STIM1L is a new actin-binding splice variant involved in fast repetitive Ca\(^{2+}\) release

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**Abstract**

Cytosolic Ca\(^{2+}\) signals encoded by repetitive Ca\(^{2+}\) release rely on two processes to refill Ca\(^{2+}\) stores: Ca\(^{2+}\) reuptake from the cytosol and activation of a Ca\(^{2+}\) influx via store-operated Ca\(^{2+}\) entry (SOCE). However, SOCE activation is a slow process. It is delayed by >30 s after store depletion because stromal interaction molecule 1 (STIM1), the Ca\(^{2+}\) sensor of the intracellular stores, must form clusters and migrate to the membrane before being able to open Orai1, the plasma membrane Ca\(^{2+}\) channel. In this paper, we identify a new protein, STIM1L, that colocalizes with Orai1 Ca\(^{2+}\) channels and interacts with actin to form permanent clusters. This property allowed the immediate activation of SOCE, a characteristic required for generating repetitive Ca\(^{2+}\) signals with frequencies within seconds such as those frequently observed in excitable cells. STIM1L was expressed in several mammalian tissues, suggesting that many cell types rely on this Ca\(^{2+}\) sensor for their Ca\(^{2+}\) homeostasis and intracellular signaling.

**Introduction**

Ca\(^{2+}\) signals are involved in many cell processes from early cell differentiation to cell death. They are encoded in frequency, amplitude, duration, and localization of cytosolic Ca\(^{2+}\) transient increases (Berridge et al., 2003). Two Ca\(^{2+}\) sources enable these signals: influxes from extracellular fluids and releases from internal stores. These two sources are interdependent, as the Ca\(^{2+}\) content of the stores is regulated by an influx from the extracellular environment by a process termed store-operated Ca\(^{2+}\) entry (SOCE).

Cellular events connecting Ca\(^{2+}\) store depletion to SOCE activation have been clarified recently with the identification of two major SOCE components: the stromal interaction molecules (STIMs) STIM1 and STIM2 and the Orai family of channels Orai1–3. STIMs are single-pass transmembrane ER Ca\(^{2+}\) sensors that activate SOCE upon Ca\(^{2+}\) store depletion (Liou et al., 2005; Roos et al., 2005; Brandman et al., 2007; Wang et al., 2009; Zhou et al., 2009). When Ca\(^{2+}\) decreases in the stores, STIMs slowly redistribute into clusters where they colocalize with Orai channels to form elementary subunits of SOCE (Liou et al., 2005; Luik et al., 2006; Mercer et al., 2006; Treves et al., 2010). Recent publications describe specific sequences of STIMs involved in oligomerization, cluster formation, and Orai activation (Liou et al., 2005; Baba et al., 2006; Zeng et al., 2008; Fahrner et al., 2009; Muik et al., 2009; Park et al., 2009; Yuan et al., 2009).

In mammalian skeletal muscle cells, single-twitch contractions are controlled by ER Ca\(^{2+}\) releases without noticeable contribution of Ca\(^{2+}\) influxes (Lamb, 2000; Launikonis et al., 2010). However, during repetitive twitches, SOCE is required, thus providing an excellent model for studying Ca\(^{2+}\) store regulation during repetitive Ca\(^{2+}\) releases (Stiber et al., 2008; Darbellay et al., 2010). Accordingly, STIM1-deficient human patients and mice present an abnormal muscular fatigability (Stiber et al., 2008; Feske, 2009, 2010; Picard et al., 2009; Feske et al., 2010). STIM1 has been shown to be permanently localized within the triad junction of muscle fibers (Stiber et al., 2008). This may be part of the reason for the fast SOCE activation in muscle (<1 s) when compared with that of other cell types (~30 s to >1 min; Parekh and Putney, 2005; Wu et al., 2006; Launikonis and Rios, 2007; Liou et al., 2007; Navarro-Borelly et al., 2008; Stiber et al., 2008; Calloway et al., 2009;
A new 115-kD STIM1 named STIM1L (Fig. 1A). STIM1L was detected in all tested skeletal muscles (gluteus, vastus lateralis, gastrocnemius, diaphragm, and tongue) and in the three tissues belonging to the central nervous system (brain, cerebellum, and brain stem). STIM1L was also detected in the spleen, lungs, liver, and heart but not in the kidney. Note that, as previously described (Berna-Errro et al., 2009), STIM1S is ubiquitously expressed in mouse tissues. Because STIM1L is highly expressed in all skeletal muscles (and not in T lymphocytes; Fig. 1A), we decided to use cultured multinucleated myotubes to decipher its molecular and functional characteristics.

We first confirmed that STIM1L is expressed in adult human muscle fibers and in in vitro–differentiated myotubes. The time course of STIM1 expression during the first 52 h of myotube differentiation is illustrated in Fig. 1B. STIM1L appeared during the second day of differentiation and is abundantly expressed in adult human semitendinous muscle. The two 90- and 115-kD proteins were recognized by three different anti-STIM1 antibodies and were both silenced by different siRNAs directed against STIM1 (siSTIM1; Figs. 1C [left] and S1A). Western blot quantification showed that siSTIM1 reduced the expression of the 90-kD protein by 88 ± 6% and that of the 115-kD protein by 90 ± 10% (n = 3, and P < 10⁻⁶ for both conditions; Fig. 1B, right). Collectively, these results identify STIM1L as a new member of the STIM1 protein family.

