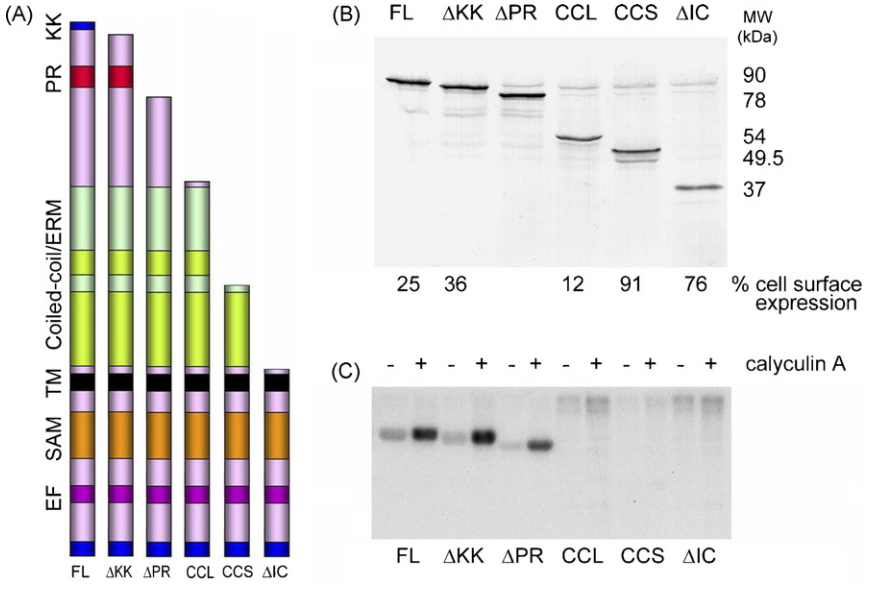

Fig. 4. (A) Diagrammatic representation of STIM1 protein fragments expressed from a full length *STIM1* cDNA construct (FL) and constructs with progressive C-terminal deletions: deleted lysine-rich region (ΔKK, at D666), deletion of proline-rich and lysine-rich regions (ΔPR, at M597), deletion downstream of the coiled-coil/ERM region (CCL, at H395), deletion downstream of the first coiled-coil region (CCS, at S340), and deletion of entire intracellular region (ΔIC, at R235). (B) Western blot analysis of cell lysates from 293T cells expressing the STIM1 cDNA constructs shown in A, using an antibody recognising an N-terminal peptide of STIM1. Molecular weights of STIM1 proteins are shown on the right. The cell surface expression was determined by FACS analysis. (C) Autoradiograph showing phosphorylation of full length and truncated STIM1 proteins expressed in 293T cells. Cells were incubated in ^{32}P phosphate for 2 h followed by further incubation for 1 h in the presence (+) or absence (−) of the phosphatase inhibitor calyculin A (as in [4]). STIM1 proteins were immunoprecipitated with STIM1 antibodies prior to gel electrophoresis.

interacting partners, dependent on the conformational status of the entire region. Thus, while in a store-replete state these coiled-coils form dimers with other STIM proteins (STIM1 or STIM2) they might undergo a conformational change after store-depletion to enable interactions with other STIM1 dimers, other proteins, and with coiled-coils in Orai1. Detailed structural analysis of the coiled-coil region will be required to understand exactly how the dynamic state in STIM1 interactions might be regulated, and this is an important area for further research.

The interaction between STIM1 and STIM2 has been shown to have a powerful inhibitory effect on SOC activation in a variety of cell types [25] suggesting that STIM1 and STIM2 may have a coordinate role in regulating SOC entry. Even though STIM2 has an EF hand in the extracellular region, it does not appear to act as an ER Ca^{2+} sensor, and is not redistributed within the ER after store depletion when overexpressed [25]. Only when STIM1 levels are high enough does store depletion result in translocation of STIM2 to punctae at the ER-plasma membrane junction, presumably through association with STIM1. However, these STIM1–STIM2 aggregates are not able to activate SOC entry [25]. STIM2 can also inhibit the function of the constitutively active STIM1 EF hand mutant that pre-exists in punctae, indicating that it can interfere downstream of punctae formation, presumably by competing positively with other protein interactions that make up functional complexes between the ER and SOC channels. However, high levels of STIM2 expression can result in constitutive activation of Ca^{2+} entry without any redistribution within the ER, which is enhanced by co-expression of Orai1 [42]. Thus, it is not yet clear what actual role STIM2 has in regulating Ca^{2+} entry *in vivo*, and further research is required in this area.

