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Different binding property of STIM1 and its novel splice variant STIM1L to Orai1, TRPC3, and TRPC6 channels

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ABSTRACT

Stromal interaction molecule 1 (STIM1) is the endoplasmic reticulum (ER) Ca^{2+} sensor to control ER Ca^{2+} levels. A recent study has shown that STIM1L, a new splice variant of STIM1, is expressed in various tissues of rodent and in human skeletal muscle, and that the interaction of STIM1L with actin filament allows rapid activation of store-operated Ca^{2+} entry (SOCE) mediated through Orai1 channels. Here, we characterize mRNA expression and function of human STIM1 and STIM1L, and compare their binding property to Orai1 functioning as store-operated Ca^{2+} channels (SOCCs), and TRPC3 (transient receptor potential canonical 3) and TRPC6 channels functioning as endothelin type A receptor (ET_AR)-operated Ca^{2+} channels (ROCCs). Although mRNA for STIM1 was ubiquitously expressed in human tissues, STIM1L was detected only in skeletal muscle. STIM1L augmented thapsigargin- and endothelin-1-induced SOCE more strongly than STIM1 in human embryonic kidney 293 cells stably expressing ET_AR, whereas, it tends to suppress ET_AR-operated Ca^{2+} entry (ROCE) via TRPC3 and TRPC6 more strongly than STIM1. Coimmunoprecipitation experiments have revealed that when compared with STIM1, STIM1L binds more abundantly to Orai1 and also to TRPC3 and TRPC6. These results suggest that the higher binding capacity of STIM1L to SOCCs and ROCCs plays an important role in the regulation of Ca^{2+} signaling such as the augmentation of SOCE via Orai1 and the inhibition of ROCE via TRPC3 and TRPC6.

1. Introduction

Ca^{2+} is a universal second messenger that regulates a wide variety of physiological and pathophysiological events, including cell constriction, cell proliferation, cell differentiation, and activation of immune cells [1]. Store-operated Ca^{2+} entry (SOCE) and receptor-operated Ca^{2+} entry (ROCE) from the extracellular space are important mechanisms for Ca^{2+} influx in many non-excitable cells, and are mediated through store-operated Ca^{2+} channels (SOCCs) and receptor-operated Ca^{2+} channels (ROCCs), respectively [2]. Several lines of evidence suggest that Orai1 acts as the molecular component of SOCCs, and also that some members of the transient receptor potential canonical (TRPC) channel subfamily consisting of seven isoforms (TRPC1 to TRPC7) act as SOCCs, whereas others act as ROCCs [1,2].

SOCE is well-known to be triggered by depletion of ER resulting from activation of the inositol 1,4,5-trisphosphate (IP_3) cascades associated with G_q protein-coupled receptors (G_qPCRs) including endothelin type A receptor (ET_AR) and the blockade of the ER Ca^{2+}-ATPase by its inhibitors like thapsigargin [1–3]. Stimulation of G_qPCR activates phospholipase C (PLC) that hydrolyzes phosphatidylinositol bisphosphate to form IP_3 and diacylglycerol (DAG) [1]. The binding of IP_3 to its receptors on ER triggers Ca^{2+} release from ER, leading to depletion of ER [1]. The Ca^{2+}-depletion of ER activates SOCCs via stromal interaction molecule 1 (STIM1) which is a sensor of ER Ca^{2+} levels [2,4]. STIM1 is a single transmembrane-spanning protein that is predominantly localized on the ER [4]. Upon depletion of ER, STIM1 is multimerized and translocated to the plasma membrane, where it associates with either Orai1, TRPC1, TRPC4, or TRPC5 to make them function as SOCCs, but STIM1 does not interact with TRPC3 and TRPC6 [5]: hence TRPC3 and TRPC6 are destined to

Abbreviations: AM, acetoxymethyl ester; BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; ET-1, endothelin-1; ET_AR, endothelin type A receptor; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; G_qPCR, G_q protein-coupled receptor; HA, influenza hemagglutinin; HEK293, human embryonic kidney 293; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cell; IP_3, inositol 1,4,5-trisphosphate; PASMC, pulmonary arterial smooth muscle cell; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; ROCC, receptor-operated Ca^{2+} channel; ROCE, receptor-operated Ca^{2+} entry; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; SOCC, store-operated Ca^{2+} channel; SOCE, store-operated Ca^{2+} entry; STIM1, stromal interaction molecule 1; TG, thapsigargin; TRPC, transient receptor potential canonical.

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function as ROCCs [6]. We have provided evidence that TRPC3- and TRPC6-mediated ROCE following activation of ET_AR with its agonist, endothelin-1 (ET-1), is regulated by various signaling molecules but not initiated by depletion of ER [6,7].