STIM1L results from an alternative splicing on exon 11

We performed an RNA ligase–mediated rapid amplification of 5' and 3' cDNA ends (RACE) followed by PCR amplification.
using STIM1-specific primers (see Materials and methods) to evaluate any possible alternative splicing on STIM1 mRNA. Only one band of the expected size (1.5 kb) was revealed by 5’ end amplification of mRNA from differentiating myotubes (Fig. S1 B, left). On the other hand, 3’ end amplification identified one band in mRNA from myoblasts but two bands in myotubes (Figs. 2 A and S1 B). Sequencing of both bands confirmed the specific amplification of STIM1 cDNA and identified a new sequence inserted between exons 11 and 12 of the known stim1 human gene (available from GenBank/EMBL/DDBJ under accession no. NG_016277). This 318-bp segment aligned in silico with a sequence immediately after exon 11 in the stim1 human gene. From these results, we conclude that during human myoblast differentiation, an alternative splicing occurs on exon 11 of STIM1, producing a new STIM1 protein characterized by a longer exon 11 encoding a longer cytosolic C-terminal tail (Fig. 2, B and C). Fig. 2 D shows the amino acid sequence of the additional segment displaying threonine- and serine-rich domains (the nucleotide sequence is shown in Fig. S1 C; available from GenBank under accession no. HQ317451). The sequence is highly conserved in mammalians, suggesting that it is involved in essential functions (Fig. S2 A). Additionally, we confirmed these findings by performing PCR using primers targeting exons 10 and 12 of STIM1 (Fig. S2 B).

**STIM1L activates Orai1 upon store depletion and is involved in endogenous SOCE**

To evaluate the role of STIM1L during thapsigargin (Tg)-induced SOCE, we overexpressed STIM1L in STIM1S-silenced myoblasts. Cells were cotransfected with a plasmid encoding the ORF of STIM1L and an siRNA targeting 3’ untranslated noncoding sequences of STIM1 mRNA (siSTIM1L). 2 d after transfection, Ca2+ handling was assessed using the fluorescent Ca2+ indicator Fura-2. As shown in Fig. 3 A, STIM1S silencing decreased the amplitude of SOCE evoked by readdition of 1.8 mM Ca2+ after depletion of the Ca2+ stores with 1 µM Tg (red curve). Overexpression of STIM1L in STIM1S-silenced myoblasts more than compensated for the depletion of STIM1S and caused a 312 ± 20% increase of the Tg-induced SOCE when compared with control myoblasts (Fig. 3 A, green curve vs. red curve). Western blots were performed on extracts obtained from the cells used for Fura-2 measurements to correlate as accurately as possible the protein levels with the Ca2+ influxes shown in Fig. 3 A (Fig. 3 B). As shown, proliferating myoblasts only express STIM1S, and STIM1S is silenced by 74 ± 7% (n = 3) 48 h after siSTIM1L transfection. As expected, in myoblasts transfected with the STIM1L plasmid and siSTIM1L, the 115-kD band was strongly expressed, whereas the amount of STIM1S decreased by 70 ± 11% (n = 3). From these results, we conclude that STIM1L is able to generate SOCE independently of the well-characterized SOCE partner STIM1S.

The additional sequences present on STIM1L are located near regions known to interact with Orai1 (Fig. 2). We thus wondered whether Orai1 channels would still be the main partner of STIM1L. To test this point, we quantified SOCE in

![Figure 2](image-url)

**Figure 2.** STIM1L alternative splicing. (A) Identification of an alternative splicing variant of STIM1 mRNA by RACE. Products of the 3’ RACE before amplification by nested PCR are shown. 3’ RACE of STIM1 mRNA was performed in proliferating myoblasts and in myotubes after 120 h of differentiation. One band of the expected size of ~2.6 kbp was detected in myoblasts and myotubes, and an additional band of ~2.9 kbp was detected in myotubes [also see Fig. S1 B]. (B) Exon/intron map of the human STIM1 gene (available from GenBank/EMBL/DDBJ under accession no. NG_016277). This 318-bp segment aligned in silico with the specific amplification of STIM1 cDNA and identified a new splicing variant of STIM1 mRNA by RACE. Products of the 3’ RACE before amplification by nested PCR are shown. 3’ RACE of STIM1 mRNA was performed in proliferating myoblasts and in myotubes after 120 h of differentiation. One band of the expected size of ~2.6 kbp was detected in myoblasts and myotubes, and an additional band of ~2.9 kbp was detected in myotubes [also see Fig. S1 B]. (C) A schematic of the proteins STIM1S and -L. ABD, actin-binding domain; CAD, Ca2+ release–activated Ca2+ channel activation domain; CC1, coiled coil 1; CC2, coiled coil 2; K, leucine-rich domain; SAM, sterile 

**STIM1L-oversexpressing myoblasts deprived of endogenous STIM1S and treated with an siRNA directed against Orai1 (siOrai1).** Fig. 3 C shows that most of the gain of function conferred by the overexpression of STIM1L in STIM1S-silenced myoblasts was reversed by Orai1 silencing (compare blue and cyan curves). This result identifies Orai1 as the main partner of STIM1L. However, as the residual SOCE in Orai1-silenced myoblasts overexpressing STIM1L (cyan curve) is approximately sevenfold larger than that observed in Orai1-silenced myoblasts not overexpressing STIM1L (green curve), our results do not exclude that STIM1L may also activate Ca2+ channels other than Orai1. The Western blot in Fig. 3 D illustrates Orai1 silencing efficiency. A 65 ± 5% decrease of Orai1 expression was observed in myoblasts transfected with siOrai1 (n = 3).

