Modulation of STIM1 and capacitative Ca\(^{2+}\) entry by the endoplasmic reticulum luminal oxidoreductase ERp57

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STIM1 is an endoplasmic reticulum (ER) membrane Ca\(^{2+}\) sensor responsible for activation of store-operated Ca\(^{2+}\) influx. We discovered that STIM1 oligomerization and store-operated Ca\(^{2+}\) entry (SOC) are modulated by the ER oxidoreductase ERp57. ERp57 interacts with the ER luminal domain of STIM1, with this interaction involving two conserved cysteine residues, C\(^{49}\) and C\(^{56}\). SOC is accelerated in the absence of ERp57 and inhibited in C\(^{49}\) and C\(^{56}\) mutants of STIM1. We show that ERp57, by ER luminal interaction with STIM1, has a modulatory role in capacitative Ca\(^{2+}\) entry. This is the first demonstration of a protein involved in ER intraluminal regulation of STIM1.

Keywords: calcium signalling; oxidoreductase; endoplasmic reticulum; STIM


INTRODUCTION

Intracellular Ca\(^{2+}\) is an important second messenger in processes as varied as muscular contraction, gene transcription and apoptosis. Ca\(^{2+}\) is released from the endoplasmic reticulum (ER) into the cytoplasm and the loss of ER Ca\(^{2+}\) stores necessitates refilling by store-operated Ca\(^{2+}\) entry (SOC; Putney & McKay, 1999; Hogan et al., 2010), mediated by STIM1, a transmembrane ER protein that senses the Ca\(^{2+}\) filling state of the ER (Roos et al., 2005), and Orai1, a plasma membrane Ca\(^{2+}\) channel that, on the extracellular medium (Soboloff et al., 2006). STIM1 has an amino-terminal, ER luminal portion containing a Ca\(^{2+}\)-sensing EF-hand motif (Stathopoulos & Ikura, 2009). At rest, Ca\(^{2+}\) is bound to the ER hand of STIM1 in the lumen of ER and the protein is held as an inactive dimer (Covington et al., 2010; Hogan et al., 2010). In response to Ca\(^{2+}\) depletion in the ER, Ca\(^{2+}\) dissociates from STIM1, leading to oligomerization of STIM1 and its translocation to subplasmalemmal punctae (Liou et al., 2005; Roos et al., 2005; Muilk et al., 2008; Covington et al., 2010). Although STIM1 and Orai1 alone can reconstitute SOC activity, other associated proteins might be involved, including SERCA (Sampieri et al., 2009; Manjarres et al., 2010), EB1 (Grigoriev et al., 2008) and CRACR2A (Srikanth et al., 2010).

Here, we discovered that ERp57, an ER luminal oxidoreductase, modulates STIM1 function in the lumen of the ER. ERp57 bound to STIM1, depending on the presence of two conserved cysteine residues (C\(^{49}\) and C\(^{56}\)) in the ER luminal domain of STIM1. SOC was accelerated in the absence of ERp57 and inhibited in C\(^{49}\) and C\(^{56}\) mutants of STIM1. This is the first demonstration of a protein involved in ER intraluminal modulation of STIM1.

RESULTS

ERp57 interacts with the luminal domain of STIM1

Our initial goal was to identify molecules that bind to the ER luminal domain of STIM1, and thus we used surface plasmon resonance (BIACore) to screen for ER-resident proteins that might interact with STIM1 within the ER lumen. The recombinant ER luminal domain of STIM1, encompassing the EF-hand and sterile-\(\alpha\) motif domains (Fig 1A), was immobilized to BIACore sensor chips to test whether ER-luminal-resident proteins including ERp57, protein disulphide isomerase, calreticulin, calsequestrim and the ER luminal domain of calnexin would bind to STIM1. Of all tested proteins, only ERp57 bound to the immobilized STIM1 luminal domain (Fig 1B). We concluded that the oxidoreductase ERp57 interacts in vitro with the STIM1 ER luminal domain.

ERp57 is a 58-kDa thiol oxidoreductase and a member of the protein disulphide isomerase-like family (Coe & Michalak, 2010) that promotes disulphide bond formation and isomerization (Frickel et al., 2004). ERp57 is widely expressed in mouse tissues and has been most studied for its role in quality control in the secretory pathway (Coe & Michalak, 2010). The protein is a required component of the peptide-loading complex of major histocompatibility complex class I molecules (Wearsch & Cresswell, 2007), affects STAT3 signalling (Coe et al., 2010) and modulates SERCA function (Li & Camacho, 2004).

