ARTICLE
Journal of Cellular Biochemistry 112:147–156 (2011)

Journal of Cellular Biochemistry

Identification of Functional Domains and Novel Binding Partners of STIM Proteins

Norimitsu Saitoh,[1] Kenji Oritani,[1*] Kazunobu Saito,[2,3] Takafumi Yokota,[1] Michiko Ichii,[1] Takao Sudo,[1] Natsuko Fujita,[1] Koichi Nakajima,[4] Masato Okada,[3] and Yuzuru Kanakura[1]

[1] *Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, Osaka, Japan*

[2] *DNA-Chip Development Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

[3] *Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka, University, Osaka University, Osaka, Japan*

[4] *Department of Immunology, Osaka City University Graduate School of Medicine, Osaka, Japan*

ABSTRACT

With a signal trap method, we previously identified stromal interaction molecule (STIM: originally named as SIM) as a protein, which has a signal peptide in 1996. However, recent works have accumulated evidences that STIM1 and STIM2 reside in endoplasmic reticulum (ER) and that both mainly sense ER Ca^{2+} depletion, which plays an essential role in store operated calcium entry. In the present study, we extensively analyzed the domain functions and associated molecules of STIMs. A STIM1 mutant lacking the coiled-coil domains was massively expressed on the cell surface while mutants with the coiled-coil domains localized in ER. In addition, STIM1 mutants with the coiled-coil domains showed a longer half-life of proteins than those without them. These results are likely to indicate that the coiled-coil domains of STIM1 are essential for its ER-retention and its stability. Furthermore, we tried to comprehensively identify STIM1-associated molecules with mass spectrometry analysis of co-immunoprecipitated proteins for STIM1. This screening clarified that both STIM1 and STIM2 have a capacity to bind to a chaperone, calnexin as well as two protein-transporters, exportin1 and transportin1. Of importance, our result that glycosylation on STIM1 was not required for the association between STIM1 and calnexin seems to indicate that calnexin might function on STIM1 beyond a chaperone protein. Further information concerning regulatory mechanisms for STIM proteins including the data shown here will provide a model of Ca^{2+} control as well as a useful strategy to develop therapeutic drugs for intracellular Ca^{2+}-related diseases including inflammation and allergy. J. Cell. Biochem. 112: 147–156, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: STIM; ENDOPLASMIC RETICULUM; COILED-COIL; HALF LIFE; CALNEXIN

Changes of intracellular calcium (Ca^{2+}) levels provide universal signals, which regulate various cellular events, such as gene expression, secretion, and differentiation [Berridge et al., 2000, 2003]. In electrochemically non-excitable cells, endoplasmic reticulum (ER) acts to store an intracellular pool of Ca^{2+}, which is released soon after the activation of receptors for cytokines or hormones. In addition, Ca^{2+} depletion from ER leads to the rapid activation of plasma membrane Ca^{2+} channels in a process known as store-operated Ca^{2+} (SOC) entry [Putney, 1986, 2007]. This Ca^{2+} entry is essential for the refill of Ca^{2+} in ER and the cytokine/hormone-induced sustained increase of intracellular Ca^{2+} as well. Recent important findings in this area are the identification of stromal interaction molecule (STIM) and orai proteins; STIM1 and STIM2 as sensors of ER luminal Ca^{2+} changes and Orai1, Orai2, and Orai3 as components of highly Ca^{2+}-selective SOC channels [Liou et al., 2005; Roos et al., 2005; Vig et al., 2006; Zhang et al., 2006].

STIM1 and STIM2 are single-pass, type-I transmembrane proteins, which mainly localize in ER [Williams et al., 2001; Soboloff et al., 2006a; Stathopulos et al., 2008]. Both proteins contain a helix-tern-helix EF-hand Ca^{2+}-sensing domain followed by a sterile α motif (SAM) in luminal side of ER. In addition, their cytoplasmic segment has two stretches of the coiled-coil domains and a highly acidic stretch. Both STIM1 and STIM2 have been proposed to sense ER-luminal Ca^{2+} levels. Indeed, luminal Ca^{2+} depletion results in the redistribution of STIM1 from ER homogeneity to discrete "puncta" in close proximity to the plasma membrane where it can activate SOC channels [Zhang et al., 2005;

*Correspondence to: Dr. Kenji Oritani, MD, Department of Hematology and Oncology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. E-mail: oritani@bldon.med.osaka-u.ac.jp
Received 24 March 2010; Accepted 28 September 2010 • DOI 10.1002/jcb.22910 • © 2010 Wiley-Liss, Inc.
Published online 4 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

Luik et al., 2006; Wu et al., 2006; Liou et al., 2007]. During this puncta formation, Ca^{2+} depletion induces the oligomerization of STIM1 through its EF-SAM regions [Stathopulos et al., 2008]. Its coiled-coil regions also mediate constitutive homotypic interactions, leading to the stabilization of the EF-SAM-triggered oligomers as well as the translocation of STIM1.

Although available information about biochemical and physiological properties of STIM proteins, little is known about the domain functions of STIMs and their association molecules, which could help us understand not only why STIMs can display their specific functions but also what molecules control the localization and protein content of STIMs in cells. In the present study, we have found that the coiled-coil domains of STIM1 are essential for its ER-localization and protein stabilization. In addition, we have identified calnexin, exportin1, and transportin1 as novel associated molecules of STIMs with mass spectrometry analysis of co-immunoprecipitated proteins for STIM1.

MATERIALS AND METHODS

PLASMID CONSTRUCTS

To produce FLAG-tagged STIM1 and its deletion mutants, mouse STIM1 cDNA was amplified by PCR using a F4-9/pEFBOS plasmid as a template [Oritani and Kincade, 1996]. The PCR primers are as follows: 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-TTCTTCGGAGAATTCTTCGAGCTCCCC-3′ for STIM1(Full), 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-GTACCGGATCTGTTCCGGGAGCTCCCC-3′ for STIM1(EX + TM + CC), 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-GTGTACTTCTTTTACTACGAGCTCCCC-3′ for STIM1(EX + TM), 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-TGAGCCGTATTAGTGGACGAGCTCGGG-3′ for STIM1(EX). Each PCR product was then digested with BamH1 and Xho1, and ligated into FLAG-pcDNA 3 expression vector (Addgene, Cambridge, MA) (STIM1(Full)-FLAG, STIM1(EX + TM + CC)-FLAG, STIM1(EX + TM)-FLAG, STIM1(TM)-FALG, respectively). In these constructs, a FLAG tag was inserted into C-terminal of each cDNA. For STIM2(Full)-FLAG, the plasmid was constructed in a similar way to STIM1 constructs by using pENTR11 mSTIM2 myc (Addgene) as a template and 5′-GGGGGATCCACGGAAACAATGAAC-3′ and 5′-TTCTTCTTCAGATTCGAGCTCGGG-3′ as primers. To produce STIM1 lacking two Asn-linked glycosylation sites (STIM1(delGly)-FLAG), site-directed mutagenesis was performed. To substitute two ''Asn''s at positions 131 and 171 for ''Ala''s, site-directed mutagenesis using STIM(Full)-FLAG as a template was performed. The PCR primersare as follows: 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-AGTCTTCACATGCGGACCTGACACCTA-3′ as well as 5′-TCAGAAGTGTACGCCTGGACTGTGGAT-3′ and 5′-TTCTTCGGAGAATTCTTCGAGCTCCCC-3′ for the substitution of N131A, 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-GATCGTCATTGGCGGTGGTGGTACTGT-3′ as well as 5′-CTAGCAGTAACCGCCACCACCATGACA-3′ and 5′-TTCTTCGGAGAATTCTTCGAGCTCCCC-3′ for the substitution of N171A. The final PCR product (primers: 5′-GGGGGATCCACCGTCATGGATGTGTGCGCC-3′ and 5′-TTCTTCGGAGAATTCTTCGAGCTCCCC-3′) was digested with BamH1 and Xho1, and ligated into FLAG-pcDNA3 expression vector. Nucleotide sequences of all constructs were verified by sequencing.