To assess the endogenous function of STIM1L, we used differentiated skeletal muscle cells (myotubes), as these cells strongly express both STIM1 variants (Fig. 1 A). To silence STIM1L, we designed three siRNAs that targeted sequences specific to the new spliced fragment of exon 11. As shown in Fig. 3 E, the three siRNAs efficiently silenced STIM1L (90 ± 6, 91 ± 7, and 91 ± 5% inhibition for siSTIM1L-1, -2, and -3, respectively) without significantly affecting STIM1S. Fig. 3 F illustrates that Tg-induced SOCE amplitude was reduced by 56 ± 9% in STIM1L-silenced myotubes (n = 4 and P < 0.003; siSTIM1L-1 was used in this and all following experiments).
Figure 3. **STIM1L activates Orai1 and is involved in endogenous SOCE.** (A, left) Cytoplasmic Ca\(^{2+}\) was assessed with Fura-2 in proliferating myoblasts 2 d after siRNA transfection (siSTIM1L or control siRNA) and 1 d after plasmid electroporation [STIM1L or pcDNA3]. As siSTIM1L targets the 3' untranslated region of STIM1 mRNA, endogenous STIM1 was silenced, whereas STIM1 vector expression was unaffected. Intracellular Ca\(^{2+}\) stores were depleted using 1 µM Tg in a medium containing 250 nM Ca\(^{2+}\), and, subsequently, 1.8 mM Ca\(^{2+}\) was added to the external medium to reveal SOCE (traces represent the mean ± SEM of 10 cells; one representative experiment out of three that were performed in three different clones). (right) Mean ± SEM peak SOCE amplitude out of three experiments. Results were normalized to control peak SOCE. (B, left) STIM1 protein expression. The same myoblasts were used for the Western blot shown and for the cytoplasmic Ca\(^{2+}\) recordings illustrated in A. (right) Mean ± SD of three experiments. Results were normalized to control STIM1S expression and corrected for loading differences using α-tubulin (α-tub). (C) Same protocol as in A, except that STIM1L-YFP was used instead of STIM1L vector and EGFPN3 instead of pcDNA3 (control plasmid). Error bars represent mean ± SEM. (D) Orai1 protein expression. The same myoblasts were used for the Western blot shown and for the cytoplasmic Ca\(^{2+}\) recordings illustrated in C. (right) Mean ± SD of three experiments. Results were normalized to control Orai1 expression and corrected for loading differences using α-tubulin. Orai1 silencing efficiency was similar in myoblasts expressing either EGFPN3 control plasmid or STIM1L-YFP plasmid (not depicted). (E, left) STIM1 expression in myotubes 96 h in differentiation medium and transfected with siRNAs (siSTIM1L-1, -2, and -3) that target STIM1-specific sequences. (right) Mean ± SD of STIM1L expression out of three experiments. Results were normalized to control STIM1 expression and corrected for loading differences using α-tubulin. (F, left) Cytoplasmic Ca\(^{2+}\) assessed with Fura-2 in myotubes...
To specifically silence STIM1S, we designed and tested three siRNAs that overlapped the junction between exon 11 and 12 of STIM1 mRNA. As this sequence is absent in STIM1L mRNA, these siRNAs should silence STIM1S without modifying STIM1L expression. Two siRNAs silenced STIM1S efficiently (73 ± 5 and 66 ± 4% inhibition for siSTIM1S-1 and -2, respectively), whereas one had no effect. STIM1L expression was only slightly reduced (17 ± 3 and 19 ± 4% inhibition for siSTIM1S-1 and siSTIM1S-2, respectively) by both siRNAs that were efficiently silencing STIM1S. Fig. 3 (G and H) illustrates the effects of siSTIM1S-1 (the siSTIM1S that was used in all subsequent experiments) on STIM1S expression and on Tg-induced SOCE. SOCE was reduced by 47 ± 12% in STIM1S-silenced myotubes. Note that, as formerly described, STIM1 was silenced without impeding differentiation by triggering myoblast differentiation immediately after siSTIM1S transfection (Darbellay et al., 2009, 2010). As the degree of store depletion may determine the amplitude of SOCE, we compared maximum amplitude (and also time to peak) of myoplasmic Ca\(^{2+}\) increases upon Tg addition in all conditions described in Fig. 3. No statistical difference was noted between the various conditions. Lastly, being able to specifically silence STIM1S allowed us to confirm that endogenous Orai1 is the main partner of endogenous STIM1L (Fig. S3 A).

Repetitive Ca\(^{2+}\) releases rely on the immediate activation of the SOCE triggered by STIM1L

Experiments described in Fig. 3 (F and H) show that STIM1L and STIM1S contributions to Tg-induced SOCE amplitude were similar. As muscle cells are known to perform high frequency, high amplitude, and high duration cytosolic Ca\(^{2+}\) increases relying on release from the internal stores (Miledi et al., 1977, 1982; Gorassini et al., 2000), we compared the respective role of STIM1L and STIM1S during repetitive Ca\(^{2+}\) releases in myotubes. To simulate the excitation/contraction coupling mechanism normally occurring during skeletal muscle contraction, we increased the K\(^+\) concentration of the superfusion solution (Stiber et al., 2008; Darbellay et al., 2010). Each extracellular 65-mM KCl pulse generated large cytosolic Ca\(^{2+}\) transients that were visualized with Fura-2. Fig. 4 A illustrates that control myotubes exposed to seven consecutive pulses of 65 mM KCl were able, after an initial decrement, to perform six similar cytosolic Ca\(^{2+}\) peaks. In contrast, STIM1L-silenced myotubes were unable to sustain successive Ca\(^{2+}\) peaks (Fig. 4, B and D). After the seven KCl pulses, internal Ca\(^{2+}\) store content was assessed using 2 μM Tg and 10 mM caffeine in 250 nM of external Ca\(^{2+}\). Compared with control myotubes (Fig. 4 A), Ca\(^{2+}\) store content was reduced by 87 ± 6% in STIM1L-silenced myotubes (P < 10\(^{-3}\); Fig. 4 B). Finally, STIM1S silencing inhibited neither KCl-induced Ca\(^{2+}\) releases nor Ca\(^{2+}\) store content, although the mean Ca\(^{2+}\) peak in STIM1S-silenced myotubes was slightly reduced (Fura-2 ratio was 1.05 ± 0.05 in STIM1S-silenced myotubes vs. 1.23 ± 0.09 in control myotubes; P < 10\(^{-3}\); Fig. 4, C and D). From these results, we concluded that STIM1S-dependent SOCE is too slow to compensate for the Ca\(^{2+}\) extrusion occurring during repetitive cytosolic Ca\(^{2+}\) increases. Indeed, in the absence of STIM1L, few releases lasting 15 s were sufficient to totally empty internal Ca\(^{2+}\) stores.

