The regulation of STIM1 by phosphorylation

Eulalia Pozo-Guisado and Francisco Javier Martin-Romero*

Department of Biochemistry and Molecular Biology; School of Life Sciences; University of Extremadura; Badajoz, Spain

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Calcium ion (Ca^{2+}) concentration plays a key role in cell signaling in eukaryotic cells. At the cellular level, Ca^{2+} directly participates in such diverse cellular events as adhesion and migration, differentiation, contraction, secretion, synaptic transmission, fertilization, and cell death. As a consequence of these diverse actions, the cytosolic concentration of free Ca^{2+} is tightly regulated by the coordinated activity of Ca^{2+} channels, Ca^{2+} pumps, and Ca^{2+}-binding proteins. Although many of these regulators have been studied in depth, other proteins have been described recently, and naturally far less is known about their contribution to cell physiology. Within this last group of proteins, STIM1 has emerged as a major contributor to Ca^{2+} signaling by means of its activity as Ca^{2+} channel regulator. STIM1 is a protein resident mainly, but not exclusively, in the endoplasmic reticulum (ER), and activates a set of plasma membrane Ca^{2+} channels termed store-operated calcium channels (SOCs) when the concentration of free Ca^{2+} within the ER drops transiently as a result of Ca^{2+} release from this compartment. Knowledge regarding the molecular architecture of STIM1 has grown considerably during the last years, and several structural domains within STIM1 have been reported to be required for the specific molecular interactions with other important players in Ca^{2+} signaling, such as Ca^{2+} channels and microtubules. Within the modulators of STIM1, phosphorylation has been shown to both activate and inactivate STIM1-dependent Ca^{2+} entry depending on the cell type, cell cycle phase, and the specific residue that becomes modified. Here we shall review current knowledge regarding the modulation of STIM1 by phosphorylation.

Background

The cytosolic free Ca^{2+} concentration ([Ca^{2+}_i]) in eukaryotic cells is in the low nanomolar range (100 nM), and transient modifications of this concentration serve as an intracellular pathway by means of the activation of phospholipase C. This activation is responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the subsequent generation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (DAG). Soluble Ins(1,4,5)P_3 binds to its receptor (Ins(1,4,5)P_3R) in the endoplasmic reticulum (ER), whereas the more lipophilic DAG accumulates in the plasma membrane. Ins(1,4,5)_R is a receptor-operated Ca^{2+} channel that activates by binding to its endogenous ligand Ins(1,4,5)P_3, leading to transient Ca^{2+} release from the ER and thereby transiently increasing [Ca^{2+}_i]. Many Ca^{2+}-sensitive effectors are found in the cytosol, which contribute to the diversity of the intracellular signaling initiated by the Ca^{2+} release from the ER. Alternatively, other Ca^{2+} channels located in the plasma membrane such as the ligand-gated plasma membrane P2X receptor and the voltage-operated Ca^{2+} channels families regulate the influx of extracellular Ca^{2+} to the cytosol upon binding to ligands or upon plasma membrane depolarization conditions, respectively. We highlight these examples to draw attention to the fact that there are two major sources of Ca^{2+} that may increase [Ca^{2+}_i], - the extracellular milieu, and the intracellular compartments including the most important intracellular Ca^{2+} store, the ER and the specialized sarcoplasmic reticulum (SR) in muscle cells. In all cases the increase of [Ca^{2+}_i] is transient, and the high cytosolic Ca^{2+} levels are rapidly cut down by the activation of Ca^{2+} pumps in the plasma membrane and the ER. Whereas the plasma membrane Ca^{2+}-ATPase (PMCA) extrudes Ca^{2+} into the extracellular milieu, the sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} into the lumen of the ER. Other active Ca^{2+} transport systems are also involved in the restoration of cytosolic Ca^{2+} basal levels, such as the Na’/Ca^{2+} exchanger with a lower affinity for Ca^{2+} compared with Ca^{2+} pumps, and the mitochondrial Ca^{2+} uniporter (MCU) which transport Ca^{2+} into the mitochondria matrix. The combination of the activities of all Ca^{2+} transport systems defines specific Ca^{2+} transients in terms of amplitude, intensity, and duration which vary according to the initial stimulus and the cell type. Thus, the number, type and molar ratios of the molecules involved in the origin and maintenance of the Ca^{2+} mobilization define the spatio-temporal pattern of Ca^{2+} signaling. For instance, Ca^{2+} signaling during fertilization of mammalian oocytes is initiated by the sperm-specific phospholipase C zeta (PLCζ) that triggers the phosphoinositide pathway once PLCζ has been released into the oocyte after the fusion with the sperm. This pathway induces an initial increase of [Ca^{2+}_i] in the oocyte which lasts for 1–2 min, which is followed by repetitive and transient increases of [Ca^{2+}_i], required for the exit from cell cycle arrest of oocytes in the metaphase of the
second meiotic division. Another classical example of Ca²⁺ mobilization required to trigger downstream effects is found in skeletal muscle cells. Depolarization of plasma membrane induces a conformational change in dihydropyridine receptors (DHPRs, voltage-dependent, L-type, plasma membrane Ca²⁺ channels) which activate ryanodine receptors located in the SR, enabling the Ca²⁺ release from this intracellular store. These are just two classical examples of how Ca²⁺ fluxes regulate cell physiology, which also indicate the importance of intracellular Ca²⁺ stores for this signaling. To ensure the durability and maintenance of the signaling, a plasma membrane Ca²⁺ transport system regulates intracellular Ca²⁺ stores refilling. This Ca²⁺ entry pathway is called store-operated Ca²⁺ entry (SOCE), a ubiquitous mechanism and one of the most important pathways for Ca²⁺ entry in non-excitable cells. Consistent with its descriptive name, SOCE is regulated by the Ca²⁺ concentration within the ER.