A recent study has shown a novel splice variant of STIM1, named STIM1L [8]. STIM1L as well as STIM1 (from now on, we refer to this protein as STIM1S) are single-pass transmembrane proteins, with STIM1L having a longer cytosolic C-terminal tail because of insertion of 106 amino acids [8]. STIM1L is reported to be expressed in most murine tissues and human skeletal muscle and to function as a Ca^{2+} sensor of ER like STIM1S [8]. Notably, the STIM1L-specific sequence, which is a serine/threonine-rich domain, strongly binds to actin filament [8]. Through binding to actin filament, STIM1L on ER membrane is localized in close proximity to Orai1 channel on the plasma membrane, allowing rapid activation of SOCE [8].

When the previous work on murine and human STIM1L was published [8], we had also been successful in cloning this molecule from human skeletal muscle and noticed that human STIM1L showed totally different tissue distribution from murine STIM1L: that is, mRNA expression for human STIM1L was detected only in skeletal muscle. Based on recent findings showing the strong interaction of STIM1L with actin, we wondered whether human STIM1L possesses the binding and functional properties different from STIM1S. Therefore we determined to examine its functional role in SOCE and ROCE and also molecular interaction of human STIM1L with plasma membrane channel molecules such as Orai1 and TRPCs. Here, we provide evidence that (1) in human tissues, STIM1L is detected only in skeletal muscle, (2) both STIM1S and STIM1L bind to the molecular entity of SOCE such as Orai1 and the molecular entity of ROCE such as TRPC3 and TRPC6, (3) the binding capacity of STIM1L to these proteins is far higher than that of STIM1S, and (4) STIM1L augments SOCE more strongly than STIM1S, whereas it tends to suppress ROCE more strongly than STIM1S.

2. Materials and methods

2.1. Materials

The following drugs and reagents were used in the present study: synthetic human ET-1 (Peptide Institute, Osaka, Japan); fura-2/acetoxymethyl ester (fura-2/AM) (Dojindo Laboratories, Kumamoto, Japan); thapsigargin (TG), aprotinin, leupeptin, pepstatin, sodium deoxycholate, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), Na_3VO_4, NaF, bovine serum albumin (BSA) (Sigma–Aldrich Co., St. Louis, MO, USA). All cell culture media and supplements except fetal calf serum (FCS; Invitrogen Corp., Grand Island, NY, USA) were obtained from Sigma–Aldrich. Antibodies for an influenza hemagglutinin (HA) epitope tag, FLAG peptide, Myc-tag, green fluorescent protein (GFP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase (HRP) conjugated FLAG peptide (HRP-FLAG) were obtained from Covance Inc. (Emeryville, CA, USA), Sigma–Aldrich, Cell Signaling Technology Inc. (Beverly, MA, USA), Clontech Laboratories Inc. (Mountain View, CA, USA), Santa Cruz Biotechnology Inc. (Delaware Avenue Santa Cruz, CA, USA), and Medical and Biological Laboratories Co., Ltd. (Aichi, Japan), respectively. Clean-Blot IP Detection Reagent HRP was obtained from PIERCE Biotechnology Inc. (Rockford, IL, USA). The other regents used were of the highest grade in purity.

2.2. Detection of mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs prepared from human skeletal muscle, brain, placentae, leukocyte were obtained from Clontech Laboratories. Total RNA from human left ventricle was from BioChain Institute (Newark, CA, USA). Other total RNAs were extracted and purified from human pulmonary arterial smooth muscle cells (PASMC), human umbilical vein endothelial cells (HUVEC), Hela cells, and human embryonic kidney 293 (HEK293) cells by using total RNA purification kit (RNeasy Mini Kit, QIAGEN, Tokyo, Japan) following the instructions of the manufacturer. RT-PCR was performed by using both SuperScript II First-Strand Synthesis System (Invitrogen Corp.) for RT and PfuUltra II fusion HS DNA polymerase (Agilent Technologies, Inc., Santa Clara, CA, USA) with gene-specific primers for STIM1S and STIM1L (forward, 5′-GCGGGATCCCGCCACCATG-GATGTATGCGTCCGTCTTGCCCTGTG-3′ and reverse, 5′-CGACCGG TGCCTTCTTAAGAGGCTTCTTAAAGATTTTGAGGGGAA-3′), and for β-actin (forward, 5′-ATCCTGCGTCTGGACCTGGCTG-3′ and reverse, 5′-CCTGCTTGCTGATCCACATCTGCTG-3′) for PCR. Synthesized cDNA was heated for 10 min at 94 °C, then amplified by 35 cycles (94 °C for 20 s, 60 °C for 20 s, 72 °C for 90 s) followed by 10 min of extension at 72 °C. The PCR products were separated by electrophoresis on 1.0% or 2.0% agarose gels and visualized by ethidium bromide staining.