To test whether STIM1L could be responsible for a rapid SOCE activation that would without delay compensate Ca\(^{2+}\) extrusion, the onset of SOCE was evaluated by assessing Mn\(^{2+}\) quench of Fura-2 (Hoth and Penner, 1992; Fasolato et al., 1993). Mn\(^{2+}\) enters cells through Ca\(^{2+}\) channels and quenches Fura-2 fluorescence. The decrease of Fura-2 emission at 360 nm is thus directly linked to the Ca\(^{2+}\) influx. Fig. 4 E (top) illustrates ER Ca\(^{2+}\) release triggered by Tg and caffeine in the presence of 0.5 mM Mn\(^{2+}\). Fig. 4 E (bottom) represents, in the same cells, Fura-2 quench by Mn\(^{2+}\) entering through Ca\(^{2+}\) channels activated by the ER depletion. As shown, in the presence of STIM1L (myotubes), Fura-2 emission at 360 nm strongly decreased immediately after Tg and caffeine addition, whereas in the absence of STIM1L (myoblasts), a similar decrease occurred but was delayed by ~2 min. In addition, Ca\(^{2+}\) influx activation was also delayed in STIM1L-silenced myotubes (Fig. 4 E, red curve). However, this delay did not occur in STIM1S-silenced myotubes (Fig. 4 E, green curve).

The bar graph (Fig. 4 F) illustrates that, in the presence of STIM1L (control myotubes), 47 ± 6% of the maximum slope of the Fura-2 quench trace was induced immediately after store depletion, whereas this fraction dropped to 14 ± 2% in STIM1L-silenced myotubes (STIM1S present only) and to 11 ± 5% in control myoblasts (STIM1S present only). As expected, in STIM1L-silenced myotubes (STIM1L present only), the fraction of the maximum slope induced immediately was large (83 ± 21%). Fig. S3 B shows that double silencing of STIM1L and STIM1S totally inhibited the influx triggered by the Tg and caffeine, confirming that this influx was purely STIM1 dependent. Results from experiments in which STIM1L is exogenously expressed in three cell types that do not have endogenous STIM1L are in full agreement with the previous findings (shown in Fig. 4, E and F). Fig. 4 (G and H) illustrates that exogenous STIM1L-YFP expression in myoblasts, a human umbilical vein endothelial cell–derived cell line (EA.hy926), and HeLa cells allows immediate SOCE activation after Tg. These results have to be compared with STIM1S-YFP expression that, in the same cells, does not allow immediate SOCE activation. Fluorescence resonance energy transfer (FRET) increase between STIM1S-CFP
and -YFP after stimulation with 2 μM Tg and 10 mM caffeine had a t1/2 of 20 ± 3 s (Fig. S3 C), which excludes that the delay of SOCE activation in STIM1S-expressing cells was caused by a slow action of Tg and caffeine. From these results, we conclude that fast SOCE activation in myotubes depends on STIM1L expression and that fast SOCE activation is required to perform repetitive store-dependent Ca2+ signals.

**STIM1L interacts with actin to form permanent clusters**

The delay that characterized the activation of the STIM1S-dependent SOCE is linked to the process of STIM1S migration and cluster formation that precedes the activation of Orai1 channels (Liou et al., 2007). We thus wondered whether STIM1L clusters could form faster (or using a different mechanism) than STIM1S clusters. To examine this hypothesis, we overexpressed STIM1L- and STIM1S-YFP into myoblasts and compared their cellular distribution before and after Tg-induced store depletion. As expected, STIM1S was diffusely distributed in resting myoblasts with filled Ca2+ stores and in myotubes 48 h after transfections with various STIM1L. Ca2+ stores were depleted with 10 mM caffeine and 2 μM Tg in 1.8 mM of external Ca2+ and 0.5 mM of external Mn2+. (bottom) Fura-2 fluorescence at a 360-nm excitation. Cytoplasmic Ca2+ and Fura-2 fluorescence quench were measured simultaneously. No statistical difference was noted between maximum amplitude (and also time to peak) of myoplasmic Ca2+ increases upon Tg/caffeine addition. a.u., arbitrary unit.

(F) Fraction of the maximum slope of Fura-2 quench traces that occurred immediately after Ca2+ store depletion with 2 μM Tg and 10 mM caffeine. Immediate activation slopes were measured between times a and b and maximum slopes between times b and c in E. Mean ± SEM of six experiments using 0.5–1 mM Mn2+. (13 cells were recorded in each experiment; also see Fig. S3 B). (G and H) Fura-2 fluorescence quench at 360 nm measured in myoblasts, Hela cells, and EA.hy926 (human endothelial) cells with 1 mM Mn2+ in the external medium. For HeLa and EA.hy926 cells, Ca2+-stores were depleted with 2 μM Tg in 1.8 mM of external Ca2+. The squares represent the mean of six to eight cells out of three to four experiments. The thin lines represent individual cell traces, n.s., not significant. The vertical and horizontal dashed lines represent the application of Tg ([caffeine] at time 0 and initial Fura-2 fluorescence at 360 nm, respectively.

As STIM1L forms linear clusters, we assessed a possible involvement of actin filaments in the anchoring and permanent clustering of STIM1L. Figs. 5 C and S4 C show that STIM1L exons 11 and 12 formed linear clusters in all observed cells (>100 cells in eight experiments). The level of Ca2+ in stores had no influence on these results, as these small constructs lack the Ca2+ sensor (unpublished data). Fig. S4 (A and B) shows that GFP-tagged exons 11 and 12 of both STIM1L and STIM1S can be expressed in myoblasts and that these fragments have a dominant-negative effect on Tg-induced SOCE amplitude.

