Original Paper

Calsequestrin-1 Regulates Store-Operated Ca^{2+} Entry by Inhibiting STIM1 Aggregation

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Key Words

CSQ1 • STIM1 • Orai1 • SOCE • STIM1 Aggregation

Abstract

Background/Aims: Stromal interacting molecule-1 (STIM1) aggregation and redistribution to plasma membrane to interact with Orai1 constitute the core mechanism of store-operated Ca^{2+} entry (SOCE). Previous study has revealed that calsequestrin-1 (CSQ1) regulates SOCE in HEK293 cells through interacting with STIM1 and inhibiting STIM1/Orai1 interaction. Here, we further investigate how CSQ1/STIM1 interaction affects SOCE. **Methods:** Using confocal microscopy, STIM1 aggregation and co-localizations with CSQ1 or Orai1 upon Ca^{2+} store depletion by thapsigargin were measured and quantified by Imaris software in HeLa cells transfected with different CSQ1 mutants. The interactions of CSQ1/STIM1 and STIM1/Orai1, and internal Ca^{2+} changes were detected by co-immunoprecipitation and Fura2, respectively. **Results:** Wt-CSQ1 overexpression significantly reduced STIM1 clustering in the perimembrane and cytosolic regions, whereas over-expression of a C-terminal amino acid 362-396 deletion mutant (C35) did not. Consistently, a significant depression of SOCE, increased CSQ1 monomerization and CSQ1/STIM1 interaction, and a reduced STIM1/Orai1 association were observed in wt-CSQ1 but not in C35-transfected cells. Additionally, mutant lacking C-terminal AA 388-396 deletion exerted weaker potency in inhibiting STIM1 aggregation and association with Orai1 than wt-CSQ1. **Conclusions:** Our results demonstrate that CSQ1 monomers suppress SOCE by interacting with STIM1 and attenuating STIM1 aggregation via its C-terminal amino acid 362-396.

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Introduction

Almost all cell types rely on Ca^{2+} signals to maintain homeostasis at resting state and trigger specific cell responses upon stimulation. Store-operated Ca^{2+} entry (SOCE) is one of the mechanisms to control cytosolic Ca^{2+} signaling and endoplasmic reticulum (ER) Ca^{2+} storage [1, 2]. The core machinery of SOCE includes stromal interacting molecule-1 (STIM1)

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and plasma membrane (PM) Ca^{2+} release-activated Ca^{2+} channel pore forming subunit 1 (Orai1) proteins [3-5]. STIM1 is an ER transmembrane protein with a long, substantially α -helical cytosolic domain ending in a polybasic C-terminal membrane-binding domain. It triggers SOCE by sensing ER store depletion, translocating as oligomers to an ER membrane compartment closely juxtaposed to the adjacent PM, forming STIM1 clusters called 'puncta', and binding with Orai1 [4-6]. By a direct protein-protein interaction, the STIM1-Orai1 pathway enables Orai1 gating and Ca^{2+} influx through such an effectively demarcated microdomain for Ca^{2+} signaling [7-9].

Calsequestrin (CSQ) is the most abundant Ca^{2+} buffering protein within the sarcoplasmic reticulum (SER). CSQ type 1 (CSQ1) is the only isoform expressed in fast-twitch skeletal muscle, while equal amounts of CSQ1 and the so-called cardiac or CSQ2 isoforms are expressed in slow-twitch muscle [10, 11]. In addition, recent studies have demonstrated that CSQ1 also expresses in non-excitable cells and plays functional roles in buffering ER Ca^{2+} and controlling Ca^{2+} release [12, 13]. CSQ molecules exist as monomers when luminal Ca^{2+} concentration is lower than 10 μM . As luminal Ca^{2+} concentration increases, it undergoes molecule compaction, dimerization and polymerization, a stable form to bind with more Ca^{2+} [14, 15]. In addition to buffering Ca^{2+} inside SER, studies have shown that CSQ down-regulates SOCE in skeletal muscle [16-19] and in non-excitable cells [12, 13].