Residues within the coiled-coil domain are required not only for redistribution of STIM1 in the ER but are also required for retention of STIM1 within the ER rather than sequestration to the plasma membrane. Deletion of the cytoplasmic region C-terminal of the coiled-coil domain (CC long

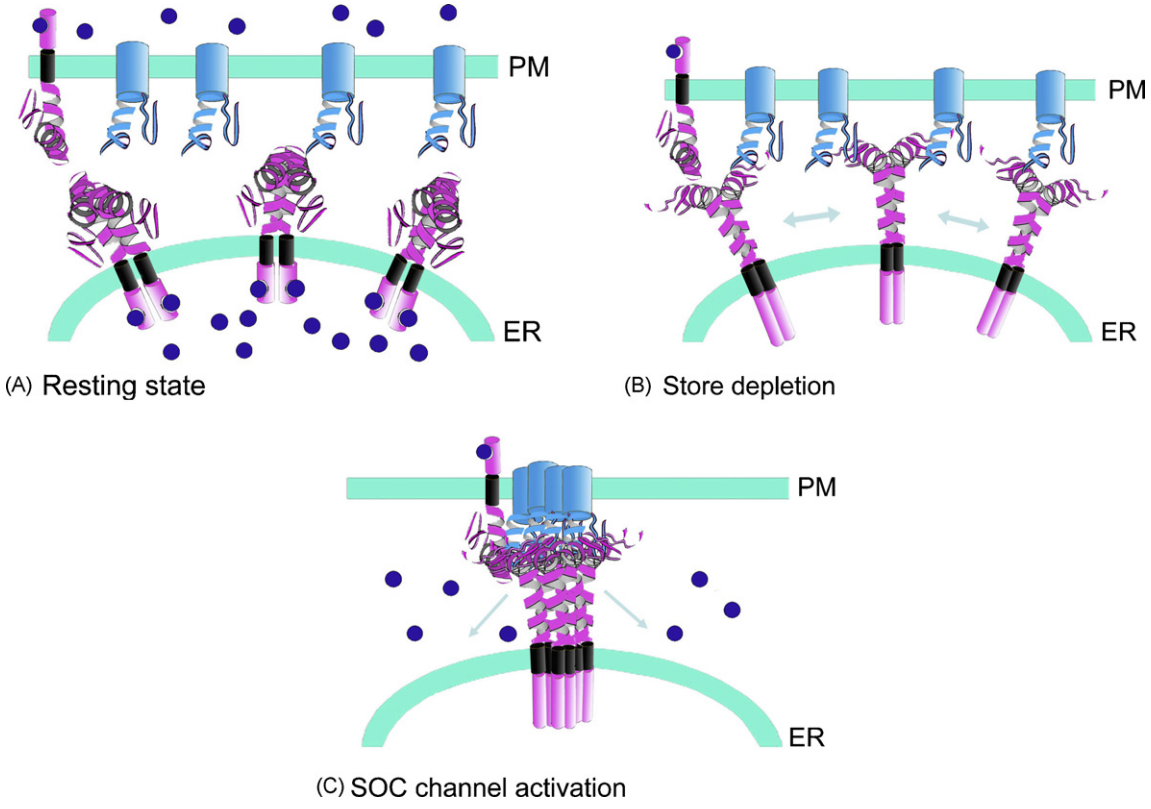

Fig. 5. A proposed model for conformational changes in STIM1 protein (pink) in the ER that mediate interactions with Orai1 protein (blue) and STIM1 in the plasma membrane (PM) to form functional SOC channels. (A) In resting, store-replete cells, binding of Ca^{2+} (circles) in the ER lumen maintains the extracellular region of STIM1 in a compact, monomeric conformation. STIM1 nevertheless exists as dimers, mediated through associations between the cytoplasmic coiled-coil/ERM domains, which are distributed uniformly thoughout the ER. Lateral aggregation of dimers is prevented by a mechanism involving the Ca^{2+}-dependent conformation of the extracellular region. We propose that the conformational state of the coiled-coil/ERM region determines its potential to promote STIM1 aggregation and/or interaction with other proteins. (B) After store depletion, the extracellular region of STIM1 responds to the absence of Ca^{2+} binding by assuming an extended conformational change that promotes STIM1 dimerisation and oligomerisation through this region. We propose that this, in turn, induces a conformational change in the cytoplasmic region that promotes lateral aggregation of STIM1 dimers within the ER to specific sites beneath the PM. The cytoplasmic domains of STIM1 associate with the cytoplasmic domains of Orai1 and STIM1 proteins in the PM. (C) Lateral aggregation of STIM1 in the ER causes clustering of Orai1 and STIM1 in the PM to form a complex of proteins (punctae) that couple the ER and PM to establish functional SOC channels. Specific protein interactions in these complexes regulate SOC channel activity and Ca^{2+} entry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

form, Fig. 4) that contains the proline-rich region (PRR) and the lysine-rich tail (KK tail) does not markedly influence the amount of overexpressed STIM1 localised to the cell surface when compared to full-length STIM1, determined by FACS analysis in HEK293 cells (12% cf. 25%, respectively). This data indicates that the lysine-rich region is not an ER retention signal in STIM1. However, deletion of the C-terminal coiled-coil segment (CC short, Fig. 4) or the entire cytoplasmic region (ΔIC, Fig. 5) causes 91% and 76% of STIM1 to be localised at the cell surface. It is possible that protein–protein interactions between STIM1 and other proteins mediated by the coiled-coil domain are required for retention of STIM1 within the ER, but this has not been determined.