2.3. Construction of retrovirus vectors encoding human STIM1S, STIM1L, and Orai1

The full-length cDNA clones of human STIM1S, STIM1L, and Orai1 were generated from the single-stranded cDNA for mRNA of human skeletal muscle (STIM1S and STIM1L) and HEK293 cells (Orai1) by a PCR reaction with gene-specific primers containing the BamHI and AgeI sites for STIM1S and STIM1L (forward, 5′-GCGGGATCCCGCCACCATGGATGTATGCGTCCGTCTTGCCCTGTG-3′ and reverse, 5′-CGACCGGTGCCTTCTTAAGAGGCTTCTTAAAGATTTT-GAGGGGAA-3′), and for Orai1 (forward, 5′- GCGGGATCCCGC CACCATGCATCCGGAGCCCGCCCCGCCCCCGAG-3′ and reverse, 5′-C GACCGGTGCGGCATAGTGGCTGCCGG-3′) to subclone into the pCR-Blunt II-TOPO vector (Invitrogen Corp.). The pCR-Blunt II-TOPO vectors encoding these full-length cDNA clones were digested with two restriction enzymes, BamHI and AgeI, simultaneously. The resulting cDNA fragments and tags (GFP, FLAG, and Myc) were ligated into the pMXrmv5 retrovirus vector to yield STIM1S, STIM1L, and Orai1 fused with GFP, FLAG peptide, or Myc-tag at their C terminus. All of the constructs were verified by DNA sequencing.

2.4. Cell culture

HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v v^{-1}) FCS, penicillin (100 units ml^{-1}), and streptomycin (100 μg ml^{-1}) at 37 °C in humidified air with 5% CO_2.

2.5. Stable coexpression of human ET_AR and STIM1S, STIM1L, or Orai1 in HEK293 cells

HEK293 cells stably expressing human ET_AR fused with a HA epitope tag at the N terminus (HA-ET_AR) were generated as mentioned previously [6,7]. To generate HA-ET_AR-positive HEK293 cells (HA-ET_AR/HEK293) stably coexpressing STIM1S-myc or STIM1L-myc, these genes were introduced into HA-ET_AR/HEK293 cells by retroviral gene transfer [6,7]. The resulting cells were subsequently used to introduce the genes of Orai1-FLAG, TRPC3-FLAG, and TRPC6-FLAG. The genes of STIM1S-FLAG and STIM1L-FLAG were also introduced into HA-ET_AR/HEK293 cells stably coexpressing TRPC3-GFP and TRPC6-GFP [6,7] to estimate their functional role in ET_AR-operated Ca^{2+} entry via TRPC3 and TRPC6.

2.6. Measurement of $[Ca^{2+}]_i$

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by using a fluorescent Ca^{2+} indicator, fura-2/AM, as described [6,7]. Changes of $[Ca^{2+}]_i$ in cells were measured at 30 °C using CAF-110 spectrophotometer (JASCO, Tokyo, Japan) with the excitation wavelengths of 340 and 380 nm, and emission wavelength of 500 nm.

2.7. Confocal microscopy

Confocal microscopy was carried out using FluoView FV300 (Olympus Corporation, Tokyo, Japan) with a 63 × oil-immersion lens.

2.8. Coimmunoprecipitation

Cells were solubilized with lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 6.8), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 20 mM NaF, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, and 10 μg ml^{-1} pepstatin) supplemented with EDTA-free, protease inhibitor cocktail (PIERCE Biotechnology Inc.). The cell lysates were sonicated for 10 s on setting 10 of a handy sonicator (UR-20P, TOMY SEIKO Co., Ltd., Tokyo, Japan) and centrifuged at 20,000×*g* for 20 min at 4 °C. Protein content of supernatant was measured according to the method of Bradford [9] using BSA as standard.

Coimmunoprecipitation was carried out using Dynabeads Protein G (Invitrogen Corp.). Briefly, the Dynabeads were incubated with a primary antibody (anti-FLAG, 1:100 dilution) for 1 h at room temperature. The Dynabeads-antibody complexes were washed twice with washing buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 6.8), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) by gentle pipetting, and then incubated with lysates containing equal protein amounts for 1 h at room temperature. The resulting Dynabeads were washed three times with the washing buffer. The proteins bound to the Dynabeads were eluted by adding 50 mM Glycine (pH 2.8) admixed with 62.5 mM Tris–HCl, 10% glycerol, 5% 2-mercaptoethanol, 2.5% SDS, and 0.1% bromphenol blue, followed by incubation at 37 °C for 30 min.

2.9. Western blot analysis

Western blot analysis was carried out as described [6,7]. Briefly, the proteins in immunoprecipitated samples and whole cell lysates were separated on a 5–20% or 12.5% polyacrylamide gel (SuperSep, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and electrotransferred to a polyvinylidene fluoride membrane (Immobilon-P, pore size 0.45 μm, Millipore Corp., Bedford, MA, USA) with a semi-dry electroblotter. The resulting membranes were incubated with a primary antibody overnight at 4 °C. The primary antibody except anti-FLAG HRP antibody was detected with a secondary HRP-conjugated anti-mouse IgG antibody or Clean-Blot IP Detection Reagent HRP (PIERCE Biotechnology Inc.). These HRP-conjugated