Our previous study has demonstrated a molecular mechanism underlying this negative regulation of CSQ1 on SOCE in HEK293 cells [13]. Upon ER store depletion, CSQ1 monomers increase and are able to interact with STIM1. Meanwhile, STIM1 is oligomerizing and clustering because of store empty and translocate to PM to interact with Orai1. However, the CSQ1 sequestration of STIM1 then somehow reduces STIM1 interacting with Orai1 and SOCE, providing a brake to prevent exaggerated activation of SOCE in physiological conditions. In this study, we further analyzed the aggregation and redistribution of STIM1 after depletion of Ca^{2+} stores by thapsigargin (TG) in HeLa cells, another commonly used cell line in the study of SOCE, and found that CSQ1 regulates SOCE by interacting with STIM1, causing inhibition of STIM1 aggregation and redistribution towards cell membrane.

Materials and Methods

Cell culture and transfection

HeLa cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in dulbecco's modified eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum in a humidified incubator at 37°C with 5% CO_2 . Cells were transfected with Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol using 2 $\mu\text{g}/\text{ml}$ in DMEM of each construct. HA-tagged human wild-type CSQ1 and its mutant cDNA were cloned into the p-EGFP-C2 expressing vector. The deletion mutants were C9 (deletion of wild-type amino acids 388-396) and C35 (deletion of wild-type amino acids 362-396). Nucleotide sequences of these constructs were verified by sequencing. These constructs (20 μg each) were transfected into HeLa cells for 36 h and protein expressions were confirmed by Western blotting assay.

Immunofluorescence experiment

HeLa cells were fixed in 4% formaldehyde for 20 min and permeabilized with HEPES-Triton (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl_2 , 0.5% Triton X-100) for 30 min at room temperature. For nuclear staining, cells were incubated with Hoechst 333242 for 10 min at room temperature. For the measurement of CSQ1 and STIM1 aggregation, HeLa cells expressing empty vectors or exogenous wt-CSQ1, C9 or C35 mutants were suspended in Ca^{2+} -free HBSS and then stimulated with 1 μM TG plus 5 μM ionomycin for 5 min. Then the cells were immune-labeled with mouse anti-STIM1 mAb diluted by 1:750. Alexa Fluor 594 goat-anti-mouse secondary antibody was used at 1:750 dilution ratio. The confocal image was taken by Leica SP8 microscopy, the pinhole 1.0 μm . The selection of STIM1 particles between 0.5 to 1.0 μm in radius were calculated automatically by Imaris Bitplane software program (Switzerland), as previously described [20].

Western blotting

HeLa cells were lysed in the ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, and 1% Noidet P-40, with a protease inhibitor cocktail). The protein sample (30 µg) was separated by sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE, 10%), and then transferred onto PVDF membrane. The membrane was blocked with 5% milk, incubated with the horseradish-conjugated secondary antibody, and reacted with enhanced chemiluminescence reagents.

Measurement of intracellular Ca^{2+}

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the fluorescent probe Fura2. HeLa cells suspensions were loaded with Fura2/AM in culture medium at 37°C for 30 min. After washing three times with 1.8 mM Ca^{2+} -containing HEPES-buffered saline solution (HBSS) and then Ca^{2+} -free HBSS, cells were suspended in Ca^{2+} free HBSS for subsequent experiments. The fluorescence was monitored in a stirred cuvette with fluorescence spectrophotometer HITACHI F-7000 at 37°C. The excitation wave-lengths were alternated between 340 and 380 nm and emission was measured at 510 nm. Changes in $[Ca^{2+}]_i$ were expressed as 340/380 nm fluorescence ratio (F340/F380) or Ca^{2+} concentration calibrated according to the method as previously described [21].

Chemical cross-linking

As previously described [22, 23], after transfected with three CSQ1 depletion mutants for 36 h, HeLa cells were suspended in Ca^{2+} -free PBS, and cross-linked with 2% (w/v) formaldehyde solution for 5 min, and finally terminated by the addition of a 1 M glycine stock solution, pH 8.0, to a final concentration of 0.1 M and incubation continued for 5 min on ice. Then, HeLa cells were collected and washed 3 times with ice-cold PBS. Finally, cells were lysed with lysis buffer supplemented with 1% SDS, 0.1% Triton-X 100, 1 mM PMSF, 10 µl protease inhibitor cocktail under constant agitation for 5 min at 4°C. The lysates were then centrifuged at 4°C, 15,000 g for 15 min and the supernatants were collected for Western blotting.