**STIM1 Regulates Ca²⁺ Entry**

The mechanism that links luminal Ca²⁺ levels with plasma membrane Ca²⁺ entry is mediated by STIM1 (stromal interaction molecule 1), a type I transmembrane protein that senses Ca²⁺ within the luminal space of the ER, and activates plasma membrane Ca²⁺ channels (SOCs) upon Ca²⁺ depletion conditions in the intracellular stores. Human STIM1 protein consists of 685 amino acids, with an SAM domain (sterile α motif) and an EF-hand domain that acts as a Ca²⁺ sensor within the luminal space of the ER. Upon depletion of Ca²⁺ within the ER, the SAM-EF domain mediates the oligomerization of STIM1, triggering this oligomerization in clusters of ~1 μm diameter. The clustering favors the relocalization of STIM1 in ER-plasma membrane (PM) junctions required for the activation of SOCE. STIM1 directly binds to store-operated calcium channels (SOCs), activating a highly Ca²⁺-selective, non-voltage-gated, inwardly rectifying current known as the Ca²⁺ release activated Ca²⁺ current (ICRAC). This current is reconstituted by both STIM1 and ORAI1 (also known as CRACM1), a plasma membrane protein that constitutes a major Ca²⁺ channel regulated by STIM1. In addition to ORAI1, some of the transient receptor potential canonical (TRPC) channels can function in a STIM1-dependent mode. STIM1 directly binds and activates TRPC1, TRPC4 and TRPC5 channels that can therefore act as SOCs. However, the STIM1/TRPC molar ratio determines the STIM1-dependent or STIM1-independent mode of action of the channels. In addition, TRPC1 also associates with ORAI1 to produce TRPC1-ORAI1-STIM1 ternary complexes that act as SOCs.

It was early found that the C-terminal portion of STIM1 is sufficient for the activation of ORAI1, and that both proteins physically interact through a coiled-coil domain in the C-terminus of ORAI1. The minimal domain of STIM1 that activates ORAI1 is a conserved CRAC activation domain (CAD) of STIM1 of ~100 amino acids that binds directly to ORAI1. An alternative given name for this domain is STIM1-ORAI1 activation region (SOAR). The cytosolic domain (residues 234–685) contains three coiled-coil domains, CC1, CC2, and CC3. CC2 and CC3 overlap with the SOAR/CAD region required to activate Ca²⁺ channels (amino acids 334–442). Because CC1 interacts with CC2/CC3 at resting state, keeping STIM1 in a closed conformation, the activation of STIM1 by store depletion and the binding of STIM1 with ORAI1 requires an intramolecular transition of STIM1 into an open conformation, in order to expose CC2 and CC3 so they are able to activate ORAI1 by a physical interaction of coiled-coil domains. Investigation of the mechanism of TRPC1 gating led to the proposal that it involves an electrostatic mechanism through the interaction of two negative charged aspartates in TRPC1 and the conserved polybasic domain, enriched in lysine residues, at the C-terminus of STIM1. An additional Ca²⁺ channel that is regulated by STIM1 belongs to the voltage-operated Ca²⁺ channels family. STIM1 binding to Ca₁,2 channels suppresses the activation of these channels, an action that is mediated also by the SOAR/CAD domain of STIM1, causing long-term internalization of the channel from the membrane. Thus, multiple actions are triggered by the cytosolic domain of STIM1, including the activation and inactivation of diverse channels in response to different stimuli. It is plausible that the cytosolic domain encompasses additional domains that modulate the Ca²⁺ channel-regulation activity of STIM1, by controlling STIM1 subcellular localization and/or the selectivity of the Ca²⁺ channel to be activated or inhibited. In this regard, STIM1 has a serine/proline-rich domain (or S/P)-rich domain which could be acting as a modulator of the STIM1 activity.