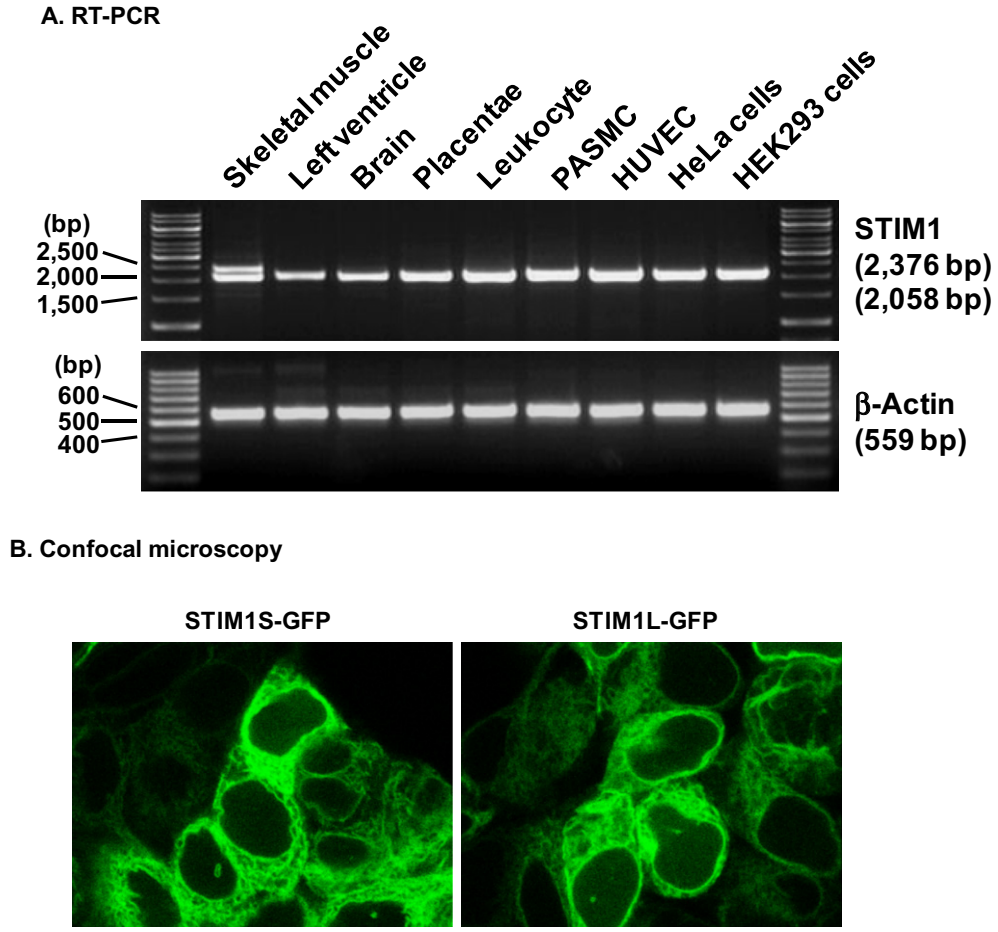

Fig. 1. mRNA expression of STIM1S and STIM1L in human skeletal muscle, left ventricle, brain, placentae, leukocyte, pulmonary arterial smooth muscle cells (PASMC), umbilical vein endothelial cells (HUVEC), Hela cells, and human embryonic kidney 293 (HEK293) cells (A), and subcellular localization of human STIM1S and STIM1L expressed in HEK293 cells (B). (A) The size of full-length cDNAs of STIM1S and STIM1L were 2058 bp and 2376 bp, respectively (top panel). The predicted size of GAPDH was 559 bp (bottom panel). The positions of size markers are indicated to the left in bp. (B) Subcellular localization of STIM1S (left panel) and STIM1L (right panel) proteins fused with GFP at the C-terminus. The subcellular localization of STIM1S-GFP and STIM1L-GFP expressed in HEK293 cells was visualized by a fluorescence confocal microscopy.