To further analyse the interaction between ERp57 and STIM1, we carried out fluorescence resonance energy transfer (FRET) analysis.
We generated STIM1−/− mice and mouse embryonic fibroblasts (MEFs), as well as ERp57−/− cell lines. STIM1−/− MEFs were transfected with expression vectors encoding yellow fluorescent protein (YFP)-tagged STIM1 (YFP–STIM1) and cyan fluorescent protein (CFP)-tagged ERp57 (ERp57–CFP). In resting cells, YFP–STIM1 was distributed throughout the ER and colocalized with ERp57–CFP (Fig 1C, iii). Coexpression of YFP–STIM1 and ERp57–CFP produced a robust FRET signal under resting conditions (Fig 1C, iv). FRET signal remained present after thapsigargin-induced store depletion (supplementary Fig S1 online). These observations supported our in vitro observations (Fig 1B) and indicated that ERp57 and STIM1 form complexes in vivo and in vitro.

Enhanced SOC in the absence of ERp57

We next tested whether binding of ERp57 to STIM1 had an effect on the ability of STIM1 to activate SOC. To do this, we used ERp57-deficient MEFs and Fura2 imaging of intracellular Ca2+ dynamics (Coe et al, 2010). We used thapsigargin, a SERCA inhibitor, to deplete ER Ca2+ stores, followed by addition of extracellular Ca2+ to initiate SOC. As expected, there was robust SOC in wild-type cells, whereas STIM1−/− MEFs showed minimal SOC (Fig 2A,B; extent of SOC: wild type: 233 ± 10 nM, n = 3; STIM1−/−: 140 ± 6 nM, n = 4; P = 0.004). In contrast, ERp57−/− MEFs showed enhanced SOC when compared with wild-type cells (extent of SOC: wild type: 233 ± 10 nM, n = 3; ERp57−/−:...
**Fig 2** Store-operated Ca\(^{2+}\) entry in ERp57\(^{+/−}\) cells. (A) Representative traces of Fura2 fluorescence in wild-type (wt), STIM1-deficient cells (STIM1\(^{−/−}\)), ERp57-deficient cells (ERp57\(^{−/−}\)) and ERp57-deficient cells expressing recombinant ERp57 (ERp57\(^{−/−}\) + ERp57). Endoplasmic reticulum (ER) store Ca\(^{2+}\) depletion was induced with 2 μM thapsigargin (TG), followed by activation of store-operated Ca\(^{2+}\) entry (SOC) with the addition of 2 mM Ca\(^{2+}\). Ca\(^{2+}\) concentrations were calculated by calibration steps as described in supplementary methods online. (B) Maximum extent of SOC for wild-type (WT), STIM1\(^{−/−}\), ERp57\(^{−/−}\), and ERp57\(^{−/−}\) cells expressing recombinant ERp57 (ERp57\(^{−/−}\) + ERp57). Inset, western blot analysis of wild-type, ERp57\(^{−/−}\), and ERp57\(^{−/−}\) cells expressing recombinant ERp57 probed with anti-ERp57 antibodies. (C) Yellow fluorescent protein (YFP)–STIM1 punctae formation in STIM1\(^{−/−}\) cells, ERp57\(^{−/−}\) cells, and ERp57\(^{−/−}\) cells expressing recombinant ERp57. Under resting conditions, YFP–STIM1 shows a reticular pattern when expressed in STIM1\(^{−/−}\) cells (i). Under resting conditions in ERp57\(^{−/−}\) cells, in addition to reticular distribution of YFP–STIM1, there were also several punctae forming as indicated by the arrows (ii), with this abolished in ERp57\(^{−/−}\) cells expressing recombinant ERp57 (iii). Thapsigargin-dependent depletion of Ca\(^{2+}\) stores induced YFP–STIM1 relocation to subplasmalemmal punctae in all cells investigated. Scale bar, 16 μm. (D) YFP–STIM1 punctae formation in STIM1\(^{−/−}\) cells without (i–iv) or with (v–viii) concomitant expression of ERp57–cyan fluorescent protein (CFP). Three minutes after thapsigargin-induced depletion of Ca\(^{2+}\) stores, there was evidence of punctae formation in cells without ERp57–CFP but none in cells overexpressing ERp57–CFP (compare ii and vi). After 6 min, extensive punctae can be seen in cells without ERp57–CFP (iii) but minimal punctae are present in cells overexpressing ERp57–CFP (vii). Even 10 min after store depletion, most YFP–STIM1 signal remains non-punctate in cells overexpressing ERp57–CFP (viii).
Therefore, we examined whether increased SOC in the absence of ERp57. Wild-type and ERp57–/– cells had comparable rates of basal Ca2+ entry (that is, without store depletion), further supporting a role for ERp57 in SOC (data not shown). Next, we expressed recombinant ERp57 in ERp57–/– cells (Coe et al, 2010) to test for the specificity of the ERp57 effect on SOC. ERp57–/– cells expressing recombinant ERp57 showed significantly reduced SOC to a similar extent to that seen in wild-type cells (extent of SOC: ERp57+/–: 336 ± 22 nM, n = 3; ERp57–/– + ERp57: 232 ± 26 nM, n = 3; P = 0.05; Fig 2A,B), demonstrating the specificity of the effect of ERp57 on SOC. Interestingly, ERp57–/– cells had lower thapsigargin-releasable ER Ca2+ stores than did wild-type cells (wild-type: 427 ± 26 nM, n = 3; ERp57–/–: 283 ± 1 nM, n = 3; P = 0.03; Fig 2A; supplementary Fig S2 online). This could have contributed to altered SOC dynamics in ERp57-deficient cells. However, the expression of recombinant ERp57 in ERp57–/– cells did not return thapsigargin-releasable ER Ca2+ stores to wild-type levels (ERp57–/–: 283 ± 1 nM; ERp57–/– + ERp57: 281 ± 14 nM; Fig 2A; supplementary Fig S2 online), supporting a role for ERp57 on SOC by interaction with STIM1 rather than its effects on the size of ER Ca2+ stores.