5.3. The cytoplasmic proline-rich region and lysine-rich tail regulate SOC activity

The C-terminal half of the cytoplasmic regions of STIM1 and STIM2 contain dissimilar proline-rich regions and lysine-rich tails. The phosphorylation state of STIM1 and STIM2 is significantly enhanced in the presence of the serine/threonine phosphatase inhibitor calyculin A [4], indicating that the phosphorylation of STIMs undergoes dynamic change in cells. Quantitative analysis of STIM1 phosphoamines in store-replete cells using thin layer chromatography has demonstrated that STIM1 is phosphorylated predominantly on serine residues [7], all of which are C-terminal to the coiled-coil domain (Fig. 4). An estimated 70% of phosphorylated residues are within the proline-rich region while 30% are located within the C-terminal region of the coiled-coil ERM domain, where they could potentially function to regulate the conformation of the extracellular region.

Deletion of the proline-rich and lysine-rich domains does not inhibit SOC activation, but appears to influence the rate of transport of STIM1 from the general ER to the punctae at the plasma membrane contacts [30], and influences the pharmacology of the SOC activation and inactivation response to store depletion [24]. The proline-rich region thus appears to have a role in regulating the dynamics of both STIM1 aggregation and SOC channel activity, presumably through mediating protein–protein interactions within the complexes formed at the ER-plasma membrane interface. This fine-tuning function as opposed to a critical role in SOC channel activity is consistent with the absence of a proline-rich and a lysine-rich domain in D-Stim and imply a much simpler molecular control over Ca^{2+} entry in *Drosophila* cells. The presence of a second STIM protein in vertebrates that can inhibit STIM1-mediated SOC activation after store depletion also supports a more complex control of SOC channels in vertebrate cells. The proline-rich region in human STIM1 contains a motif (HSPSSP) for binding of the SH3 containing protein 14-3-3, but this sequence is not conserved in the murine protein. We have not been able to demonstrate an interaction between 14-3-3 and STIM1 in store-replete cells, which may indicate that this is not a critical functional site in STIM1, or that the site is functional only after store-depletion in human cells. Identification of proteins that interact with the proline-rich domains of STIM1 and STIM2 will be critical for elucidating the mechanisms by which these domains function in the regulation of SOC channels.

6. Conclusions

The biological significance of the unique structural domains of STIM1 is now becoming clear. A good working model can be proposed for how these domains contribute to the function of STIM1 as a Ca^{2+} sensor in the ER and the sequential changes in aggregation, translocation within the ER, formation of punctae at sites of contact with the plasma membrane and activation of SOC channels (Fig. 5). The biochemical and biophysical data suggest that the extracellular region of STIM1 exists in a monomeric compact conformational state when Ca^{2+} is bound to the EF hand, while the intracellular coiled-coil domain mediates dimeric STIM1–STIM1 interactions. Upon Ca^{2+} depletion, the extracellular EF hand/SAM domain undergoes a conformational change that promotes dimerisation and oligomerisation of the extracellular region. The intracellular coiled-coil/ERM region of STIM1 mediates lateral aggregation of STIM1 and translocation within the ER either after Ca^{2+} depletion or when the extracellular region is absent or mutated and unable to bind Ca^{2+}. This default conformational state is prevented in the Ca^{2+} replete state by the Ca^{2+}-dependent conformation of the extracellular region. We thus propose that in normal cells, the Ca^{2+}-dependent conformation of the extracellular region of STIM1 influences the conformation of the intracellular coiled-coil/ERM domain that permits aggregation into higher order protein complexes. These complexes are translocated to sites beneath the plasma membrane where they are able to form complexes with Orai1 and regulate the activity of SOC channels through specific protein–protein interactions mediated by the intracellular STIM1 domains.

There are still many unanswered mechanistic questions which will undoubtedly be addressed over the next few years: (1) How does Ca^{2+} binding to the extracellular region influence oligomerisation of the cytoplasmic region? (2) What mechanisms cause STIM protein aggregates to be redistributed and translocated into punctae within the ER at contact sites with the plasma membrane? (3) Which proteins make up the protein complexes at these punctae? (4) What proteins constitute the SOC channel in addition to Orai1? (5) Are interactions between ER- and plasma membrane-localised STIM1 required for SOC function? (6) How do protein interactions influence SOC channel pharmacokinetics? (7) What role, if any, does STIM1 phosphorylation play in regulation of SOC channel activity? (8) What other functions does STIM1 have at the plasma membrane? (9) What is the role of STIM2 in SOC inhibition and activation *in vivo*?

An understanding of what actual role these mechanisms play in the regulation of SOC function *in vivo*, during tissue development, homeostasis and disease, will require further

studies in animal models. Such studies are also likely to reveal whether regulation of SOC entry mediated by STIM proteins has a role in cell proliferation and survival, the function initially proposed for STIM1.

Conflict of interest

There are no conflicts of interest associated with this submission.

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