CELL CULTURE AND TRANSFECTION

A human embryonic kidney carcinoma cell line (293T) and a human cervix carcinoma cell line (HeLa) were cultured in Dubecco's modified Eagle's medium (DMEM); (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS); (Equitec-Bio, Kerrville, TX). STIM1-deficient DT40 B cell line, a kind gift from Dr. Baba and Dr. Kurosaki (Osaka University, Osaka, Japan), was cultured in RPMI (Nacalai Tesque) supplemented with 10% FBS, 1% chiken serum (Biowest, Nuaille, France), 2 mM L-glutamine, and 50 μM 2-Mercaptoethanol. Transient transfection of plasmids into 293T and HeLa cells was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer protocols. Stable transfection into DT40 cells was performed with Amaxa (Amaxa Biosystems, Gaithersburg, MD) according to manufacturer protocols.

IMMUNOPRECIPITATION AND WESTERN BLOT

The immunoprecipitation and Western blotting assays were performed as described previously [Sekine et al., 2005]. In some experiments, the transfectants were lysed after their surface proteins were labeled with biotin (EZ-Link Sulfo-NHS-SS-Biotin; Pierce, Rockford, IL). The antibodies used in this study were listed as follows: ANTI-FLAG® M2-Agarose Affinity Gel (Sigma–Aldrich, St. Louis, MO), anti-FLAG antibody (Sigma), anti-STIM1 (Abnova, Taipei, Taiwan), anti-calnexin (Stressgen, Ann Arbor, MI), anti-exportin1 (Bethyl Laboratories, Montgomery, TX), anti-transportin1 (ImmuQuest, Cleveland, UK), and anti-calreticulin (Affinity BioReagents, Golden, CO) as well as anti-Mouse IgG HRP conjugate (Promega, Madison, WI), anti-Rabbit IgG HRP conjugate (Promega), and Streptavidin-HRP (Pierce).

CONFOCAL MICROSCOPY

After HeLa cells were transfected with the indicated plasmids, cell fixation, permeablization, and immunofluorescence staining was performed as described previously [Muromoto et al., 2006]. The following antibodies were used: mouse anti-FLAG antibody (Sigma), rabbit anti-PDI antibody (Cell Signaling, Danvers, MA) as well as Alexa Fluor® 568 goat anti-mouse IgG and Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen). The nuclei of cells were stained with DAPI (Invitrogen, Eugene, OR). The stained samples were visualized with LSM 5 PASCAL (Carl Zeiss, Tokyo, Japan) according to manufacturer protocols.

ANALYSIS OF PROTEIN STABILITY

Twenty-four hours after transient transfection with indicated plasmids, HeLa cells were plated out onto 24 well plates. The cells were starved for 5 h in 500 μl of cysteine and methionine free medium (Invitrogen) supplemented with 10% methionine free FBS (Sigma), pulse-labeled in 200 μCi/ml of ^{35}S-methionine (MP Biomedicals, Solon, OH) for 1 h. After rinsing with chase medium (DMED supplemented with 10% FBS and 20 mM methionine and 20 mM cysteine), the cells were cultured for 0, 1, 2, and 3 days. The cultured cells were lysed with lysis buffer and precipitated with ANTI-FLAG® M2-Agarose Affinity Gel (Sigma) for 4 h. The eluted proteins were separated by 5–20% SDS–polyacrylamide gels.

The gels were dried and autoradiographed with BAS-2500 (FUJIFILM, Tokyo, Japan). The radioactivity of each specific band was calculated using Image Gauge V4.22 (FUJIFILM) software.

CALCIUM MEASUREMENT

For Ca^{2+} measurement, 1×10^7 DT40 cells were incubated with 5 μM Fluo4-AM (Invitrogen) and 1.25 mM Probenecid (Sigma) in 500 μl of RPMI medium containing 10% FBS and 1% chiken serum for 60 min at 37°C. Then cells were washed several times with Ca^{2+}-free Tyrode buffer. Before Ca^{2+} measurement, cells were pretreated with Tyrode buffer containing 0.5 mM EGTA (Sigma). Cells were treated with 10 μM thapsigargin (TG; Sigma) for 300 s, then the extracellular Ca^{2+} concentration was restored to 2 mM. Changes in fluorescence intensity were monitored using FACS Calibur (BD, Tokyo, Japan).

IDENTIFICATION OF STIM1-ASSOCIATED MOLECULES

After 293T cells were transfected with STIM1(Full)-FLAG, the cells were lysed with lysis buffer (1% digitonin was used as a detergent), and subject to immunoprecipitation with ANTI-Flag® M2-Agarose Affinity Gel for over night at 4°C. The immunoprecipitated proteins were separated by electrophoresis on 5–20% SDS–polyacrylamide gels. Then, proteins on the gels were visualized with silver-staining (Wako, Osaka, Japan) according to manufacturer protocols.

After the completion of staining, the bands of interest were excised from the gel and digested with TrypsinGold (Promega). The digested samples were analyzed by nanocapillary reversed-phase LC-MS/MS using a C18 column (φ 75 μm) on a nanoLC system (Ultimate, LC Packing, Kent City, MI) coupled to a quadrupole time-of-flight mass spectrometer (QTOF Ultima, Waters, Milford, MA). Direct injection data-dependent acquisition was performed using one MS channel for every three MS/MS channels and a dynamic exclusion for selected ions. Proteins were identified by database searching (Mascot Daemon, Matrix Science, Boston, MA). Search parameters were as follows: MS tolerance of 0.2, MS/MS tolerance of 0.4 Da, protease specificity allowing for one missed cleavage site, fixed modification of carbamidomethylation of cysteine residues.

RESULTS

THE CYTOPLASMIC REGION OF STIM1 IS ESSENTIAL FOR ITS LOCALIZATION IN ER

Because STIM1 does not have a typical ER-retention sequence [Soboloff et al., 2006b; Baba and Kurosaki, 2009], it is a riddle that STIM1 mainly localizes in ER. To analyze the peculiar manner of its intracellular distribution, we constructed a series of deletion mutants as illustrated in Figure 1A. HeLa cells were transfected with each plasmid of STIM1(EX, EX + TM, EX + TM + CC, Full)-FLAG, and the protein synthesis was confirmed with Western blot using anti-FLAG antibody. Each deletion mutant protein was detected at the predicted sizes although the band for a mutant deleted both transmembrane and cytoplasmic domains was very faint (Fig. 1B, lower panel). We then analyzed protein trafficking of each mutant. After the transfection of plasmids into HeLa cells, their supernatant and cell lysates were prepared and subjected to Western blot. A specific band was detected in supernatant of HeLa cells transfected with STIM1(EX)-FLAG, but the bands for other transfectants is not clearly detected (Fig. 1B, upper panel). After surface proteins on each transfectant were labeled with biotin, the cell lysates were analyzed with Western blot using HRP-streptavidin or anti-FLAG antibody. As shown in Figure 1C, all STIM1 mutants excluding STIM1(EX) were efficiently expressed (detected by anti-FLAG antibody), but only STIM1(EX + TM) was massively expressed on the cell surface (detected by HRP-avidin). In addition, surface expressed STIM1(EX + TM + CC) was only faintly detected similar to STIM1(Full). These results indicate that the cytoplasmic segment (AA 246-593) containing the coiled-coil domains may determine the intracellular distribution of STIM1. In other words, STIM1 lacking its cytoplasmic region is likely to traffic from ER to golgi apparatus, resulting in the surface expression or secretion. In contrast, STIM1 with its cytoplasmic region tends to be restored in cytoplasm. This possibility was confirmed by experiments with a confocal microscopy. When STIM1(EX) lacking a transmembrane domain was expressed, only small amount of the protein was detected (Fig. 2), confirming that it was secreted as shown in Figure 1B. The other mutants, STIM1(Full) and STIM1(EX + TM + CC), were restored in ER, which was oriented by PDI-staining. In contrast, the distribution of STIM1(EX + TM) was not restricted to ER. Similar distribution patterns of STIM1 mutants were also observed in 293T cells (data not shown). Therefore, the coiled-coil domains of STIM1 critically contribute to its localization in ER where STIM1 mainly works as a Ca^{2+}-sensor.