Fig 3I Store-operated Ca2+ entry in ERp57–/– cells with knockdown of STIM1. (A) Representative traces of Fura2 fluorescence in wild-type (WT) cells, ERp57–/– cells and in ERp57–/– cells transfected with small interfering RNA (siRNA) targeting STIM1, or scrambled siRNA (sc siRNA). Endoplasmic reticulum (ER) store Ca2+ depletion was induced with 2 μM thapsigargin (TG), followed by activation of store-operated Ca2+ entry (SOC) with the addition of 2 mM Ca2+. Ca2+ concentrations were calculated by calibration steps as described in supplementary methods online. (B) Extent of SOC in wild-type, ERp57–/– cells and ERp57–/– cells transfected with siRNA targeting STIM1, or scrambled siRNA. (C) Western blot (WB) analysis of ERp57–/– cells treated with siRNA using anti-STIM1 and anti-GAPDH antibodies (loading control). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

336 ± 22 nM, n = 3; P = 0.02; Fig 2A,B), indicating that STIM1-dependent activation of SOC was enhanced in the absence of ERp57. Wild-type and ERp57–/– cells had comparable rates of basal Ca2+ entry (that is, without store depletion), further supporting a role for ERp57 in SOC (data not shown). Next, we expressed recombinant ERp57 in ERp57–/– cells (Coe et al, 2010) to test for the specificity of the ERp57 effect on SOC. ERp57–/– cells expressing recombinant ERp57 showed significantly reduced SOC to a similar extent to that seen in wild-type cells (extent of SOC: ERp57+/–: 336 ± 22 nM, n = 3; ERp57–/– + ERp57: 232 ± 26 nM, n = 3; P = 0.05; Fig 2A,B), demonstrating the specificity of the effect of ERp57 on SOC. Interestingly, ERp57–/– cells had lower thapsigargin-releasable ER Ca2+ stores than did wild-type cells (wild-type: 427 ± 26 nM, n = 3; ERp57–/–: 283 ± 1 nM, n = 3; P = 0.03; Fig 2A; supplementary Fig S2 online). This could have contributed to altered SOC dynamics in ERp57-deficient cells. However, the expression of recombinant ERp57 in ERp57–/– cells did not return thapsigargin-releasable ER Ca2+ stores to wild-type levels (ERp57–/–: 283 ± 1 nM; ERp57–/– + ERp57: 281 ± 14 nM; Fig 2A; supplementary Fig S2 online), supporting a role for ERp57 on SOC by interaction with STIM1 rather than its effects on the size of ER Ca2+ stores.

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Ca2+ store content, substantiating a direct role for ERp57 binding to STIM1 (supplementary Fig S2 online). We concluded that ERp57, by binding to STIM1 in the lumen of ER, might serve as a brake on the initiation of SOC.