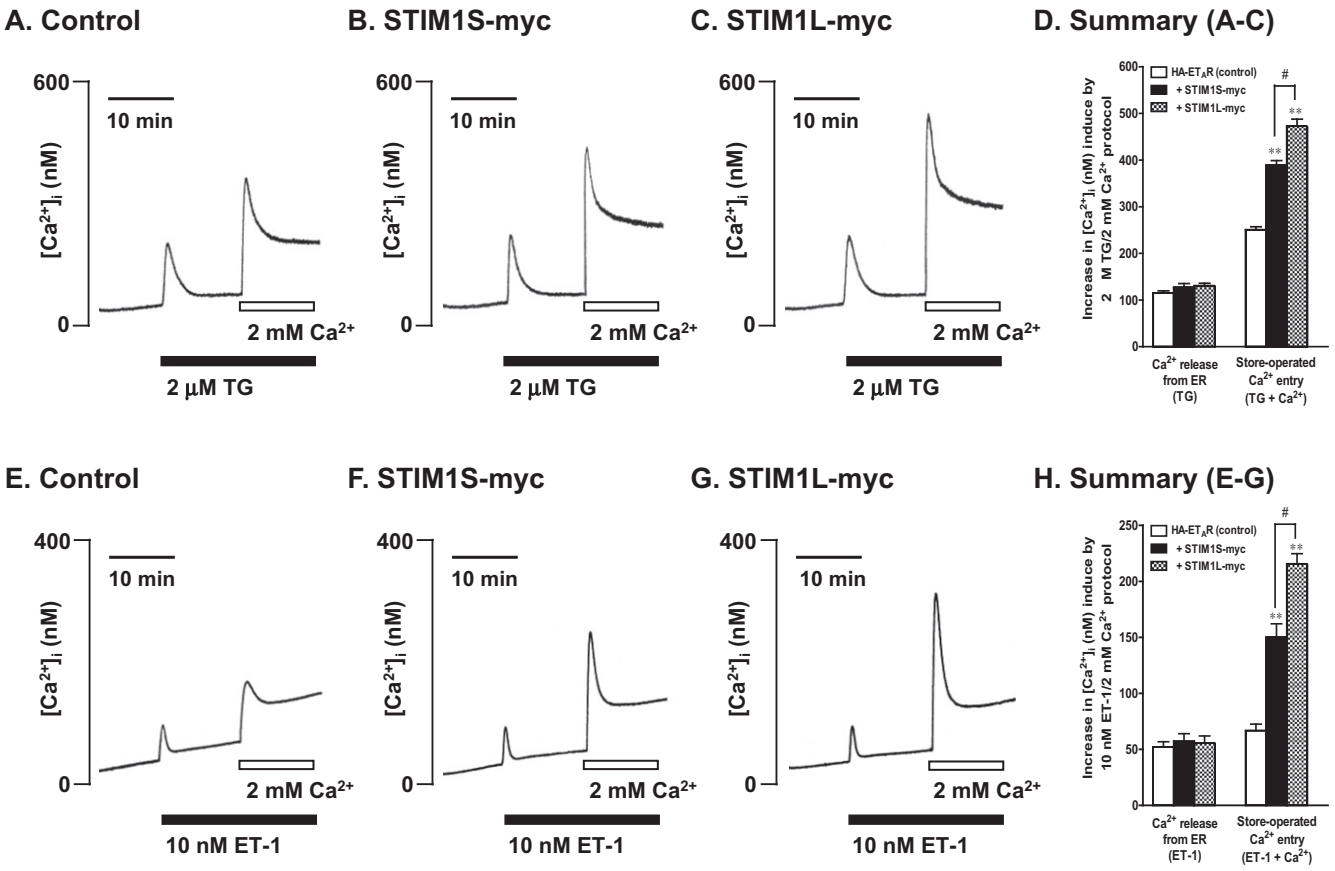

Fig. 2. Pharmacological evaluation of SOCE after store depletion triggered by TG (A–D) or ET-1 (E–H) in HEK293 cells stably expressing human ET_AR alone (A and E), or coexpressing human ET_AR and STIM1S-myc (B and F), or STIM1L-myc (C and G). Representative traces for 2 μM TG- or 10 nM ET-1-induced Ca^{2+} release from ER in nominally Ca^{2+}-free medium followed by SOCE upon restoration of 2 mM extracellular Ca^{2+}. (D and H) Histograms show increases in $[Ca^{2+}]_i$ due to Ca^{2+} release from ER and SOCE induced by TG- or ET-1-induced Ca^{2+}-depletion/Ca^{2+}-restoration protocol. Data are presented as means ± S.E.M of the results obtained from 4–6 experiments. $^{**}P < 0.01$, versus its control, $^{\#}P < 0.05$, between indicated columns.

antibodies and the reagent were detected with ECL Western blotting Analysis System (GE Healthcare Limited, Little Chalfont, Buckinghamshire, UK) or ImmunoStar LD (Wako Pure Chemical Industries, Ltd.). The amounts of immunoprecipitated proteins were analyzed with National Institutes of Health Image J1.37 Software.

2.10. Data analysis

Data for $[Ca^{2+}]_i$ measurement were collected and analyzed by using a MacLab/8s with Chart (v. 3.5) Software (ADInstruments Japan, Tokyo, Japan). All data are presented as means ± S.E.M, where *n* refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad PRISM (version 3.00, GraphPad Software Inc., San Diego, CA, USA) by Student's unpaired *t*-test. A *P* value less than 0.05 was considered to indicate significant differences.