THE CYTOPLASMIC REGION OF STIM1 YIELDS ITS PROTEIN STABILITY

Figure 2 showed that HeLa cells transfected with STIM1(Full)-FLAG and STIM1(EX + TM + CC)-FLAG, but not those with STIM1(EX + TM)-FLAG, changed the shape of ER to "the distended form," where STIM1 proteins seemed to be massively accumulated in ER. This might indicate that the cytoplasmic region corresponding to the coiled-coil domains has some specific effects, such as STIM1-stabilization. To clarify this possibility, we analyzed a half-life of each STIM1 mutant. After the transfection of plasmids for the deletion mutants into HeLa cells, the newly synthesized proteins were labeled with ^{35}S-methionin. The cells were lysed and subjected to immunoprecipitation using anti-FLAG antibody after the indicated periods of cultures. As shown in Figure 3, the half-life of STIM1(Full) was approximately 19 h. Although STIM1(EX + TM + CC) exhibited similar half-life to STIM1(Full), STIM1(EX + TM) disappeared more rapidly. Similar results were obtained in 293T cells as well as HeLa cells (data not shown). Therefore, the coiled-coil domains have an ability to stabilize STIM1 proteins.

THE CYTOPLASMIC REGION OF STIM1 IS ESSENTIAL FOR ACTIVATING SOCE

To validate physiological roles of cytoplasmic region of STIM1 in activating SOCE, we established STIM1-deficient DT40 B cells stably expressing STIM1(Full) and STIM1(EX + TM) (Fig. 4A), and examined whether these stably transfected cells have an ability to restore SOCE. As shown in Figure 4B, the reconstitution of SOCE was observed in DT40 cells expressing STIM1(Full), but not in those expressing STIM1(EX + TM). This data indicate that cytoplasmic region of STIM1 is essential for activating SOCE.

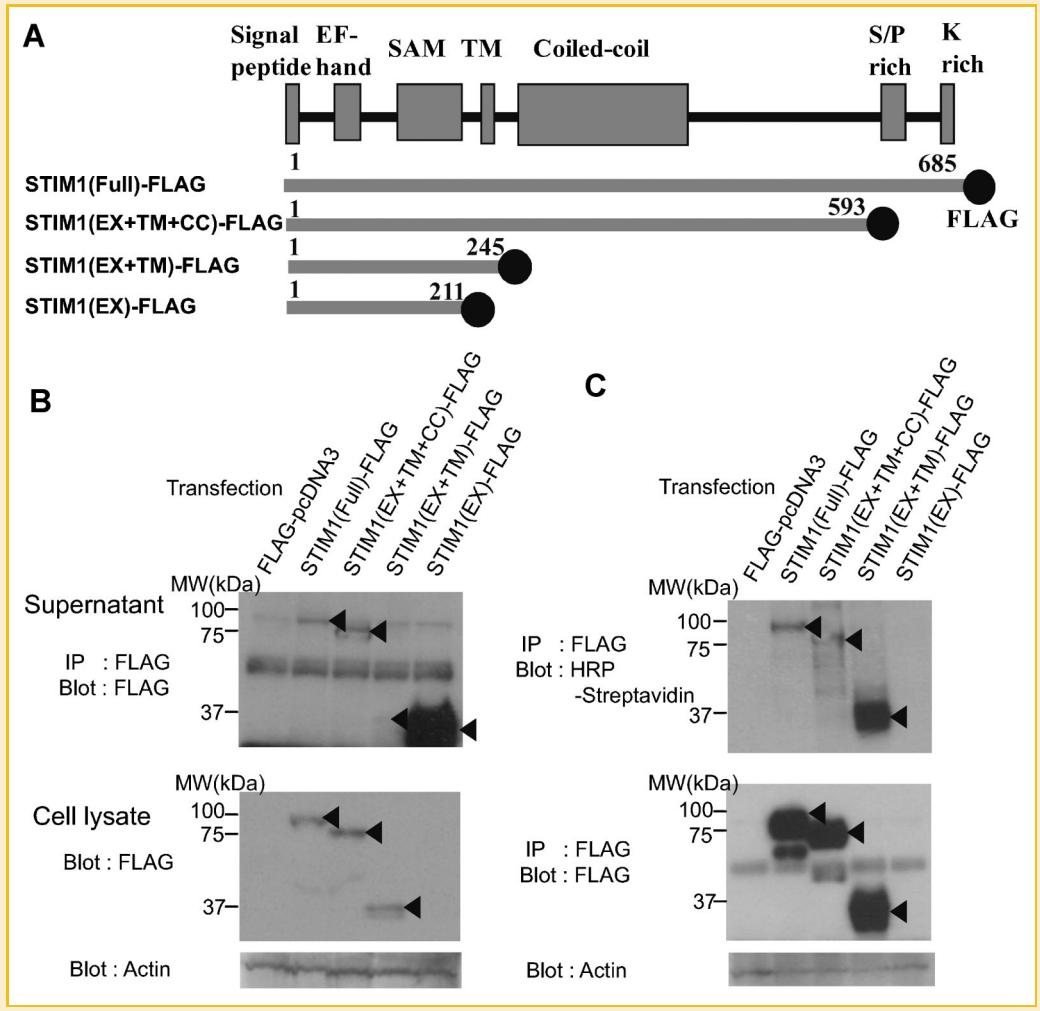


Fig. 1. The coiled-coil domains of STIM1 contribute ER-retention. A: Schematic representation of STIM1 functional domains and STIM1 mutants. TM, transmembrane domain; S/P rich, Ser/Thr-rich domains; K rich, Lysine-rich C-terminal domain. B: HeLa cells were transfected with the indicated STIM1 mutant expression plasmids, and their supernatant and cell lysates were collected after 24 h of cultures. Each supernatant was precipitated with ANTI-FLAG® M2-Agarose Affinity Gel. The precipitates as well as cell lysates were subjected to Western blot using an anti-FLAG antibody. One representative of three independent experiments is shown. Specific bands for each STIM1 mutant were shown by arrows. The membrane was deprobed and stained with anti-actin antibody for a loading control. C: Twenty-four hours after transient transfection of the indicated STIM1 mutant expression plasmids to HeLa cells, their cell surface proteins were biotinylated. Then, the biotin-labeled HeLa cells were lysed, precipitated with ANTI-FLAG® M2-Agarose Affinity Gel, and Western blotted with anti-FLAG and HRP-streptavidin. Specific bands for each STIM1 mutant were shown by arrow heads. The membrane was deprobed and stained with anti-actin antibody for a loading control. Similar results were obtained in three independent experiments.