ER luminal cysteines of STIM1 form a disulphide bond

ERp57 is an ER luminal oxidoreductase; therefore, we identified two highly conserved cysteine amino-acid residues (C49 and C56)
upstream of the EF-hand Ca\(^{2+}\)-binding domain as potential ERp57-binding sites. We carried out site-specific mutagenesis of these residues and SDS–PAGE analysis of STIM1 and STIM1 mutants under reducing and non-reducing conditions to examine whether C49 and C56 form an intramolecular disulfide bond in the ER luminal domain of STIM1. Under non-reducing conditions, wild-type YFP–STIM1 migrated more slowly than under reducing conditions, supporting the presence of an intramolecular disulfide bond in STIM1 (supplementary Fig S3A online). In contrast, the YFP–STIM1–C49/56A mutant showed the same mobility in SDS–PAGE (supplementary Fig S3A online), indicating that ER luminal C49 and C56 form a disulfide bond in STIM1. Analysis of YFP–STIM1 expressed in \(\text{ERp57}^{-/-}\) cells indicated that ERp57 was not necessary for disulfide bond formation (supplementary Fig S3B online). Similar experiments using lysates from wild-type and \(\text{ERp57}^{-/-}\) cells expressing Orai1–YFP showed shifts in apparent size, indicating the lack of a role for ERp57 on Orai1 itself (supplementary Fig S3C online).

Cysteines of STIM1 are required for ERp57 binding
To test the role of STIM1 ER luminal domains and luminal cysteines, we used our YFP–STIM1–C49/56A mutant for analysis of punctae formation, FRET interactions between YFP–STIM1 and ERp57–CFP, and SOC activity. The YFP–STIM1–C49/56A mutant expressed in \(\text{STIM1}^{-/-}\) cells showed ER-like reticular localization with no visible punctae (Fig 4A, i). Importantly, thapsigargin-dependent Ca\(^{2+}\) store depletion did not result in oligomerization and punctae formation by the YFP–STIM1–C49/56A mutant (Fig 4A, v, after store depletion). We concluded that ER luminal C49 and C56 have a role in STIM1 subplasmalemmal punctae localization.

We next examined whether STIM1 ER luminal cysteines are involved in ERp57 binding to STIM1. \(\text{STIM1}^{-/-}\) cells were co-transfected with expression vectors encoding YFP–STIM1–C49/56A and ERp57–CFP, followed by FRET analysis. Fig 5A shows that YFP–STIM1–C49/56A and ERp57–CFP produced robust FRET at rest (Fig 4A, iv and viii), although the signal was at a
lower level than that observed between ERp57–CFP and wild-type YFP–STIM1. Although ER luminal C^49 and C^56 might have a role in ERp57 binding to STIM1, the FRET signal was unaffected by depletion of ER Ca^{2+} stores (Fig 4A, after store depletion). When ERp57^{+/−} cells were co-transfected with the same expression vectors, we again observed robust FRET signal between YFP–STIM1 and ERp57–CFP (supplementary Fig S4A online, i–iv) and reduced FRET between YFP–STIM1–C^{49/56A} and ERp57–CFP (supplementary Fig S4A online, v–viii).

To quantify, we calculated N_{FRET} values for FRET between ERp57–CFP and YFP–STIM1 or YFP–STIM1–C^{49/56A} to account for differences in relative expression of YFP and CFP fluorophores (Xia & Liu, 2001). N_{FRET} was significantly reduced (33%) with YFP–STIM1–C^{49/56A} as compared with wild-type YFP–STIM1 (N_{FRET} for wild type: 0.274 ± 0.011, n = 70; N_{FRET} for C^{49/56A}: 0.182 ± 0.007, n = 64; P = 9.02 × 10^{−8}; Fig 5B). Expression of ERp57–CFP and YFP–STIM1 or YFP–STIM1–C^{49/56A} in ERp57^{−/−} cells yielded similar results, with a decrease in N_{FRET} by 28% for YFP–STIM1–C^{49/56A}, significant at P = 1 × 10^{−6} (supplementary Fig S4B online). We concluded that STIM1 ER luminal cysteines were involved in binding to ERp57.