3. Results and discussion

3.1. Comparison of mRNA expression for STIM1S and STIM1L in human tissues

First, we examined expression of STIM1L mRNA in various human tissues using RT-PCR with primers corresponding to the exons 1 and 12. In sharp contrast with ubiquitous expression of STIM1L in mouse tissues, human splicing variant STIM1L (2376 bp) was detected only in skeletal muscle (Fig. 1A). Conversely, the well-known STIM1S (2058 bp) was expressed ubiquitously, as reported previously [4]. We have confirmed that the splicing variant isolated in the present study is identical with human STIM1L described by Darbellay et al. [8]. Indeed, the additional segment (106 amino acids) of isolated splicing variant was inserted between positions 514 and 515 in the STIM1S amino acid sequence, and was identical with a new actin-binding domain in human STIM1L [8]. Our data, taken together with the previous observations [8], indicate that unlike murine STIM1L, human STIM1L is a skeletal muscle-specific splice variant of STIM1S.

3.2. Subcellular localization of human STIM1S and STIM1L expressed in HEK293 cells

As reported recently [4], a confocal laser-scanning microscopic approach revealed that in resting cells with filled Ca^{2+} stores, STIM1S and STIM1L tagged with GFP at the C-terminus were diffusely distributed in the cytoplasm of HEK293 cells, presumably on ER (Fig. 1B). Unlike the data in human myoblasts [8], there seems to be no apparent difference in subcellular localization between STIM1S and STIM1L expressed in HEK293 cells.

3.3. Augmentation of SOCE by STIM1S and STIM1L

It is reported that binding of STIM1L-specific segment to actin enables close interaction of STIM1L with Orai1, resulting in rapid activation of SOCE after Ca^{2+}-store depletion in human myoblasts [8]. To compare the ability to enhance SOCE between STIM1S and STIM1L, we employed the 2 μM TG-induced Ca^{2+}-depletion/2 mM Ca^{2+}-restoration protocol to measure SOCE [6,7].

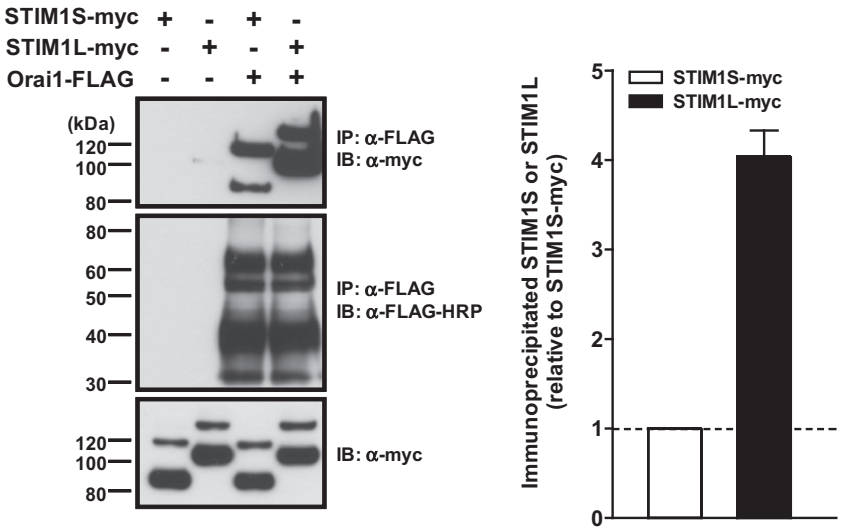

Fig. 3. Binding property of STIM1S and STIM1L to Orai1. The combinations of proteins were expressed in HEK293 cells as indicated on the top. Top panels: FLAG-tagged Orai1 proteins were immunoprecipitated with anti-FLAG antibody (IP: α-FLAG). Immunoprecipitates were probed with the anti-myc monoclonal antibody (IB: α-myc) to estimate coimmunoprecipitated STIM1S-myc and STIM1L-myc. Middle panels: FLAG-tagged Orai1 proteins were immunoprecipitated with anti-FLAG antibody (IP: α-FLAG), and the immunoprecipitates were probed with the anti-FLAG-HRP antibody (IB: α-FLAG-HRP) to normalize the quantity of coimmunoprecipitated STIM1S-myc and STIM1L-myc. Bottom panels: the expressed STIM1S-myc and STIM1L-myc were detected with anti-myc monoclonal antibody (IB: α-myc). The histograms represent the relative ratio of the quantity of coimmunoprecipitated STIM1L-myc to that of coimmunoprecipitated STIM1S. The ratio was normalized by the quantity of proteins immunoprecipitated with anti-FLAG antibody (middle panels). Data are presented as means ± S.E.M of the results obtained from four experiments.

As expected, STIM1S and STIM1L overexpressed in HA-ET_AR/HEK293 cells augmented the TG-induced SOCE, compared with that in control cells (Fig. 2A–D). The augmentation of TG-induced SOCE by STIM1L was significantly larger than that by STIM1S (Fig. 2D). Recently, we have demonstrated that activation of ET_AR induces SOCE but not ROCE in HA-ET_AR/HEK293 not transfected with TRPCs [6]. Like the TG-induced SOCE (Fig. 2A–D), 10 nM ET-1-induced SOCE was potentiated by STIM1L more strongly than STIM1S (Fig. 2E–H). We have confirmed that the differences in SOCE between STIM1S and STIM1L are not due to differences in their expression levels (Fig. 3). These results suggest that STIM1L acts more effectively to trigger SOCE than STIM1S.