As STIM1L forms linear clusters, we assessed a possible involvement of actin filaments in the anchoring and permanent clustering of STIM1L. Figs. 5 C and S4 C show that STIM1L exons 11 and 12–GFP and STIM1L-GFP formed linear clusters that perfectly colocalized with actin filaments in unstimulated myoblasts. In contrast, STIM1S clusters did not colocalize with actin filaments (Fig. 5 D). The distinct patterns of Fig. 5 (C and D) suggest that STIM1S and STIM1L clusters did not colocalize after store depletion. However, overexpression of STIM1S-mCherry and STIM1L-GFP in the same cells shows a little overlap of STIM1S/L clusters, suggesting that a fraction of
STIM1S may be recruited in STIM1L clusters after store depletion (unpublished data). Treatment with 1 µM cytochalasin D (3 h at room temperature) largely disrupted the actin organization in 60% of the myoblasts, and, in these cells, the linear organization of STIM1L exons 11 and 12–GFP disappeared, although STIM1L exons 11 and 12–GFP and disrupted fragments of actin still colocalized perfectly (Fig. 5 E). A similar result was obtained in STIM1L-expressing cells using either 1 µM cytochalasin D or 1 µM latrunculin B, and a partial effect was obtained using 0.1 and 0.5 µM cytochalasin D (Figs. S4 C and S5 A). We also verified that STIM1S clusters still formed and remained stable in actin-disrupted cells after Tg-induced Ca^{2+} store depletion (unpublished data). As shown in Fig. 5 F, endogenous STIM1L also forms linear clusters that colocalize with actin in STIM1S-silenced myotubes. In addition, Fig. 5 G shows that FRET occurs between actin-phalloidin-TRITC and STIM1L-GFP (detected 5 min after treatment with 2 µM Tg (D)), and distribution of actin (detected by phallolidin—orbit) and STIM1S exons 11 and 12–GFP in myoblasts treated with 1 µM cytochalasin D for 3 h at room temperature (E). Bars, 20 µm. (B) Distribution of endogenous STIM1L (detected with an antibody against both STIM1 isoforms) and actin (detected by phallolidin–Atto 390) in STIM1S-silenced myotubes. Bar, 5 µm. (B–F) One representative cell out of >40 cells in three experiments. (G) Acceptor photobleaching method to measure FRET between actin (detected by phallolidin–TRITC) and STIM1L-GFP in fixed myoblasts 24 h after electroporation. The blue and red circles indicate regions used for GFP fluorescence measurements, and white squares indicate 561-nm bleached regions. The bar graph illustrates the mean ± SD of GFP fluorescence increases when acceptor (actin-phallolidin–TRITC) is bleached. Measurements were normalized to GFP fluorescence variation in control regions (fluorescence ratio of red regions [2:1] was divided by the fluorescence ratio of blue regions [2:1]). Bar, 10 µm. (H) Western blot illustrating coimmunoprecipitation (IP) of endogenous STIM1L using an actin antibody. One representative experiment out of three. All images are shown in pseudocolors.

Figure 5. STIM1L interacts with actin and forms permanent clusters that colocalize with Orai1 in store-filled cells. (A) Distribution of STIM1S-YFP and STIM1L-YFP overexpressed in myoblasts before and after treatment with 2 µM Tg. The bar graph represents the total surface area of clusters in the various conditions. Clusters were detected with an automatic detection of fluorescence intensity variations called “by edges” (mean ± SD and n = 5 experiments; MetaMorph 7.5.6; Molecular Devices). Bar, 20 µm. (B) Distribution of STIM1S exons 11 and 12–GFP and STIM1L exons 11 and 12–GFP in store-emptied cells using 2 µM Tg. Red rectangles show enlarged images. Bar, 20 µm. (C–E) Distribution and colocalization of actin (detected by phallolidin–TRITC) and STIM1L detected by phallolidin–TRITC and STIM1L-YFP 5 min after treatment with 2 µM Tg (D), and distribution of actin (detected by phallolidin–TRITC) and STIM1L exons 11 and 12–GFP in myoblasts treated with 1 µM cytochalasin D for 3 h at room temperature (E). Bars, 20 µm. (F) Distribution of endogenous STIM1L (detected with an antibody against both STIM1 isoforms) and actin (detected by phallolidin–TRITC) and STIM1L-GFP 5 min after treatment with 2 µM Tg (D), and distribution of actin (detected by phallolidin–TRITC) and STIM1L exons 11 and 12–GFP in myoblasts treated with 1 µM cytochalasin D for 3 h at room temperature (E). Bars, 20 µm. (B–F) One representative cell out of >40 cells in three experiments. (G) Acceptor photobleaching method to measure FRET between actin (detected by phallolidin–TRITC) and STIM1L-GFP in fixed myoblasts 24 h after electroporation. The blue and red circles indicate regions used for GFP fluorescence measurements, and white squares indicate 561-nm bleached regions. The bar graph illustrates the mean ± SD of GFP fluorescence increases when acceptor (actin-phallolidin–TRITC) is bleached. Measurements were normalized to GFP fluorescence variation in control regions (fluorescence ratio of red regions [2:1] was divided by the fluorescence ratio of blue regions [2:1]). Bar, 10 µm. (H) Western blot illustrating coimmunoprecipitation (IP) of endogenous STIM1L using an actin antibody. One representative experiment out of three. All images are shown in pseudocolors.
Figure 6. Actin fiber disruption dissociates STIM1L-Orai1 preformed complexes and delays SOCE activation. (A) Distribution and colocalization of STIM1L-GFP, Orai1-RFP, and actin in resting myoblasts (filled Ca$^{2+}$ stores). Actin was detected with phalloidin–Atto 390. One representative cell out of >30 cells in three experiments. The red box outlines the enlarged image shown on the bottom. Bar, 5 µm. (B) Acceptor photobleaching method to measure FRET between Orai1-RFP and either STIM1L or STIM1S-GFP in fixed myoblasts 24 h after electroporation. The bar graph shows the mean ± SD, and the horizontal dashed line represents the control FRET level. FRET fluorescence increases when acceptor (Orai1-GFP) is bleached (n = 8 for each condition). Analysis was performed as described in Fig. 5. G. The blue and red circles indicate regions used for GFP fluorescence measurements, and white squares indicate 561-nm bleached regions. Bar, 5 µm. (C) Cytoplasmic Ca$^{2+}$ assessed with Fura-2 to evaluate maximum SOCE in control and STIM1S-silenced myotubes in the absence or presence of 1 µM cytochalasin D. Ca$^{2+}$ stores were depleted with 2 µM Tg and 10 mM caffeine in 250 nM of external Ca$^{2+}$; and, subsequently, 1.8 mM Ca$^{2+}$ was added to the external medium to reveal SOCE (traces represent the mean ± SEM of 76 cells; n = 3 experiments in three clones). (D) Fura-2 fluorescence quench at 360 nm measured in myotubes (with 1 mM Mn$^{2+}$ in the external medium) in the absence or presence of 0.5 and 1 µM cytochalasin D. Ca$^{2+}$ stores were depleted with 2 µM Tg and 10 mM caffeine in 1.8 mM of external Ca$^{2+}$ (mean ± SEM of 16 cells; n = 3 experiments). (E) Same experiment as in D, except that myoblasts overexpressing STIM1L-YFP and Orai1-RFP were used instead of myotubes and that actin depolymerization was obtained using 1 µM latrunculin instead of cytochalasin D (mean ± SEM of 13 cells; n = 3 experiments). (F) Myoblasts at 24 h after electroporation with STIM1L-Δ589–599-GFP before and after treatment with 2 µM Tg for 1.5 min (one representative experiment out of three). Bar, 5 µm. (G) Fura-2 fluorescence quench at 360 nm measured in STIM1S-silenced myoblasts (with 1 mM Mn$^{2+}$ in the external medium and 48 h after siSTIM1 transfection) expressing either STIM1L-Δ589–599-GFP or STIM1LGFP. Ca$^{2+}$ stores were depleted with 2 µM Tg and 10 mM caffeine in 1.8 mM of external Ca$^{2+}$ (mean ± SEM of 12 cells; n = 5 experiments). (D, E, and G) Vertical dashed lines represent the application of Tg and caffeine at time 0, and horizontal dashed lines represent the initial Fura-2 fluorescence at 360 nm. (H) SOCE activation by STIM1L and S. In resting [store filled] cells, STIM1L is already associated with Orai1 and forms permanent clusters via its interaction with actin filaments. Upon store depletion, STIM1L triggers SOCE without delay. STIM1L-dependent SOCE requires homomerization, migration, and cluster formation of STIM1S molecules, a process that needs ~2 min to take place. This second process, which occurs during a long-lasting decrease of the Ca$^{2+}$ store content, results in a global increase of SOCE amplitude. All images are shown in pseudocolors. ABD, actin-binding domain of STIM1L; a.u., arbitrary unit.

These two molecules are located in close proximity (Fig. 6 B, left images and right blue bars) and that this close proximity is disrupted by 1 µM cytochalasin D (Fig. 6 B, red bars). The absence of FRET between STIM1S-GFP and Orai1-RFP in store-filled myoblasts was assessed as a control (Fig. 6 B, green bars).

Finally, we assessed the impact on SOCE of actin disruption and transfection of an STIM1L mutant missing amino acids 589–599 (STIM1L-Δ589–599-GFP), a mutant that did not form permanent clusters in store-filled cells. Fig. 6 C shows that actin disruption with 1 µM cytochalasin D had no effect on endogenous maximal SOCE amplitude in normal or STIM1S-silenced myotubes. This result suggests that neither STIM1L nor STIM1S relies on actin integrity to control maximal SOCE amplitude. However, and as expected, endogenous SOCE activation was clearly delayed in myotubes after actin depolymerization with 1 and 0.5 µM cytochalasin D (Fig. 6 D). Accordingly, the acceleration of SOCE activation conferred by STIM1L overexpression in myoblasts (Fig. 4 G) was reversed by actin disruption (Fig. 6 E). Fig. 6 F shows the distribution in myoblasts of STIM1L-Δ589–599-GFP, a mutant missing an amino acid sequence located within the actin-binding domain. Neither clusters nor lines were observed in store-filled myoblasts transfected with this mutant. Fig. 6 F also shows that, although store depletion with Tg still induced cluster formation, these clusters did not colocalize with actin fibers. Lastly, Fig. 6 G illustrates that Mn$^{2+}$ quench of the Fura-2 fluorescence was severely delayed in STIM1S-silenced myoblasts expressing STIM1L-Δ589–599-GFP, an expected result in the absence of permanent STIM1L clusters in store-filled cells. From these results, we conclude that actin fibers stabilize inactivated STIM1L–Orai1 complexes in naive store-filled cells and, by this mechanism, allow fast SOCE activation upon Ca$^{2+}$ store depletion in STIM1L-expressing cells.
Discussion

Here, we identified a longer form of the STIM1 protein, STIM1L, which allows immediate activation of SOCE and is required to trigger repetitive cytosolic Ca\(^{2+}\) releases. STIM1L is the product of an alternative splicing on exon 11 of the STIM1 gene and is expressed in many mammalian tissues. Live cell imaging showed that STIM1L–Orai1 clusters were already present at rest when Ca\(^{2+}\) stores were full. In contrast, STIM1S molecules were diffusely distributed at rest, and it took >1 min for STIM1S clusters to fully form after Ca\(^{2+}\) store depletion (Liou et al., 2007). As the main cause of the STIM1S-related SOCE activation delay appears to be the time required for STIM1S to migrate, form clusters, and bind to Orai1 (Liou et al., 2007; Navarro-Borelly et al., 2008; Calloway et al., 2009), the presence of permanent STIM1L–Orai1 clusters could explain the rapid SOCE activation in STIM1L-expressing cells. In myoblasts, STIM1S oligomerized within 10 s of Tg and caffeine application (Fig. S3 C), whereas SOCE activation was delayed >1 min (Fig. 4 G). A similar result was obtained in myotubes. These observations confirm that the delay characterizing STIM1S-related SOCE activation is a result of the overall process of STIM1S oligomerization, migration, and binding to Orai1. The kinetics of STIM1S oligomerization after store depletion (<10 s; Fig. S3 C) also suggests that a difference between STIM1S and STIM1L luminal Ca\(^{2+}\) affinity, if present, will not play a major role in the overall delay of STIM1S-dependent SOCE activation. Here, we also showed that the formation of permanent clusters is directly linked to the interaction of the added sequence of STIM1L with actin, as STIM1L-GFP, the GFP-tagged exons 11 and 12 of STIM1, and endogenous STIM1L form linear clusters that colocalize with actin and disappear upon actin depolymerization. In addition, endogenous STIM1L was coimmunoprecipitated by actin. Finally, we observed that an STIM1L mutant missing a 10–amino acid sequence within the actin-binding domain forms clusters only after store depletion, does not colocalize with actin, and triggers slow SOCE activation. Although these last results do not prove a direct binding between the 589–599 amino acid fragment and actin, they clearly suggest that this fragment is required for the STIM1L–actin interaction to take place.