**Phosphorylation of STIM1 Regulates its Activity**

STIM1 is a phosphoprotein in which large-scale mass spectrometry studies have revealed potential phosphorylation sites. Subsequent initial reports of detailed analyses of phosphoresidues from immunoprecipitated STIM1 differed in their findings, mainly because the studies had focused on different cell cycle stages. During the M-phase, SOCE becomes inactivated, and STIM1 clustering remains inactive in response to store depletion. Although Yu et al. reported that STIM1 is phosphorylated during meiosis of oocytes, substitution mutations of target residues to mimic constitutive phosphorylation or dephosphorylation do not modulate the clustering of STIM1 in response to store depletion, an observation that supports the lack of any physiological function for STIM1 phosphorylation during meiosis in oocytes. Smyth et al. found that STIM1 clustering is also inactivated during mitosis of mammalian cells, and they identified specific residues, such as Ser602 and Ser608, that become dephosphorylated during that process. Other sites were initially found to be constitutively phosphorylated (Ser575, Ser620, and Ser621). Interestingly, Ser486 and Ser668 becomes phosphorylated during mitosis, but not in interphase. Ser668 belongs to a consensus sequence for cyclin-dependent kinase 1 (CDK1), and is phosphorylated by CDK1 in vitro. Also, the expression of single alanine substitution mutations (S668A or S486A) does not rescue SOCE in mitotic cells. However, expression of a double mutant S486A/S668A does show SOCE responses in mitosis, confirming the role of STIM1 phosphorylation at Ser486 and Ser668 in SOCE inactivation during mitosis.
Further evidence was that extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylate STIM1 in vitro at Ser575, Ser608, and Ser621 and that STIM1 phosphorylation at ERK1/2 target sites regulates SOCE in HEK293 cells. The phosphorylation of STIM1 at Ser575, Ser608, and Ser621 was revealed by mass spectrometry using immunoprecipitated STIM1 from asynchronous HEK293 cells, and later with phospho-specific antibodies against phosphorylated residues. This latter strategy demonstrated that STIM1 phosphorylation at ERK1/2 target sites increases during SOCE activation, and consequently the alanine substitution mutation of these sites nullifies SOCE, whereas Ser-to-Glu mutation enhances Ca$^{2+}$ entry. In contrast to the results reported in [38], phospho-specific antibodies against phosphorylated STIM1 at Ser575, phosphoSer608, and phosphoSer621 revealed a dynamic phosphorylation of STIM1 that was strongly dependent on the Ca$^{2+}$ store filling state. Thus, Ca$^{2+}$ store depletion is accompanied by an increase of STIM1 phosphorylation at ERK1/2 target sites, whereas Ca$^{2+}$ store refilling triggers STIM1 dephosphorylation at these sites. Many aspects of the molecular mechanism by which the phosphorylation of STIM1 regulates SOCE remain unclear, but the inhibition of STIM1 phosphorylation decreases STIM1 clustering in response to store depletion and impairs STIM1-ORAI1 binding, as monitored by fluorescence resonance energy transfer (FRET) and by co-immunoprecipitation. In an attempt to resolve the open question of the requirement of STIM1 phosphorylation at Ser575, Ser608, and Ser621 to activate SOCE in HEK293 cells during interphase, we recently found that phosphorylation of STIM1 at ERK1/2 target sites regulates the association of STIM1 with EB1 (end-binding protein 1), a regulator of growing microtubule ends. The role of the cytoskeleton in SOCE regulation has been studied in depth, and it was early established that STIM1 colocalizes with tubulin and that the treatment of HEK293 cells with nocodazole, which triggers microtubule depolymerization, severely reduces SOCE. Later, Sampieri et al. reported that STIM1 travels through the ER in association with EB1 under resting conditions, confirming the connection between STIM1 and microtubules, and that the association ceases upon depletion of the ER, facilitating the aggregation of STIM1 into large clusters. Those authors also observed that STIM1 re-associates with EB1 when intracellular calcium stores are replenished, i.e., STIM1-EB1 dissociation is fully reversible. Because STIM1 binds to EB1 in the resting state, STIM1 can be considered a microtubule plus-end-tracking protein (+TIP). There are two modes of direct interaction between EB1 and +TIPs - either through a cytoskeleton-associated protein glycine-rich (CAP-Gly) domain or by binding to a Ser/Thr-x-Ile-Pro (S/TxIP) consensus sequence. As a +TIP, STIM1 localization is dependent on microtubule formation, and a STIM1 sequence encompassing residues 642–645 (Thr-Arg-Ile-Pro) has been found to be essential for the binding to EB1. For those +TIPs with an S/TxIP motif, examples of regulation by phosphorylation in the vicinity of this domain, but not within this sequence, have been reported. These examples include APC, MCAK, and CLASP2, all of which are phosphorylated in the vicinity of the S/TxIP sequence, regulating their interaction with microtubules. In all cases, as with STIM1, sequences flanking the S/TxIP motif contain a high number of proline, serine, and basic residues, leading to a net positive charge in the surroundings of this EB1 binding domain. This observation might explain why phosphorylation in the vicinity of this sequence blocks the localization of +TIPs to microtubule ends. In this regard, the phosphorylation of STIM1 at residues Ser575, Ser608, and Ser621 triggers the dissociation of STIM1 from EB1. Because refilling of Ca$^{2+}$ stores is accompanied by dephosphorylation of these residues, this reversible phosphorylation constitutes a mechanism that fully explains the reversible interaction of STIM1 and EB1 (see Fig. 1). Indeed, Ser-to-Ala substitution mutants of STIM1 do not dissociate from EB1 under store depletion conditions, and Ser-to-Glu mutants remain dissociated from EB1 even under resting conditions. In accordance with the proposed model described in Figure 1, those stimuli that induce store depletion and ERK1/2 activation lead to phosphorylation of STIM1 at Ser575, Ser608, and Ser621. This specific phosphorylation triggers the dissociation of STIM1 from EB1, which in turn facilitates STIM1 clustering and the binding to SOCs to activate Ca$^{2+}$ entry. Replenishment of Ca$^{2+}$ stores induces STIM1 dephosphorylation by as yet to be described phosphatases, leading to the reassociation of STIM1 with EB1, and the microtubule localization pattern of STIM1 (Fig. 1).