3.4. Different binding property of STIM1S and STIM1L to Orai1, TRPC3, and TRPC6

Abundant evidence indicates that both STIM1S and STIM1L associate with Orai1, resulting in augmentation of SOCE after Ca^{2+} store depletion [4,10,11] and also that the STIM1L-specific amino acid sequence plays a key role in the rapid activation of SOCE via its binding to actin filaments [8]. We hypothesized that stronger augmentation of SOCE by STIM1L in comparison with STIM1S is due to STIM1L's ability to bind more strongly to Orai1 than STIM1S. As expected, the amount of STIM1L protein coimmunoprecipitated with Orai1 was approximately 4-fold larger than that of STIM1S protein (Fig. 3). This result indicates that the higher binding capacity of STIM1L to Orai1 causes larger enhancement of SOCE in comparison with STIM1S.

It is reported that STIM1S can couple to TRPC1, TRPC4 and TRPC5 exogenously expressed in HEK293 cells, whereas it does not bind to TRPC3 and TRPC6 [5]. We characterized the binding properties of STIM1L as well as STIM1S to TRPC3 and TRPC6. Unlike the previous report, STIM1S was found to interact with TRPC3 and TRPC6, although the interaction was weak (Fig. 4A, top panels). Notably, STIM1L interacted with TRPC3 and TRPC6 more strongly than STIM1S (Fig. 4), indicating the importance of STIM1L-specific sequence for the association of STIM1L with TRPC3 and TRPC6 as well as Orai1. However, it remains to be determined whether the strong interaction of STIM1L with Orai1, TRPC3, and TRPC6 in HEK293 cells results from its unique binding property to actin filament reported in human myoblasts [8].

3.5. Inhibition of ET_AR-operated Ca^{2+} influx via TRPC3 and TRPC6 by STIM1S and STIM1L

Recently, we have reported that stimulation of ET_AR with ET-1 induces ROCE via TRPC3 and TRPC6 in HEK293 cells stably expressing ET_AR together with TRPC3 or TRPC6 [6,7]. Therefore, we examined the functional significance of the interaction of STIM1S or STIM1L with TRPC3 and TRPC6 in ET_AR-operated Ca^{2+} influx.

To estimate ET_AR-operated Ca^{2+} influx, we employed the TG-induced Ca^{2+}-depletion/Ca^{2+}-restoration protocol with 10 μM Gd^{3+} to measure SOCE followed by ET_AR stimulation to measure ROCE, as described in our recent reports [6,7]. Although the transient increase in $[Ca^{2+}]_i$ due to SOCE in STIM1S- or STIM1L-overexpressing cells was higher than that in control cells, there was no significant difference in the sustained $[Ca^{2+}]_i$ level of SOCE among them (Supplementary Fig. S1). After activation of SOCE, stimulation of ET_AR with 10 nM ET-1 generated ROCE through TRPC3 (Fig. 4B) and TRPC6 (Fig. 4C). The TRPC3- and TRPC6-mediated ROCEs were diminished following expression of STIM1S and STIM1L (Fig. 4B and C). Notably, STIM1L tends to inhibit the ROCE via TRPC3 and TRPC6 more strongly than STIM1S. This may result from the higher binding capacity of STIM1L to TRPC3 and TRPC6 in comparison with STIM1S (Fig. 4A), considering that following overexpression of STIM1S and STIM1L, expression levels of TRPC3 and TRPC6 and of ET_AR are unchanged (Supplementary Fig. S2). These findings clearly indicate that STIM1S and STIM1L are negative regulators of the TRPC3- and TRPC6-mediated ROCE.

4. Conclusions

We have demonstrated that the expression of STIM1L as well as STIM1S augments SOCE via endogenously expressed Orai1 but