Co-immunoprecipitation

HeLa cells were harvested, washed with PBS and then lysed with lysis buffer, supplemented with freshly added 1mM PMSF, protease inhibitor cocktail. Then the lysates were centrifuged at 4°C, 12,000 g for 15 min. The supernatants were pre-cleared with rProtein G (for mouse monoclonal primary antibodies) or rProtein A (for rabbit polyclonal primary antibodies) beads and protein concentration was determined, using the Pierce BCA Protein Reagent Kit. The cell extracts were then adjusted to approximate 2 mg/ml total protein with lysis buffer and mixed with corresponding primary antibodies (0.2 µg/100 µg proteins) as indicated and incubated at 4°C overnight under constant agitation. Equal amounts of samples were mixed with normal IgG as negative controls. Beads were subsequently added to the mixtures (≥ 5 µl per 1 µg IgG) and agitation continued for 4 h at 4°C. The beads were then washed with lysis buffer and proteins were eluted by incubation in loading buffer at 95°C for 5 min. The eluted proteins were resolved on 10% SDS-PAGE and analyzed by Western blotting as described above.

Statistical analysis

Data are presented as the means $\pm$ SD of at least three independent measurements. Statistical comparisons between groups were carried out by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Co-localizations of endogenous CSQ1 with STIM1 and STIM1 with Orai1 after Ca^{2+} store depletion induced by TG in HeLa cells

Using Imaris Bitplane software, co-localization of two proteins can be quantified by the Pearson's coefficient value, a measurement that estimates the degree of overlap between fluorescence signals obtained in two channels. The degrees of co-localization from the Pearson's values are usually categorized as very strong (0.85~1.0), strong (0.49~0.84), moderate (0.1~0.48), weak (−0.26~0.09), and very weak (−1 to −0.27) as described previously [20, 24].

Fig. 1. Co-localization of endogenous CSQ1 and STIM1 in HeLa cells. HeLa cells cultured for 24 h were incubated in Ca^{2+} -free HBSS for 5 min and stimulated with 1 μM TG plus 5 μM ionomycin for 5 min. (A) Double immunostaining cells with specific antibody for STIM1 and Orai1, and the typical images show Orai1 and STIM1 fluorescence labeling. (B) Co-localization degrees of Orai1/STIM1, represented by Pearson's coefficient calculated using Imaris Bitplane software, are compared between control and TG-stimulated cells (see Methods). (C and D) STIM1 aggregation and redistribution in the perimembrane and cytosolic regions upon depletion of Ca^{2+} store by TG was analyzed by Imaris Bitplane software. (E) Double immunostaining cells with specific antibody for STIM1 and CSQ1, and the typical images of CSQ1 and STIM1 fluorescence labeling. (F) Analysis of the co-localization of CSQ1/STIM1 by Pearson's coefficient in control and TG-treated cells. (G) Double immunostaining cells with specific antibody for CSQ1 and Orai1, and the typical images of CSQ1 and Orai1 fluorescence labeling. (H) Analysis of co-localization of CSQ1/Orai1 by Pearson's coefficient. Scale bar: 10 μm . Arrows in panel A, E and G indicate the site of enlarged images showed below. All the data was presented by means $\pm$ SD from 40-84 cells from at least 3 independent experiments. ** $P < 0.01$ vs. control cells.