IDENTIFICATION OF STIM1-ASSOCIATED PROTEINS

In the present study, we showed essential roles of the coiled-coil domains in the ER-retention as well as the protein-stabilization of STIM1. We also indicated that cytoplasmic regions of STIM1 are critical for activating SOCE. Recent reports have suggested that the C-terminal region of STIM1 is critical for the binding to the CRAC activating domain of Orai1 [Park et al., 2009; Yuan et al., 2009]. Other reports also indicated that the polybasic C-termini of STIM1 binds to calmodulin [Bauer et al., 2008] and that the short polypeptide motif (SxIP) of STIM1 associates with the microtubule-plus-end-tracking protein end binding protein-1 (EB1) [Honnappa et al., 2009]. Thus, it is likely that STIM1 may associate with several proteins, which modulate functions and/or protein characteristics of STIM1. To identify STIM1 associated molecules comprehensively, we performed co-immunoprecipitation experiments combined with mass spectrometry. After 293T cells were transfected with STIM1(Full)-FLAG or the empty FLAG-pcDNA3 plasmid, the cell lysates from each transfectant were prepared and subjected to immunoprecipitation with anti-FLAG antibody. When the immunoprecipitated proteins in the gel were visualized with silver staining, several specific bands were detected in the lane for the STIM1(Full)-transfected samples (Fig. 5A). Each specific band was then digested in gel with trypsin and subjected to LC-MS/MS analysis. As shown in Figure 5B, the approximately 80–90 kDa band (band 2) contains STIM1 in all experiments. Of importance, calnexin, which acts as a chaperone protein and exists in ER [Ni and Lee, 2007; Caramelo and Parodi, 2008], was always identified in the band 2 at a high score. Exportin1, transportin1, and

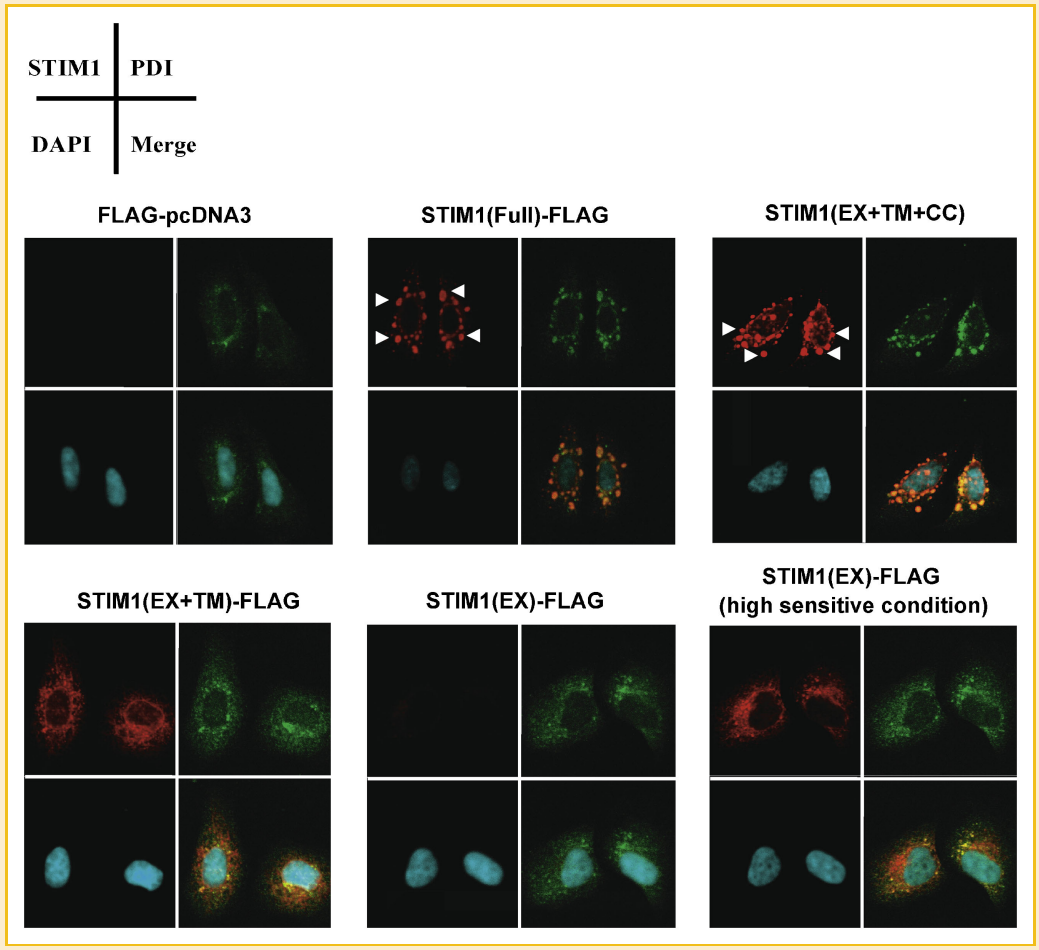


Fig. 2. Overexpression of STIM1 induces shape changes in ER. HeLa cells were transfected with the indicated STIM1 mutant expression plasmids. After 24 h, each transfectants was fixed and reacted with mouse anti-FLAG M2 and rabbit anti-PDI antibodies, and visualized with Alexa Fluor® 568 goat anti-mouse IgG and Alexa Fluor® 488 goat anti-rabbit IgG. PDI was used for the indication of ER. The same slide also was stained with DAPI for the nuclei staining. All images were taken with confocal microscopy. White arrow heads indicate the distended form of ER. Similar results were obtained in six independent experiments.

importin, all of which are involved in protein trafficking [Siomi et al., 1997; Hutten and Kehlenbach, 2007; Okada et al., 2008], were frequently scored in the approximately 100–110 kDa band (band 1). Because all bands included keratin protein, it seemed to be non-specific. Calnexin, exportin1, transportin1, or importin was never scored in the equivalent negative control samples. Therefore, we identified calnexin, exportin1, transportin1, and importin as possible STIM1-associated proteins in cells.

CALNEXIN, EXPORTIN1, AND TRANSPORTIN1 BIND TO BOTH STIM1 AND STIM2

The binding specificity between STIM1 and calnexin was confirmed with the co-immunoprecipitation studies. Using the cell lysates from 293T as well as HeLa cells transfected with STIM1(Full) or FLAG-pcDNA3 plasmid, immunoprecipitation with anti-FLAG antibody was performed. The precipitates for STIM1 contained calnexin, as expected (Fig. 6A and data not shown). Similarly, the precipitates contained exportin1 and transportin1. However, it was not clear whether the precipitates contained importin (data not shown). The specific association of STIM1 with calnexin, exportin1, and transportin1 were also observed even when we used HeLa cells (data not shown). To assess whether this association is physiological, cell lysates from HeLa were immunoprecipitated with anti-STIM1 antibody and were subjected to Western-blot using anti-calnexin, anti-exportin1, and anti-transportin1. As shown in Figure 6B, the precipitates for endogenous STIM1 in HeLa cells contained calnexin, exportin1 and transportin1, indicating that STIM1 physiologically interacts with these proteins. However, the binding of STIM1 to these molecules is unlikely to be depending on Ca^{2+} because it was observed even in the presence of EDTA (data not shown). We analyzed whether these molecules can bind to STIM2, which has a high homology with STIM1. After transient transfection of plasmid for FLAG-tagged STIM2 into 293T cells, the cell lysate was immunoprecipitated with anti-FLAG antibody and subjected to Western-blotting analysis. The immunoprecipitates for STIM2 also contained calnexin, exportin1, and transportin1, similarly to those