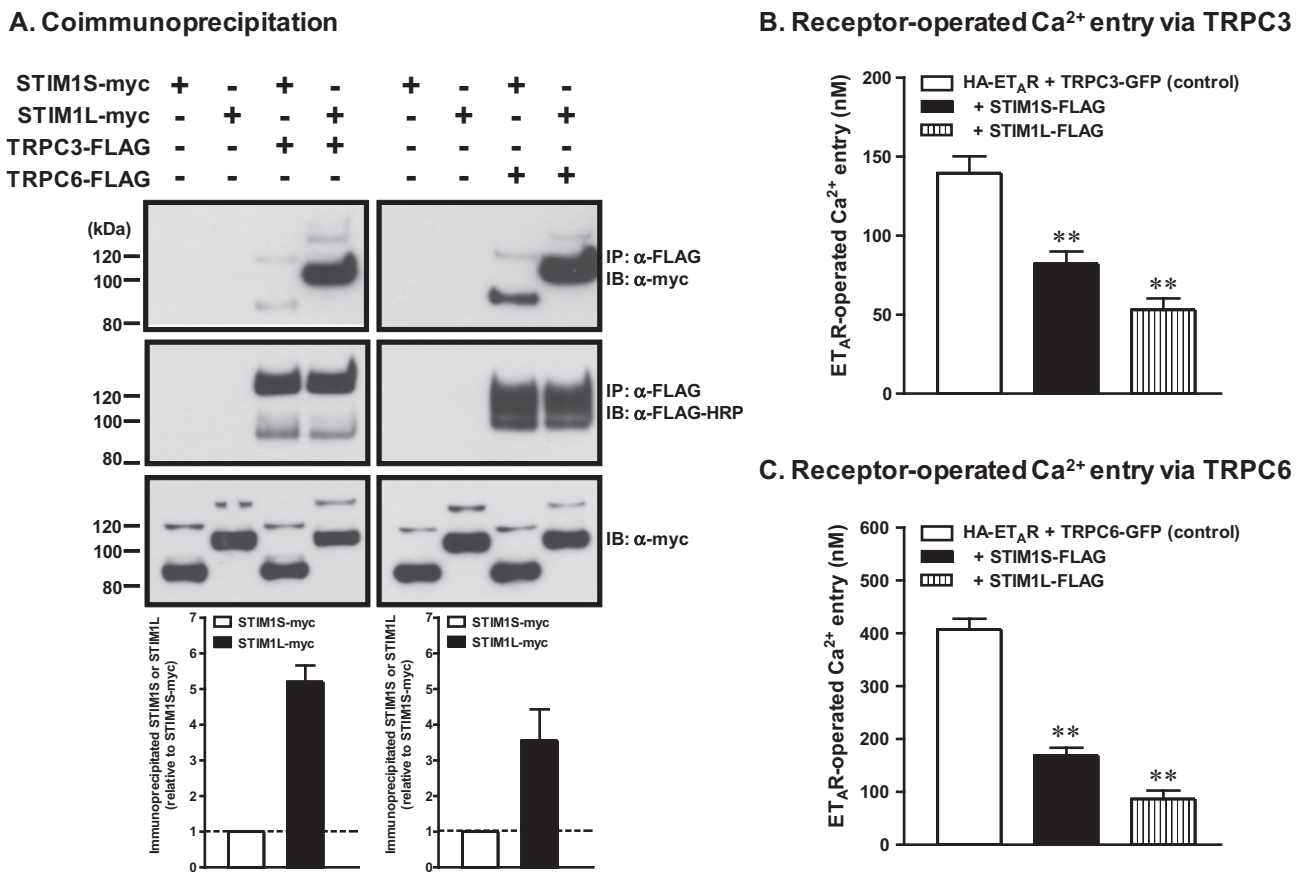

Fig. 4. Binding property of STIM1S and STIM1L to TRPC3 and TRPC6 (A) and negative regulation of ET_AR-operated Ca^{2+} influx via TRPC3 (B) and TRPC6 (C) by STIM1S and STIM1L. (A) The combinations of proteins were expressed in HEK293 cells as indicated on the top. Top panels: FLAG-tagged TRPC3 and TRPC6 proteins were immunoprecipitated with anti-FLAG antibody (IP: α-FLAG). Immunoprecipitates were probed with the anti-myc monoclonal antibody (IB: α-myc) to estimate coimmunoprecipitated STIM1S-myc and STIM1L-myc. Middle panels: FLAG-tagged TRPC3 and TRPC6 proteins were immunoprecipitated with anti-FLAG antibody (IP: α-FLAG), and the immunoprecipitates were probed with the anti-FLAG-HRP antibody (IB: α-FLAG-HRP) to normalize the quantity of coimmunoprecipitated STIM1S-myc and STIM1L-myc. Bottom panels: the expressed STIM1S-myc and STIM1L-myc were detected with anti-myc monoclonal antibody (IB: α-myc). The histograms represent the relative ratio of the quantity of coimmunoprecipitated STIM1L-myc to that of coimmunoprecipitated STIM1S. The ratio was normalized by the quantity of proteins immunoprecipitated with anti-FLAG antibody (middle panels). Data are presented as means ± S.E.M of the results obtained from 4 experiments. (B and C) Increases in $[Ca^{2+}]_i$ due to ROCE via TRPC3 and TRPC6 induced by ET_AR stimulation with 10 nM ET-1. Data are presented as means ± S.E.M of the results obtained from 4 to 6 experiments. $^{**}P < 0.01$, versus its control.

inhibits ROCE via exogenously expressed TRPC3 and TRPC6. Notably, the augmentation or the inhibition by STIM1L was larger than that by STIM1S. In addition, the binding capacity of STIM1L to Orai1 as SOCCs and TRPC3 and TRPC6 as ROCCs is significantly higher than that of STIM1S. Thus, the additional amino acid sequence of STIM1L at the cytosolic C-terminus plays an important role in the protein–protein interaction and Ca^{2+} signaling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.034.

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