First, we identified possible changes in the endogenous interactions of CSQ1/STIM1 and STIM1/Orai1 during SOCE activation in HeLa cells. We double-labeled the cells with specific antibodies in the presence or absence of TG (1 μM) + ionomycin (5 μM) to deplete ER Ca^{2+} stores, and compared their co-localizations using Imaris Bitplane software. As Fig. 1A and B shown, the co-localization of STIM1 and Orai1 dramatically increased in cell PM following TG treatment, consistent with the current notion [25, 26]. Similarly, the STIM1 particles (radius in 0.5~1.0 μm) called puncta in perimembrane region (from membrane surface to 1.0 μm inside) and clusters located in cytoplasm calculated using Imaris Bitplane software were also increased (Fig. 1C, D), indicating a noticeable conformation of STIM1 and an activation of SOCE. In addition, consistent with our previous finding in HEK293 cells [13], a significant increase in the endogenous CSQ1 co-localization with STIM1 was found mainly in the cytosolic area in HeLa cells upon depletion of Ca^{2+} stores (Fig. 1E, F), but, little co-localization of CSQ1 and Orai1 was found in resting cells and in cells after TG treatment (Fig. 1G, H). Thus, these results indicate that CSQ1 interacts with STIM1 mainly in the cytosolic area rather than in the membrane region, and has no direct association with Orai1 in HeLa cells.

Fig. 2. Effects of carboxyl-terminal deletion mutants on CSQ1 monomerization and SOCE. (A) Exogenous wt-CSQ1, C9-CSQ1 mutant and C35-CSQ1 mutant expressions were performed in HeLa cells (see Methods). (B) Quantitative analysis of exogenous CSQ1 expression by Western blotting using anti-HA antibody. (C) Typical images show the expressions of SOC-associated proteins, STIM1 and Orai1 as well as endogenous CSQ1, before and after transfection with exogenous wt-CSQ1 and deletion mutants. (D) Quantitative analysis of STIM1, CSQ1 and Orai1 expression abundances in resting cells. (E) Typical cross-linking Western blotting of CSQ1 monomerization using specific antibody for CSQ1 in over-expressed or carboxyl-terminal truncation mutant cells after Ca^{2+} store depletion by TG (1 μ M). (F) The ratio of monomer to polymer of CSQ1 under TG treatment. (G) Representative traces to illustrate the effects of exogenous CSQ1 or its truncation mutants on SOCE. (H) Average peaks of Ca^{2+} release and influx, and the plateau phase of Ca^{2+} influx. All the data is presented by means $\pm$ SD from at least 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. vector control; # $P < 0.05$, ## $P < 0.01$ vs. vector-TG cells.

Effects of CSQ1 carboxyl-terminal deletion mutants on SOCE in HeLa cells

Previous studies have demonstrated that the asp-rich region of CSQ has functional roles of binding to Ca^{2+} and STIM1, suggesting an important section in CSQ1 molecular structure in regulation of SOCE [14, 15]. To identify which motif of this region interacts with STIM1 in HeLa cells, we used the plasmids encoding HA-tagged human wild type CSQ1, C-terminal amino acid 362-396 deletion mutant (C35) and C-terminal amino acid 388-396 deletion (C9) that were constructed in our previous work [13]. After cells transfected with CSQ1 mutant for 36 h, the expressions of the three types of exogenous CSQ1 in HeLa cells were assessed by Western blotting and no significant difference was found among these groups (Fig. 2A, B). Additionally, the relative abundances of CSQ1, STIM1 and Orai1 were also detected, and none of them were altered, except for an approximate 3-fold increase in total CSQ1 content in all the three exogenous CSQ1 expression cells (Fig. 2C, D).

Then, we determined whether the carboxyl-terminal of exogenous CSQ1 plays a functional role in SOCE in HeLa cells. Previous study has shown that CSQ1 monomer but not oligomers are able to bind with STIM1 [13], thus by using cross-linking lysate with 2% formaldehyde measurement (see Methods), we detected the monomerization of CSQ1 in cells treated with different mutants. We found a significant increase in monomers with a reduction in polymer forms occurred upon TG stimulation (data not shown). Consistent with the data obtained in HEK293 cells, the proportion of monomeric CSQ1 was the highest in C9-treated cells, followed by wt-CSQ1 and then C35 cells among the three groups of transfected cells (Fig. 2E, F).