**STIM1 ER luminal cysteines are critical for SOC entry**

Finally, we tested whether mutations of cysteine amino-acid residues affected SOC. STIM1^{−/−} cells were co-transfected with expression vectors encoding Orai1–dsRed and YFP–STIM1 or YFP–STIM1–C^{49/56A}. Cells were treated with thapsigargin (TG) to empty ER Ca^{2+} stores, followed by addition of Ca^{2+} to initiate SOC. Cells expressing YFP–STIM1 (or YFP–STIM1–C^{49/56A}) and Orai1–dsRed were identified by fluorescence microscopy followed by monitoring of Fura2 fluorescence. Fig 5 shows robust SOC observed in cells coexpressing wild-type YFP–STIM1 and Orai1–dsRed. In cells coexpressing YFP–STIM1–C^{49/56A} and Orai1–dsRed, the extent of SOC was severely inhibited, as with STIM1^{−/−} cells expressing Orai1–dsRed alone (Fig 5), indicating that C^49 and C^56 of STIM1 were critical for the activation of SOC. In summary, site-specific mutation of C^49 and C^56 not only reduced ERp57 binding, but also made STIM1 unable to translocate to punctae or stimulate SOC activity.

**DISCUSSION**

In this study, we have identified two unique aspects of STIM1 function that are separate but overlapping. We discovered that ERp57, an ER-resident oxidoreductase, interacts with the ER luminal domain of STIM1 and affects SOC. Although ERp57^{−/−} cells show reduced ER Ca^{2+} stores, expression of ERp57 in ERp57^{−/−} cells inhibits SOC without restoring Ca^{2+} stores, supporting a role for ERp57 on SOC by interaction with STIM1 rather than its effects on the size of ER Ca^{2+} stores. ERp57/STIM1 interaction involves two conserved cysteines (C^49 and C^56), forming a disulphide bridge upstream from the EF-hand domain. The mutation of these residues to eliminate the disulphide bond rendered STIM1 incapable of proper punctae translocation. As the conserved C^49 and C^56 are located in close proximity to the EF-hand sensor in STIM1, these results indicated that the oligomerization behaviour of STIM1 is highly dependent on the degree of folding and unfolding of its EF-S-sterile-z motif domain, as modulated by Ca^{2+} (Stathopoulos et al, 2006, 2008; Huang et al, 2009).

While this work was in preparation for publication, Hawkins et al (2010) reported that oxidative stress promotes STIM1 redistribution and punctae formation. On induction of oxidative stress, STIM1 ER luminal C^39 undergoes S-glutathionylation, resulting in decreased Ca^{2+} binding to STIM1, its oligomerization and SOC activation (Hawkins et al, 2010). In contrast, we did not observe constitutive Ca^{2+} entry in cells expressing STIM1–C^{49/56A} (Fig 5). The discrepancy between this study and that of Hawkins et al (2010) might represent differences in cellular systems used and/or differences in the way store-operated Ca^{2+} was measured, but both studies strongly support the notion that STIM1 is regulated by redox state. Importantly, if the cysteine residues...
of STIM1 are modified, for example, by S-glutathionylation, it might not bind to ERp57. Modification of cysteine residues might be one way that STIM1, on oxidative stress, escapes ERp57-dependent inhibition.

Interestingly, Ca\(^{2+}\) release by inositol (1,4,5)-trisphosphate receptor is inhibited by another ER-resident oxidoreductase, ERp44 (Higo et al, 2004). We showed that ERp57 has a role in Ca\(^{2+}\) homeostasis by affecting STIM1 function and consequently SOC, suggesting that ER-resident oxidoreductases are critical regulators of ER Ca\(^{2+}\) homeostasis. The ER is the intersection of many signalling pathways and communication between different cellular organelles (Burns et al, 1994; Berridge et al, 2003; Higo et al, 2005; Rutkowski & Kaufman, 2007; Coe et al, 2010). Ca\(^{2+}\) is an important signalling molecule in the lumen of the ER (Corbett & Michalak, 2000; Hogan et al, 2010), and regulation of STIM1 by ERp57 might be involved in converging Ca\(^{2+}\)-dependent information from the lumen of the ER to the cytoplasm.

METHODS

Expression and purification of recombinant proteins. Complementary DNA encoding the ER luminal domain of STIM1 (amino acids 23–213) was generated by polymerase chain reaction-driven amplification and cloned into pBAD/gIII Escherichia coli expression vector. See the supplementary information online for details on recombinant protein expression and purification.

Surface plasmon resonance (BIACore) analysis and FRET analysis. Surface plasmon resonance measurements were recorded using BIACore. FRET was quantified as described (Mitra et al, 1996). See the supplementary information online for details.

Cell culture, immunoblotting, plasmid DNA, siRNA and site-specific mutagenesis. See the supplementary information online for details.

Ca\(^{2+}\) flux measurements. Ca\(^{2+}\) measurements on cells in suspension or for a single-cell Ca\(^{2+}\) imaging were recorded using Fura2-AM. See the supplementary information online for details.

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES