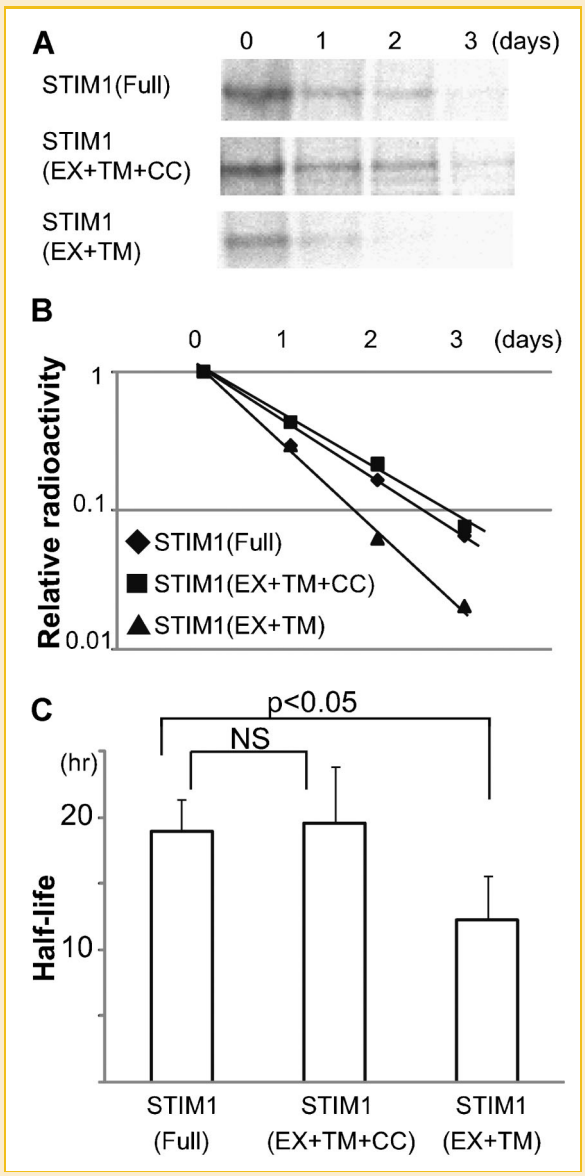


Fig. 3. The coiled-coil domains of STIM1 contribute its protein stability. A: After transient transfection of the indicated plasmids to HeLa cells, the cells were pulse-labeled with ^{35}S-methionine for 1 h and then chased for the indicated periods. The labeled cells were lysed and subjected to the precipitation with ANTI-FLAG® M2-Agarose Affinity Gel. The precipitates were separated on SDS–PAGE gels, and the gels were dried, and autoradiographed. B: The radioactivity of specific bands corresponding to each STIM1 mutant was measured with ImageGauge 4.22 software. Means of three independent experiments are shown. The plot uses logarithmic scaling on the vertical line. C: Data are shown as mean ± SD of half life of STIM1 mutant proteins in three independent experiments. NS, not significant differences.

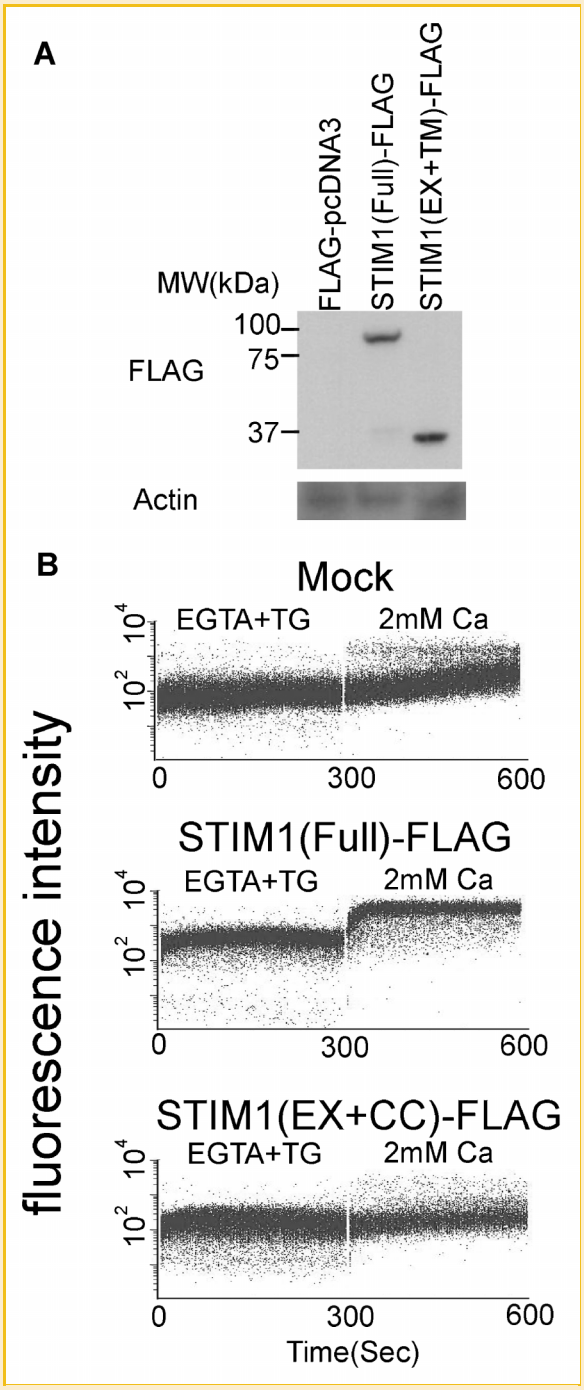
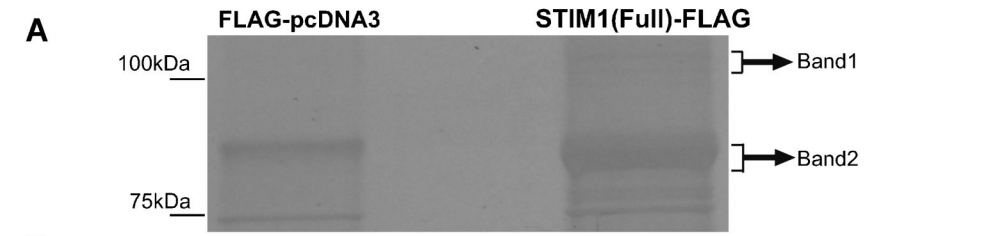


Fig. 4. The cytoplasmic region of STIM1 is essential for activating SOCE. A: Plasmids for FLAG-pCDNA3, FLAG-tagged STIM1(Full), and STIM1(EX + TM) were stably transduced in STIM1-deficient DT40 B cells, and each expressed protein was detected with Western-blot using anti-FLAG antibody. Actin was detected as a loading control. B: Intracellular Ca^{2+} mobilization was monitored by Fluo4 imaging in DT40 cells expressing STIM1(Full) and STIM1(EX + TM). The transfected DT40 cells were treated with 10 μM thapsigargin(TG) in the absence of extracellular Ca^{2+} (0.5 mM EGTA) to deplete Ca^{2+} stores. Ca^{2+} influx was monitored after adding 2 mM Ca^{2+} in each DT40 cell clone. Data are representative of three independent experiments.

for STIM1 (Fig. 6A). Therefore, both STIM1 and STIM2 can associate with calnexin, exportin1, and transportin1.