Furthermore, the classical Ca^{2+} re-addition protocol to detect SOCE was performed by stimulating Fura2 loaded cells with TG (1 μ M) in the absence of extracellular Ca^{2+} in HeLa

Fig. 3. Exogenous expression of wt-CSQ1 and CSQ1 truncation mutants affects STIM1 aggregation. (A) Double immunostaining cells with specific antibody for STIM1 and Orai1, and the typical images of Orai1 and STIM1 fluorescence labeling in different groups of cells indicate co-localization of STIM1 and Orai1 in cells with different CSQ1 mutant transfections. (B) Co-localization degrees of Orai1/STIM1, indicated by Pearson's coefficient calculated using Imaris Bitplane software, are compared between different groups of cells with or without TG + ionomycin treatment. White arrows in panel A indicates the sites of enlarged images showed below. (C and D) STIM1 aggregation and redistribution in the perimembrane area (from cell surface to 1 μm inside of cell, C) and cytosolic area upon Ca^{2+} store depletion were analyzed by Imaris Bitplane software (D). (E) Double immunostaining cells with specific antibody for STIM1 and CSQ1, and the typical images of CSQ1 and STIM1 fluorescence labeling in different groups of cells as indicated. White arrows in panel E indicates STIM1 clusters or puncta around the cell membrane, while the yellow arrows indicate the positive cells of exogenous CSQ1. It is interesting to notice that these positive-labeling cells with wt-CSQ1 over-expression has no obvious clustering of STIM1, while the native cells beside them displays noticeable STIM1 aggregation in the perimembrane area. In addition, C35 over-expressed cells also exhibit detectable STIM1 clustering. (F) Statistic data for the co-localization of CSQ1/STIM1 analyzed by Pearson's coefficient were compared between different groups of cells as indicated. Scale bar: 10 μm . All the data is presented by means $\pm$ SD from 34-76 cells from 3-4 independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. vector control, # $P < 0.05$, ## $P < 0.01$ vs. vector-TG cells.

cells. SOCE was evaluated on the peak phase and plateau phase of Ca^{2+} influx after addition of Ca^{2+} . Compared with vector control, both of the influx values induced by TG were significantly reduced by wt-CSQ1 gene or C9-CSQ1 mutant transfection. However, unlike wt-CSQ1 and C9 mutant, C35-CSQ1 mutant transfection did not produce obvious change in SOCE compared with vector control in HeLa cells. Additionally, the changes in SOCE were independent of Ca^{2+} release because no significant change in Ca^{2+} release was found among all the three groups of cells under TG treatment. Thus, these results obtained in HeLa cells confirmed the findings in HEK293 cells, providing additional evidence for the important C-terminal region of CSQ1 between amino acids 362 to 396 in suppressing SOCE.

Mutation of CSQ1 carboxyl-terminal affects STIM1 aggregation upon Ca^{2+} store depletion in HeLa cells

In order to test how the exogenous CSQ1 mutants interact with STIM1 and affect SOCE in HeLa cells, we further carried out double immuno-fluorescence labeling experiments to

Fig. 4. Interaction changes between CSQ1/STIM1 and STIM1/Orai1 in HeLa cells upon Ca^{2+} store depletion. HeLa cells cultured for 24 h were suspended in Ca^{2+} -free HBSS for 5 min and stimulated with or without 1 μM TG for 5 min until harvested. (A) Co-immunoprecipitation between CSQ1 and STIM1 in cells with or without TG treatment. (B) Association of STIM1 and CSQ1 was quantified as average protein ratios $\pm$ SD of STIM1/CSQ1. (C) Co-immunoprecipitation of exogenous wt-CSQ1 by anti-HA antibody and endogenous STIM1 in different groups of cells treated with TG. (D) Association of STIM1 and CSQ1 were quantified as average protein ratios $\pm$ SD of STIM1-HA-tagged CSQ1. (E) Reverse co-immunoprecipitation between exogenous CSQ1 and STIM1. (F) Association of CSQ1 and STIM1 was quantified as average protein ratios $\pm$ SD of HA-tagged CSQ1/STIM1. (G) Co-immunoprecipitation between STIM1 and Orai1. (H) Association of STIM1 and Orai1 was quantified as average protein ratios $\pm$ SD of Orai1/STIM1. ** $P < 0.01$ vs. vector control, ## $P < 0.01$ vs. vector-TG cells.