As has been reported for other +TIPs, the phospho-regulation of STIM1-EB1 interaction could be more complex, and other phosphoresidues might trigger a diversity of actions. For instance, the Ser-to-Ala substitution mutation of 10 residues in STIM1 leads to ER mislocalization by pulling ER tubules into the mitotic spindle. Because in wild-type STIM1-expressing cells ER tubules are excluded from this mitotic spindle, those results suggest that hyperphosphorylation of STIM1 is required to maintain ER normal structure in mitosis. However, it would be necessary to monitor the phosphorylation state of individual residues.

**Figure 1. Mechanism for the phosho-regulation of STIM1-EB1 interaction, proposed in reference 42.**
to discover which, if not all, of the residues are involved in this specific control of localization.

Finally, other phosphorylation sites at Tyr residues could be important for the initial steps of SOCE activation, because an increase of total phosphorylated Tyr has been detected in STIM1 from platelets under store depletion conditions, and the inhibition of this phosphorylation decreases the binding of STIM1 to ORAI1 and therefore SOCE.64 However, the identification of the specific modified residues and the molecular mechanism underlying this regulation remains to be clarified.

With the recent studies of the role of STIM1 phosphorylation on cell physiology, one has just started to understand the physiological relevance of this modulation. In addition to HEK293 cells, STIM1 phosphorylation, enhanced by thapsigargin, has been reported in neonatal rat ventricular myocytes.62 Also, it is known that phosphorylation of STIM1 at Ser575, one of the target sites of the ERK1/2 activity, promotes the differentiation of cultured mouse C2C12 myoblasts to myocytes.63 In parallel, it has been shown that STIM1 is necessary and sufficient for cardiomyocyte hypertrophy in vitro and in the adult heart in vivo.64,65 Taken together, these recent results indicate that the activity of STIM1 as a Ca\(^{2+}\) channels regulator is important for skeletal and cardiac muscle cells development, and that STIM1 phosphorylation constitutes a potential pharmacological target for the treatment of muscle hypertrophy.

The MEK-ERK pathway was early suggested as being involved in the activation of SOCE in human platelets, probably as a downstream effector of Ras proteins,66 and it has recently been demonstrated that constitutive dephosphorylation of STIM1 at ERK1/2 target sites impairs platelet adhesion to fibrinogen,67 supporting a physiological role for STIM1 phosphorylation by this signaling pathway.

In conclusion, phosphorylation regulates the activation of STIM1. However, the phosphorylation of different target residues has diverse effects. Because a differential phosphorylation profile can trigger opposing actions, the phosphorylation state of target residues needs to be defined under different stimuli so that one can establish molecular models capable of predicting STIM1 activities, particularly under pathophysiological conditions. In this scenario, phosphorylation target residues could be investigated as potential pharmacological targets for the treatment of diseases, since this would not alter upstream signaling cascades, and would therefore limit the intensity of side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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