In skeletal muscle fibers, SOCE has been shown to be maximally activated in <1 s after the initiation of the Ca\(^{2+}\) store release, whereas full SOCE activation takes several seconds (up to 260 s for complete cluster formation) in other cell types (Parekh and Putney, 2005; Wu et al., 2006; Launikonis and Ríos, 2007; Liou et al., 2007; Navarro-Borelly et al., 2008; Calloway et al., 2009). We confirmed that, in human myotubes, the immediate activation of SOCE relies on endogenous STIM1 and that exogenous expression of STIM1L triggers immediate SOCE activation regardless of the cell types, linking this specific feature of SOCE to STIM1L expression. As actin depolymerization disrupts STIM1L–Orai1 resting complexes and delays SOCE activation without decreasing maximal SOCE amplitude, we conclude that STIM1L–actin interaction stabilizes preformed SOCE complexes, which in turn trigger fast SOCE activation.

In STIM1L-silenced myotubes, repetitive Ca\(^{2+}\) release with a frequency faster than the time required to activate STIM1S-related SOCE rapidly emptied the Ca\(^{2+}\) content of the stores. Physiological muscle contractions involve repetitive bursts of tetanic stimulations that may last a few seconds (Miledi et al., 1977, 1982; Buchthal and Schmalbruch, 1980; Stein et al., 1988; Kiehn and Eken, 1997, 1998; Gorassini et al., 2000; Rekling et al., 2000). As cytosolic Ca\(^{2+}\) concentration remains elevated during most of the tetanic contraction, such a stimulation pattern produces a small but constant extrusion of Ca\(^{2+}\) in the extracellular space through the plasma membrane Ca\(^{2+}\) ATPase and Na\(^+\)-Ca\(^{2+}\) exchanger (Cifuentes et al., 2000; Caride et al., 2001). To compensate for this Ca\(^{2+}\) efflux, SOCE or other Ca\(^{2+}\) influxes need to be activated as soon as contraction begins. We thus propose that STIM1L may be involved in the rapid SOCE activation required to compensate for the Ca\(^{2+}\) efflux occurring during normal muscle contraction (Stiber et al., 2008). This model would explain well why skeletal muscle contraction requires functional SOCE (Kurebayashi and Ogawa, 2001; Zhao et al., 2005; Ducret et al., 2006; Lyfenko and Dirksen, 2008; Stiber et al., 2008; Dirksen, 2009; Darrellay et al., 2010; Edwards et al., 2010; Launikonis et al., 2010), even though SOCE amplitude would appear, a priori, as insufficient to significantly contribute to Ca\(^{2+}\) store refilling when compared with Ca\(^{2+}\) recycling by the sarco/ER Ca\(^{2+}\) ATPase. It would further explain why, in Stim1\(^{-/-}\) knockout mice, muscle weakness is only revealed during tetanic stimulations, whereas single-twitch contractions are normal (Stiber et al., 2008).

Based on the findings in the present and former studies (Miledi et al., 1982; Launikonis and Ríos, 2007; Stiber et al., 2008; Feske, 2009; Launikonis et al., 2009; McCarl et al., 2009; Darrellay et al., 2010; Edwards et al., 2010), we propose the model summarized in Fig. 6. The presence of a new STIM isoform, STIM1L, able to permanently cluster STIM1 molecules and thus activate SOCE immediately after store depletion, would allow any cell type to perform repetitive cytosolic Ca\(^{2+}\) releases with frequencies in seconds or faster.

Materials and methods

Cell culture

Muscle samples, cell dissociation, and clonal culture from satellite cells were prepared as previously described (Baroffio et al., 1993; Arnaudeau et al., 2006). In brief, muscle biopsies were minced and incubated for 1 h in a solution containing 0.5 mg/ml trypsin. The suspension was centrifuged and resuspended several times in a wash medium to remove muscular debris (Ham’s F10 with 15% FCS). Red blood cells were lysed with Tris–ammonium chloride buffer. Using a micropipette, single satellite cells were then manually collected (clonal culture). Human muscle samples were obtained from eight children (operated for clubfoot and <4 yr old) without any known neuromuscular disease. Plasmids (Orai1-RFP was provided by D. Al-Ansary and B. Niemeyer, Saarland University, Homburg, Germany; STIM1-YFP [19754]; Addgene; Prakriya et al., 2006), STIM1s-mCherry, STIM1L, STIM1L-YFP, STIM1S–C terminal, and STIM1L–C terminal) were transfected by electroporation with a Nucleofector II device (Lonza). Following the manufacturer’s protocol, a suspension of 5 × 10^5 myoblasts, HeLa cells, or endothelial cells (provided by C.J.S. Edgell, University of North Carolina at Chapel Hill, Chapel Hill, NC) and 2 μg plasmid DNA and/or 0.1–0.2 nM siRNA was electroporated. The transfection efficiency assessed by FACS was >80% for tagged plasmids.