assess STIM1/Orai1 and STIM1/CSQ1 co-localizations during TG stimulation as described above (Fig. 1). Compared with TG-treated vector control cells, a significant decrease in the co-localization of STIM1 and Orai1 in PM was found in wt-CSQ1 or in C9-CSQ1 transfected cells, but not in C35-CSQ1 mutated cells (Fig. 3B). In accordance, STIM1 puncta (0.5–1 μm) was less in the perimembrane area of wt-CSQ1 over-expressed cells than those in vector control cells. A similar change was also found in the cytosolic area. However, this effect on STIM1 aggregation in wt-CSQ1 cells was less pronounced in C9-treated cells and disappeared in C35-transfected cells (Fig. 3C, D). Furthermore, the co-localization between CSQ1 and STIM1, the key process of CSQ1 interfering with SOCE was evaluated. As shown in Fig. 3E and F, the strongest co-localization of CSQ1 and STIM1 occurred in C9 mutated cells, followed by wt-CSQ1 over-expressed cells, whereas no significant difference was observed between TG-treated C35-transfected cells and the vector cells. It was interesting to notice that 2 cells positively stained with anti-CSQ1 antibody displayed less STIM1 clusters in the perimembrane region than in wt-CSQ1 cells (indicated with yellow arrows), whereas the native cells beside them occurred visible STIM1 clustering in this area (indicated with white arrows in Fig. 3E). More interestingly, slight STIM1 aggregation and obvious STIM1 puncta in perimembrane region could be detected in the cells transfected with C9 and C35-CSQ1 cells (indicated with yellow arrows), respectively. These parallel changes in STIM1/Orai1 and

STIM1/CSQ1 co-localizations in different mutant-treated cells, together with the reduced STIM1 aggregation in both perimembrane and cytosolic areas demonstrate that CSQ1 down-regulates SOCE likely through interacting with STIM1, thereby inhibiting STIM1 aggregation/clustering and its interaction with Orai1 in cell membrane.

CSQ1 mutation affects the interactions of CSQ1/STIM1 and STIM1/Orai1 in HeLa cells

Finally, we investigated the interaction between endogenous and the exogenous CSQ1 with STIM1 after store depletion using co-immunoprecipitation assay. In accordance with the observations found in double-labeling experiments (Fig. 1 and 3), an increased physical interaction between endogenous CSQ1 and STIM1 was detected upon Ca^{2+} store depletion (Fig. 4B). Similarly, the strongest interaction between CSQ1 and STIM1 occurred in C9-expressing cells, followed by wt-CSQ1, but less co-immunoprecipitation was observed in C35 transfected cells (Fig. 4C-F), comparable with the observations in staining evaluations (Fig. 3). Likewise, the interaction of STIM1 and Orai1 was attenuated in both wt-CSQ1 over-expressed and C9-mutated cells, but not in C35-mutated cells, compared with vector control (Fig. 4G).

Discussion

Many studies have shown that the assembling and translocating of STIM1 to an ER membrane compartment closely juxtaposed to the adjacent PM, to interact with Orai1 in PM is the major machinery to drive SOCE [9, 27, 28]. Although the basic molecular mechanisms of SOCE activation and the contributing proteins are well understood, the regulatory mechanisms involving STIM1 or Orai1 function which is fine-tuned by various regulatory proteins, are quite complicated and obscure [29].

Numerous studies have revealed that the aspartate-rich carboxyl-terminus of CSQ is the motif to bind with Ca^{2+} and a suppressor of Ca^{2+} -induced Ca^{2+} release through binding with triadin/junctin and inhibiting ryanodine receptor [14, 18, 30-32]. Also, CSQ1 participates in the generation of retrograde signals to SOCE through the action of a Ca^{2+} -binding motif located on the carboxyl-terminal region of CSQ1 in skeletal muscle cells [16-19]. Our recent work has demonstrated that upon Ca^{2+} store depletion, CSQ1 monomers bind and interact with STIM1 through the sites lie within the carboxyl-terminal 362-387 and 362-396 residues of CSQ1, respectively [13]. This sequestration of STIM1 by CSQ1 further affects the STIM1 interacting with Orai1, thus providing a down-regulatory mechanism to prevent exaggerated activation of SOCE under physiological conditions.