CALNEXIN BINDS TO STIM1 INDEPENDENTLY OF GLYCOSYLATION STATE

Calnexin is a transmembrane protein localized in ER. Calreticulin, an ER-resident soluble protein, has high sequence homology with



B

	Sample1 protein hits	Score	Sample2 protein hits	Score	Sample3 protein hits	Score
Band1	Exportin 1 (human)	700	Keratin, type2 cytoskeletal 1 (human)	462	Importin subunit beta-1 (human)	1011
	Exportin 2 (human)	649	Importin subunit beta-1 (human)	306	Transportin 1 (human)	376
	Keratin, type2 cytoskeletal 1 (human)	346	Transportin 1 (human)	176	Keratin, type2 cytoskeletal 1 (human)	348
	Importin subunit beta-3 (human)	69				
Band2	Stromal interaction molecule 1 (mouse)	1383	Stromal interaction molecule 1 (mouse)	1755	Stromal interaction molecule 1 (mouse)	2007
	Calnexin precursor (human)	437	Keratin, type1 cytoskeletal 9 (human)	307	Calnexin (human)	295
	Keratin, type1 cytoskeletal 9 (human)	40	Calnexin (human)	214	Keratin, type1 cytoskeletal 10 (human)	262

Fig. 5. Identification of STIM1 associated molecules. A: 293T cells were transfected with STIM1(Full)-FLAG or FLAG-pcDNA3 (control). After 3 days, each transfectant (approximately 2×10^8 cells) was lysed and precipitated with ANTI-FLAG® M2-Agarose Affinity Gel. The precipitates were electrophoresed on SDS-PAGE gels, and the gels were stained with silver staining. The specific bands in the lane for STIM1(Full)-FLAG-transfected samples were indicated as band 1 and 2. B: The specific bands were excised from the gel, digested with TrypsinGold, and subjected to mass spectrometry. The analyzed data of three independent experiments for band 1 and 2 are shown with score.

calnexin [Ni and Lee, 2007; Caramelo et al., 2008]. Both proteins are sugar-binding chaperones, which recognize $Glc_1Man_9GlcNAc_2$ structure during a transient oligosaccharide-processing of newly synthesized glycoproteins. Thus, we investigated whether calreticulin, like calnexin, can bind to STIM1. In both HeLa and 293T cells, the immunoprecipitates for STIM1 contained calnexin, but not calreticulin (Fig. 6C), suggesting that the binding between STIM1 and calnexin was specific and that the binding might not be depending on glycosylation state. Thus, we analyzed the binding between STIM1 and calnexin in HeLa cells treated with tunicamycin, a glycosidase inhibitor. As shown in Figure 7A, tunicamycin-treatment of HeLa cells did not cancel the recognition of STIM1 by calnexin. In addition, calnexin could still recognize a STIM1(del-Gly) mutant whose two Asn-linked glycosylation sites were destroyed (Fig. 7B). Therefore, glycosylation on STIM1 is not required for the association between STIM1 and calnexin.

DISCUSSION

Despite a great number of recent studies showing the molecular organization of STIMs, which link Ca^{2+}-store depletion, several aspects remain to be resolved. We are now uncertain how STIM1 retains in ER, how the protein content of STIM1 is regulated, how STIM1 travels in parallel with Ca^{2+} concentration, and so on. In the present study, experiments using the deletion mutants of STIM1 revealed that the coiled-coil domains are critical for its ER-retention and protein stability, and that the cytoplasmic region is essential for activating SOCE. In addition, we identified calnexin, exportin1, and transportin1 as new STIM-associated proteins. These findings could help us understand functional mechanisms and protein characters of STIMs.

Once the nascent polypeptide chains emerge into an ER lumen, they receive a series of modifications, such as signal peptide cleavage, glycosylation, protein folding, and disulfide-bond formation [Bauer et al., 2008; Honnappa et al., 2009]. In ER, newly synthesized proteins are protected by chaperones, which are involved in the quality control of newly synthesized proteins. Only correctly folded proteins can traverse the secretory pathway leading to Golgi apparatus while misfolded and/or abnormal proteins can not exit from ER and are degraded. With regard to the trafficking of proteins between ER and Golgi, there are two ways of transports: the anterograde ER-to-Golgi transport and the retrograde Golgi-to-ER transport [Hawes et al., 2008; Spang, 2009]. Thus, the balance between anterograde and retrograde transports determines the steady-state distribution of proteins. As widely accepted, STIM1 mainly localizes in ER. With a signal trap method, we first cloned STIM1 (we originally named SIM) as a protein which has a signal peptide at N-terminus [Oritani and Kincade, 1996]. We produced and screened fusion proteins composed of cDNA-derived protein (N-terminal portion), a HPC4-tag, and a tissue factor-transmembrane. In this case, our isolated STIM1 cDNA did not contain the cytoplasmic regions. That is likely to be a reason why we recognized STIM1 as a novel cell surface protein. In general, a large number of ER-resident proteins have ER-retention signals, such as KDEL (or similar sequences) or KKXX/KXKXX, at their C-terminal position [Munro and Pelham, 1987; Nilsson et al., 1989; Jackson et al., 1990]. Of importance, STIM1 sequence does not have a possible ER-retention signal while STIM2 sequence does. Our present data strongly indicated that the coiled-coil domains were essential for the ER-retention of STIM1. It is noteworthy that some ER-resident proteins do not have ER-retention signals mentioned above. For example, an ER-localized protein, Sec71p does not have a typical ER-retention motif, but contains a Rer1p-recognized region in its transmembrane domain [Sato et al., 1997]. In the case of STIM1, there might be a hidden ER-retention sequence in the cytoplasmic region corresponding to the coiled-coil domains, similarly to the

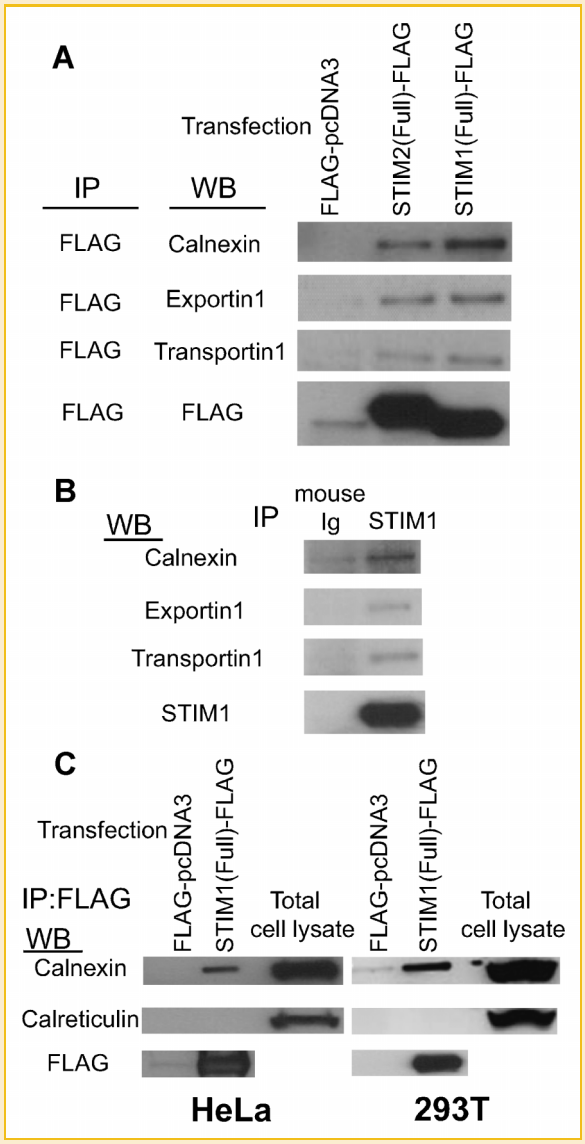


Fig. 6. Both STIM1 and STIM2 associate with calnexin, exportin1, and transportin1. A: After transient transfection of STIM1(Full)-FLAG or STIM2(-Full)-FLAG to 293T cells, each transfectant was lysed, precipitated with ANTI-FLAG® M2-Agarose Affinity Gel, and subjected to Western blot using specific antibodies against the indicated molecules. B: Cell lysates of HeLa cells were immunoprecipitated with anti-STIM1 antibody, and the precipitates were subjected to Western blot using specific antibodies against the indicated molecules. C: After transient transfection of STIM1(Full)-FLAG to 293T or HeLa cells, the transfectants were lysed and immunoprecipitated with ANTI-FLAG® M2-Agarose Affinity Gel, and subjected to Western-blotting analysis using specific antibodies against the indicated molecules. The cell lysates of 293T or HeLa cells were used as controls for positive staining. Similar results were obtained in three independent experiments.