siRNA knockdown

Myoblasts were transfected in suspension by incubating 4 × 10^5 cells in a solution containing 500 μl Opti-MEM, 3 μl Lipofectamine RNAiMax (Invitrogen), and 20 pmol of a specific siRNA (Invitrogen).
according to manufacturer protocols (Invitrogen). The transfection efficiency assessed by Alexa Fluor red fluorescent oligonucleotide (Block-T; Invitrogen) measurements was ~90%. Three different siSTIM1 siRNAs (Invitrogen) targeting the longer STIM1 isoform (STIM1L) were used (sense strand siRNA: siSTIM1-1 5'-GGCGGAUUCGUCGCGGCGU-3', siSTIM1-2 5'-UGCGCCUUCUACUGGUCU-3', and siSTIM1-3 5'-GGCCGGAUUCGUCGCGGCU-3'). These different siRNAs gave similar results on protein amount silencing analyzed by Western blots. To simplify, all results shown in this study were obtained with siSTIM1-1. STIM1 and Orai1 siRNAs were previously described as siSTIM1 (siSTIM1 in this study), siSTIM1L (targeting the 3' untranslated noncoding region of the STIM1 mRNA and called siSTIM1 in this study), and siOrai1. (Darbellay et al., 2009). Two different siSTIM1 (siRNAs: Invitrogen) targeting the shorter STIM1 isoform (STIM1S) were used (sense strand siRNA: siSTIM1S-1 5'-GGGAUUCGAGAAGGAGUUAU-G3' and siSTIM1S-2 5'-CAGAGGGAUUGGCCACAUU-G3'). siSTIM1S-1 and siSTIM1S-2 give similar results on STIM1S amount on Western blots. To simplify, all results shown in this study were obtained with siSTIM1S-1. The siRNA siMed negative control (Invitrogen) was used as a control.

Western blot measurements

Western blot measurements were performed as previously described (Konig et al., 2004). In brief, myoblasts were lysed using NP-40 extraction buffer (Invitrogen). Total proteins were separated on a SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in TBS (0.1% Tween 20, 20 mM Tris-HCl, pH 7.5, and 137 mM NaCl) and 5% nonfat milk. Blots were incubated overnight with the following primary antibodies diluted in TBS and nonfat milk: mouse monoclonal antibody anti-GOK/STIM1 (1:500; BD), rabbit antibodies anti-STIM1 (1:2,000, N and C terminal; Sigma-Aldrich), rabbit polyclonal anti-Orai1 (1:1,000, Prosci), mouse monoclonal antibody anti-a-tubulin (1:10,000, clone DM1A; Sigma-Aldrich), and mouse monoclonal anti-actin (1:50,000, clone C4; Millipore). Blots were incubated for 1 h with HRP-conjugated goat anti-mouse or -rabbit diluted antibodies (1:6,000; Bio-Rad Laboratories). Antibodies were revealed using Plus-ECL reagents (PerkinElmer) and Hyperfilm ECL (GE Healthcare). Oligonucleotide ligation, reverse transcription, and PCR were performed as previously described (Amaudeau et al., 2006). In brief, Fru-2–AM was diluted in medium to a final concentration of 2 μM from a DMSO stock solution containing 1 mM Fru-2–AM, 100 mg/ml Pluronic F127 (Invitrogen), and 0.1% acetic acid. Ratiometric images of Fura-2 fluorescence were monitored using an Axiovert microscope (S100 TV; Carl Zeiss) equipped and Orai1-RFP (or phalloidin-TRITC; Millipore) was performed on fixed cells using a confocal microscope (Eclipse Ti-Ar; Nikon) with a 100× Plan Apochromat violet-corrected 1.4 NA with a working distance of 0.12 mm. Ratios of transfected plasmids (GFP/RFP) were 1:2. Wavelengths of the excitation laser beam were 488 and 561 nm. Images were acquired and analyzed using Nikon software.

Cloning

Total RNA from 2 × 10⁶ myoblasts was extracted using TRIzol reagent (Invitrogen). In brief, chloroform was added to the TRIzol extracts and centrifuged to eliminate the chloroform solution, and the aqueous part was purified. Isopropanol was then added to the aqueous part to precipitate RNA. Precipitates were washed with 75% ethanol and finally resuspended in 20 μl diethylpyrocarbonate water. RACE was performed on 5 μg of total RNA (Gene Racer kit; Invitrogen). In brief, after RNA dephosphorylation, decapping, and 5' oligonucleotide ligation, reverse transcription was performed using 200 U Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. We amplified by PCR the cDNA ends using Platinum Taq DNA Polymerase (Invitrogen) and the following primer couples: 5' ends, GeneRacer 5' and exon 9 reverse 5'-GGAAGGGCCTAAGAATGGTG-3' and 3' ends, GeneRacer 3', and exon 6 forward 5'-CATGCTGTTGTCATCTACG-3'.

Products were further amplified by nested PCR using exon 7 reverse 5'-TCCGCTATCATCTCAGTAGC-3' and exon 7 forward 5'-GCCGGCTACTGAGAATG-3' for amplification of 5' RACE and 3' RACE products, respectively. The different PCR products obtained were cloned into the TOPO TA cloning kit (Invitrogen) for sequencing.

We cloned both full-length STIM1S and -L cDNA by PCR on reverse-transcribed mRNA from myotubes using the following primer couples: STIM1S-533F 5'-CTCCATCAAGCGCCATGTG-3' and STIM1S-2713R 5'-GGCCGATTCATGGAAGAGG-3'. Expression vectors encoding STIM1S and -L were obtained by cloning the full-length PCR products into the pcDNA 3.3-TOPO TA vector using the pcDNA 3.3-TOPO TA cloning kit according to the manufacturer’s protocol and sequence. The 115-KD STIM1 isoform tagged with YFP and YFP-STIM1L was obtained by cloning the Pml1-BgiII fragment from pcDNA-STIM1L into Pml1-BamH1 sites of the SP-YFP-STIM1 plasmid (plasmid 19754; Addgene).

DNA corresponding to exons 11 and 12 from both STIM1S and -L were amplified by PCR (exon 11, Xho1 forward 5'-TTTCTCGAGGC-3' and, exon 12, BamH1 reverse 5'-GAAGACCCACATGATGAA-3'). Two additional siRNAs were previously described as siSTIM1L1 and siSTIM1L2. The siRNA siMed negative control (Invitrogen) measurements was analyzed using Nikon software.
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