In the present study, over-expression of full length *CSQ1* gene in HeLa cells induced augmentations in CSQ1 monomerization (Fig. 2E and F) and CSQ1/STIM1 association (Fig. 3E, F and 4), but decreased in STIM1/Orai1 association (Fig. 3A, B and 4G) and SOCE as well (Fig. 2G and H), whereas the mutant lacking 35 C-terminal residues almost completely abolished the effects of over-expression of *CSQ1* gene. In accordance, STIM1 redistribution, represented as puncta positioned on the region of perimembrane, were much more in cells transfected with C35 mutant than in wt-CSQ1 over-expressed cells (Fig. 3C-F), further indicating a state of SOCE activation in C35-treated cells, but not in exogenous wt-CSQ1 transfected cells. In particular, it was clear to notice that STIM1 clustering disappeared in the perimembrane region in wt-CSQ1 positive staining cells, compared to the side by side native cells with negative wt-CSQ1 staining, while STIM1 aggregation in perimembrane area was observed in the C35 positive cells (Fig. 3E). More importantly, reduced STIM1 clusters were also found in the cytosolic area of wt-CSQ1 over-expressed cells than that in C35-mutated cells, an indication of less STIM1 aggregation due to the interaction of CSQ1/STIM1. Thus, the important point in the latter observation demonstrates that inhibition of STIM1 aggregation rather than direct interfering with STIM1/Orai1 association by CSQ1 monomers was likely the initial effect of CSQ1/STIM1 physical interaction. The summarized data further supports this notion, in which CSQ1 monomerization is positively relative with its interaction with

Fig. 5. The relationships between protein conformations and their interactions of CSQ1, STIM1 and Orai1 in HeLa cells. These data are summarized from the results presented above, which are normalized with the data obtained in vector-treated cells for easy comparisons. Here, an obvious relationship between CSQ1 monomerization with the CSQ1/STIM1 co-localization, STIM1 clustering, and STIM1/Orai1 co-localization can be found during SOCE activation and maintenance.

STIM1, and negatively relative with STIM1 aggregation in both perimembrane and cytosolic areas and STIM1/Orai1 interaction (Fig. 5).

Interestingly, although the cells transfected with the mutant lacking 9 C-terminal residues displayed more CSQ1 monomers, stronger co-localization and interaction of CSQ1/STIM1 than wt-CSQ1 and C35 mutant, the inhibitory effects of C9-CSQ1 on STIM1 aggregation, STIM1/Orai1 association and SOCE were less efficient than those in over-expressed wt-CSQ1 cells (Fig. 2-5). Hence, these observations may demonstrate that deletion of C9 enhances monomer formation thus increasing C9/STIM1 association, but also weakens its effect on STIM1 clustering, resulting in minimal change in STIM1/Orai1 interaction and SOCE. Truncation of C35 in CSQ1, however, weakens both binding and interacting with STIM1, providing a more pronounced inhibitory effect on STIM1 oligomerization and the ensuing STIM1/Orai1 association.

In summary, this study confirms the basic mechanism for CSQ1 down-regulating SOCE in HeLa cells, another non-excitable cell line. In addition, inhibition of STIM1 aggregation upon Ca^{2+} store depletion is likely the initial effect of CSQ1 monomers binding with STIM1, thereby reducing the STIM1 and Orai1 interaction and SOCE activation. This study also provides additional evidence for the site of binding/interaction of CSQ1 and STIM1 and site of interfering with STIM1 aggregation lies within, or overlaps with the carboxyl-terminal 362-387 and 362-396 residues of CSQ1, respectively. As STIM1 oligomerization is the first reaction when Ca^{2+} stores are depleted and sensed by STIM1, action on this step by CSQ1 provides a rapid and efficient mechanism for feed-back regulation of SOCE under physiological condition. Additionally, dysfunctional CSQ1 may also affect the normal SOCE response, thus causing abnormal Ca^{2+} signaling in some pathological settings [33-36].

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Disclosure Statement

All authors declare that there are no conflicts of interest in this study.

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