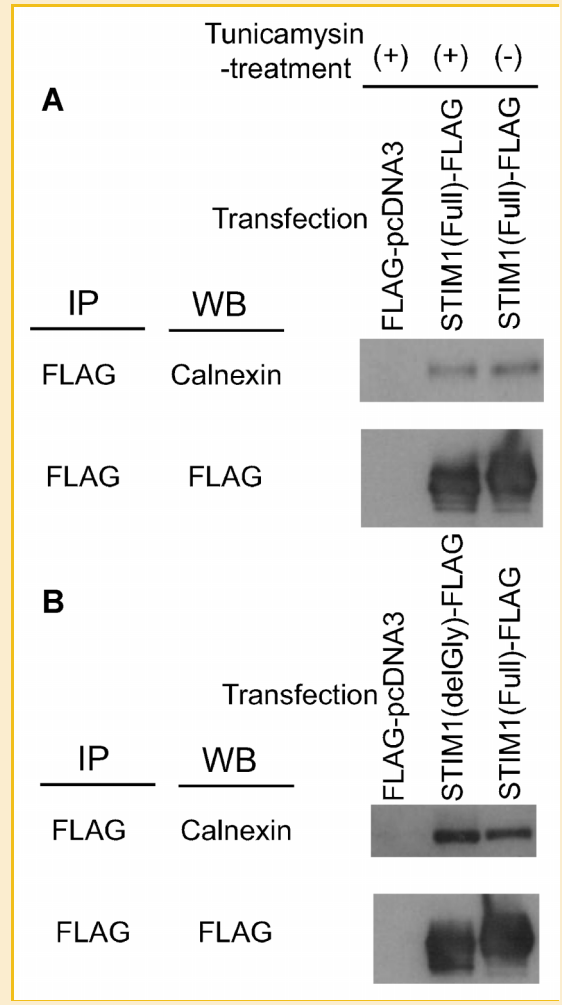


Fig. 7. Calnexin binds to STIM1 independently of glycosylation state. A: HeLa cells were transfected with STIM1(Full)-FLAG or FLAG-pcDNA3. After 12 h of cultures, the transfectants were treated with or without tunicamycin (10 μM) for 2 days. The treated cells were then lysed, precipitated with ANTI-FLAG® M2-Agarose Affinity Gel, and subjected to Western blot using anti-calnexin antibody. Similar results were obtained in three independent experiments. B: HeLa cells were transfected with STIM1(Full)-FLAG, STIM1(delGly)-FLAG or FLAG-pcDNA3. Each transfectant was lysed, precipitated with ANTI-FLAG® M2-Agarose Affinity Gel, and subjected to Western blot using anti-calnexin antibody. Similar results were obtained in three independent experiments.

case of Sec71p. Alternatively, some association molecules of the coiled-coil domains might provide ER-retention signals.

Although the distended ER might be an artifact by over-expression systems, it clearly means the capacity of the expressed proteins to be accumulated in ER. STIM1 mutants (Full and EX + TM + CC), which change the shape of ER to "the distended form," exhibited lower potential to be expressed on cell surface. In addition, both mutants had longer half-life of proteins than those without the distention of ER. Of importance, the coiled-coil domains of STIM1 are involved in both the ER-retention and the protein stability. It should be resolved how the coiled-coil domains of STIM1 exhibit these interesting roles in cells, and experiments are now in progress in this regard.

We identified exportin1, transportin1, and calnexin as novel STIM-associated proteins. Exportin1 and transportin1 belong to a family of nuclear transport receptors. These receptors recognize the nuclear localization or export signals on protein or RNA cargoes, and then transport the complexes across nuclear pores [Siomi et al., 1997; Hutten and Kehlenbach, 2007]. Of course, our data can not

completely exclude problems resulting from the nonspecific binding. However, the immunoprecipitates for STIM1 selectively contained the two kinds of the transport receptors repeatedly, suggesting that the binding of STIMs to exportin1 and transportin1 might play a physical role although STIM proteins mainly exist in ER. Together, these results might be indicating a more complex mode of interactions. With regard to calnexin, it is a type I ER membrane protein and functions as a carbohydrate-binding chaperone [Ni and Lee, 2007; Caramelo and Parodi, 2008]. Shortly after the addition of core oligosaccharide complex to nascent polypeptides, their terminal two glucose residues are removed by glycosidases I and II. This modification generates monoglycosylated oligosaccharides to which calnexin can bind. Further processing of oligosaccharides results in the release of newly synthesized glycoproteins from calnexin. Because STIM1 sequence has two putative Asn-linked glycosylation sites, STIM1, like other glycoproteins, might use calnexin for its quality control in ER. On the other hand, calnexin also has an ability to associate with several proteins independently of its glycosylation state. Calnexin variants carrying point mutations in the lectin site can not recognize oligosaccharides but retain the ability to bind to ERp57 [Leach and Williams, 2004]. Another example is the case of ER degradation enhancing α-mannosidase-like protein (EDEM). The transmembrane domain of calnexin is known to bind to EDEM [Oda et al., 2003]. As described here, the treatment of HeLa cells with a glycosidase-inhibitor could not prevent the interaction between STIM1 and calnexin. In addition, a STIM1 mutant whose Asn-glycosylation sites were destroyed could still bind to calnexin. These results seem to indicate that the binding between STIM1 and calnexin does not require glycosylation, similar to the case of ERp57 or EDEM. Thus, calnexin may function on STIM1 beyond a chaperone protein.

Targeting of the calnexin or its related molecule calreticulin gene in mice has brought severe consequences. Calreticulin-deficient mice are embryonic lethal via cardiac defects, and their phenotype was rescued by the overexpression of activated calcineurin, which is a Ca^{2+}/calmodulin-dependent protein phosphatase [Guo et al., 2002]. Calnexin-deficient mice are viable but show neurological abnormalities [Denzel et al., 2002]. In addition, its important binding partner ERp57 is known to associate with and influence the SERCA2b Ca^{2+} pump [Li and Camacho, 2004]. Thus, calnexin/calreticulin systems presumably play roles in multiple processes beyond their functions of protein folding. For example, there are possible influences in ER Ca^{2+} homeostasis as well as cell shape, adhesion, and motility. Because STIMs are important Ca^{2+} sensors in ER, the association between STIMs and calnexin might have physical roles in intracellular Ca^{2+} regulation.

To date, several STIM-associated molecules have been reported. Overexpressed STIM1 associates with microtubule cytoskeleton, especially with EB1 [Honnappa et al., 2009]. The involvement of microtubule cytoskeleton in STIM functions has also been indicated [Smyth et al., 2007]. However, these associations are limited to the restricted cell types, and can explain only a part of regulatory mechanisms for STIM functions and/or stability. In the present study, our immunoprecipitation combined with mass spectrometry analysis for STIM1-associated molecules identified calnexin, exportin1, and transportin1. Although we used digitonin as a detergent in this screening, other STIM1-interacting molecules may be identified by utilizing CHAPS as another weak detergent. The coiled-coil regions, which we here demonstrated as interesting domains, could be suitable as bait for the screening. We will continue additional rounds of screenings with minor modifications. Further information concerning regulatory mechanisms for STIM proteins will provide a model of Ca^{2+} control as well as a useful strategy to develop therapeutic drugs for intracellular Ca^{2+}-related diseases, including inflammation and allergy.

REFERENCES

Baba Y, Kurosaki T. 2009. Physiological function and molecular basis of STIM1-mediated calcium entry in immune cells. Immunol Rev 231:174–188.

Bauer MC, O'Connell D, Cahill DJ, Linse S. 2008. Calmodulin binding to the polybasic C-termini of STIM proteins involved in store-operated calcium entry. Biochemistry 47:6089–6091.

Berridge MJ, Lipp P, Bootman MD. 2000. The versatility and universality of calcium signaling. Nat Rev Mol Cell Biol 1:11–21.

Berridge MJ, Bootman MD, Roderick HL. 2003. Calcium signalling: Dynamics, homeostasis and remodeling. Nat Rev Mol Cell Biol 4:517–529.

Caramelo JJ, Parodi AJ. 2008. Getting in and out from calnexin/calreticulin cycles. J Biol Chem 283:10221–10225.

Denzel A, Molinari M, Trigueros C, Martin JE, Velmurgan S, Brown S, Stamp G, Owen MJ. 2002. Early postnatal death and motor disorders in mice congenitally deficient in calnexin expression. Mol Cell Biol 22:7398–7404.

Guo L, Nakamura K, Lynch J, Opas M, Olson EN, Agellon LB, Michalak M. 2002. Cardiac-specific expression of calcineurin reverses embryonic lethality in calreticulin-deficient mouse. J Biol Chem 277:50776–50779.

Hawes C, Osterrieder A, Hummel E, Sparkes I. 2008. The plant ER-Golgi interface. Traffic 9:1571–1580.

Honnappa S, Gouveia SM, Weisbrich A, Damberger FF, Bhavesh NS, Jawhari H, Grigoriev I, van Rijssel FJ, Buey RM, Lawera A, Jelesarov I, Winkler FK, Wüthrich K, Akhmanova A, Steinmetz MO. 2009. An EB1-binding motif acts as a microtubule tip localization signal. Cell 138:366–376.

Hutten S, Kehlenbach RH. 2007. CRM1-mediated nuclear export: To the pore and beyond. Trends Cell Biol 17:193–201.

Jackson MR, Nilsson T, Peterson PA. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J 9:3153–3162.

Leach MR, Williams DB. 2004. Lectin-deficient calnexin is capable of binding class I histocompatibility molecules in vivo and preventing their degradation. J Biol Chem 279:9072–9079.

Li Y, Camacho P. 2004. Ca^{2+}-dependent redox modulation of SERCA 2b by ERp57. J Cell Biol 164:35–46.

Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE, Jr., Meyer T. 2005. STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. Curr Biol 15:1235–1241.

Liou J, Fivaz M, Inoue T, Meyer T. 2007. Live-cell imaging reveals sequential oligomerization and local plasma membrane targeting of stromal interaction molecule 1 after Ca^{2+} store depletion. Proc Natl Acad Sci USA 104:9301–9306.

Luik RM, Wu MM, Buchanan J, Lewis RS. 2006. The elementary unit of store-operated Ca^{2+} entry: Local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. J Cell Biol 174:815–825.

Munro S, Pelham HR. 1987. A C-terminal signal prevents secretion of luminal ER proteins. Cell 48:899–907.

Muromoto R, Ishida M, Sugiyama K, Sekine Y, Oritani K, Shimoda K, Matsuda T. 2006. Sumoylation of Daxx regulates IFN-induced growth suppression of B lymphocytes and the hormone receptor-mediated transactivation. J Immunol 177:1160–1170.

Ni M, Lee AS. 2007. ER chaperones in mammalian development and human diseases. FEBS Lett 581:3641–3651.

Nilsson T, Jackson M, Peterson PA. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell 58:707–718.

Oda Y, Hosokawa N, Wada I, Nagata K. 2003. EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science 299:1394–1397.

Okada N, Ishigami Y, Suzuki T, Kaneko A, Yasui K, Fukutomi R, Isemura M. 2008. Importins and exportins in cellular differentiation. J Cell Mol Med 12:1863–1871.

Oritani K, Kincade PW. 1996. Identification of stromal cell products that interact with pre-B cells. J Cell Biol 134:771–782.

Park CY, Hoover PJ, Mullins FM, Bachhawat P, Covington ED, Raunser S, Walz T, Garcia KC, Dolmetsch RE, Lewis RS. 2009. STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. Cell 136:876–890.

Putney JW, Jr. 1986. A model for receptor-regulated calcium entry. Cell Calcium 7:1–12.

Putney JW, Jr. 2007. New molecular players in capacitative Ca^{2+} entry. J Cell Sci 120:1959–1965.

Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Veliçelebi G, Stauderman KA. 2005. STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J Cell Biol 169:435–445.

Sato K, Sato M, Nakano A. 1997. Rer1p as common machinery for the endoplasmic reticulum localization of membrane proteins. Proc Natl Acad Sci USA 94:9693–9698.

Sekine Y, Yamamoto T, Yumioka T, Sugiyama K, Tsuji S, Oritani K, Shimoda K, Minoguchi M, Yoshimura A, Matsuda T. 2005. Physical and fnctional interactions between STAP-2/BKS and STAT5. J Biol Chem 280:8188–8196.

Siomi MC, Eder PS, Kataoka N, Wan L, Liu Q, Dreyfuss G. 1997. Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. J Cell Biol 138:1181–1192.

Smyth JT, DeHaven WI, Bird GS, Putney JW, Jr. 2007. Role of the microtubule cytoskeleton in the function of the store-operated Ca^{2+} channel activator STIM1. J Cell Sci 120:3762–3771.

Soboloff J, Spassova MA, Hewavitharana T, He LP, Xu W, Johnstone LS, Dziadek MA, Gill DL. 2006a. STIM2 is an inhibitor of STIM1-mediated store-operated Ca^{2+} Entry. Curr Biol 16:1465–1470.

Soboloff J, Spassova MA, Dziadek MA, Gill DL. 2006b. Calcium signals mediated by STIM and Orai proteins—A new paradigm in inter-organelle communication. Biochim Biophys Acta 1763:1161–1168.

Spang A. 2009. On vesicle formation and tethering in the ER-Golgi shuttle. Curr Opin Cell Biol 21:531–536.

Stathopulos PB, Zheng L, Li GY, Plevin MJ, Ikura M. 2008. Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. Cell 135:110–122.

Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, Kraft S, Turner H, Fleig A, Penner R, Kinet JP. 2006. CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science 312: 1220–1223.

Williams RT, Manji SS, Parker NJ, Hancock MS, Van Stekelenburg L, Eid JP, Senior PV, Kazenwadel JS, Shandala T, Saint R, Smith PJ, Dziadek MA. 2001. Identification and characterization of the STIM (stromal interaction molecule) gene family: Coding for a novel class of transmembrane proteins. Biochem J 357:673–685.

Wu MM, Buchanan J, Luik RM, Lewis RS. 2006. Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. J Cell Biol 174:803–813.

Yuan JP, Zeng W, Dorwart MR, Choi YJ, Worley PF, Muallem S. 2009. SOAR and the polybasic STIM1 domains gate and regulate Orai channels. Nat Cell Biol 11:337–343.

Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD. 2005. STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. Nature 437:902–905.

Zhang SL, Yeromin AV, Zhang XH, Yu Y, Safrina O, Penna A, Roos J, Stauderman KA, Cahalan MD. 2006. Genome-wide RNAi screen of $Ca(2^{+})$ influx identifies genes that regulate $Ca(2^{+})$ release-activated $Ca(2^{+})$ channel activity. Proc Natl Acad Sci USA 103:9357–9362.