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An ER Locating Protein Named RCN2 Interacts With STIM1-Orai1 Complex*

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Abbreviations: ER, endoplasmic reticulum; SOC, store-operated channel; CRAC, Ca^{2+} release activating Ca^{2+}; TG, thapsigargin; TAP, tandem affinity purification; LC-MS, liquid chromatography tandem mass spectrometry

Abstract STIM1 is recognized as an ER Ca^{2+} sensor of calcium release-activated calcium (CRAC) channel that is constructed by membrane protein Orai1. However, this regulatory system may also be regulated by other proteins. Reticulocalbin 2 (RCN2) was purified and identified from STIM1-Orai1 complex. Confocal microscopy revealed that RCN2 co-localized with STIM1 in ER before and after Ca^{2+} store depletion. Single cell $[Ca^{2+}]_i$ measurements of RCN2 EF hands mutant showed slight influence on SOC electrophysiological characters. Furthermore, a novel collar form aggregation of RCN2 surrounding STIM1 clusters suggested that RCN2 potentially plays a role of structure maintenance in STIM1 clustering.

Key words RCN2, STIM1, Orai1, TAP

Cytoplasmic Ca^{2+} concentration in eukaryotic cells can be regulated in two ways: Ca^{2+} can be released from intracellular calcium stores, or can influx into cytoplasm through all kinds of Ca^{2+} channels. As one kind of these channels, store-operated Ca^{2+} (SOC) channel was vital in cells for maintaining the Ca^{2+} concentration to preserve the functional integrity of ER, such as protein folding, vesicle trafficking, and apoptosis[1]. It is also confirmed that this channel was functionally related to some diseases[2]. Two proteins, STIM1 and Orai1, were revealed to be essential for SOC activity. STIM1, which was discovered in large scale RNAi screening and identified as the sensor of SOC later[3~5], located in the lumen of ER under quiescent situation and was found having influence to SOC. Orai1 is a plasma membrane protein that was found mutated in SCID patients[6] and was then recognized as the CRAC core subunit[7, 8]. Co-expression of STIM1 and Orai1 greatly enhanced CRAC current[9], and co-immunoprecipitation signal between STIM1 and Orai1 was enhanced after TG stimulation [10]. These all revealed that STIM1 and Orai1 may form some kind of complex in certain situation, such as Ca^{2+} store depletion.

Recently, some TRPC family proteins were reported to be associated with STIM1 and Orai1 and were suggested that they may form ternary complex[11, 12]. However, SOC function was so important and complicated that there still may be some other proteins involved in the pathway. Therefore, we used a system of mammalian cell tandem affinity purification (TAP) to purify STIM1-Orai1 complex. An ER lumen protein with 6 EF hands named reticulocalbin 2 (RCN2, ERC-55)[13, 14] was then identified from purified STIM1-Orai1 complex through LC-MS/MS. Using confocal fluorescent microscopy, we found that RCN2 nicely co-localizes with STIM1. But in single cell $[Ca^{2+}]_i$ measurements, RCN2 and its EF-hands mutant had only weak influence on SOC electrophysiological characters. However, TIRF microscopy revealed a novel collar form localization of RCN2 surrounding STIM1 puncta after TG stimulation, which suggested that RCN2 may play some roles of structure constraint

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in the clustering of STIM1 following cell Ca^{2+} store depletion.

1 Materials and methods

1.1 Plasmid construction

Human STIM1, Orai1, RCN2 cDNA was amplified from human cDNA library (OriGene Technologies Inc., MD, USA). TAP tag (SBP-CBP) sequence was from pCTAP vector (Stratagene, La Jolla, CA). STIM1 with TAP tag was subcloned into pIRES2-EGFP vector (Clontech, Mountain View, CA) and Orai1 with TAP tag substituted the EGFP of pIRES2-EGFP to get plasmid pS/O-TAP. Plasmid pO/S-TAP and plasmid ppHl-S/O were constructed in similar way. EGFP and mCherry cDNA was inserted between the signal peptide and the main sequence of RCN2 cDNA and then subcloned into pCDNA3.1 zeo(+) vector (invitrogen, Carlsbad, CA) to produce correct localizing RCN2-EGFP and RCN2-mCherry. pSTIM1-mOrange (pST-mO) was constructed from pmOrange-N1, which substituted the EGFP of pEGFP-N1 with mOrange.

1.2 Cell culture, transfection and stable cell line construction

HEK293 cells were purchased from ATTC (CRL 1573) and cultured in high glucose DMEM (Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum. Experiment cells were transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA), or transfected with linear PEI (Polysciences, Warrington, PA) as Geisse *et al*. described[15]. Transfected cells were transferred onto poly-l-lysine-coated round coverslips the day before experiment. Wild type HEK293 cells and cells transfected with pS/O-TAP and pCTAP vector were processed with 1 000 mg/L G418 (sigma, St. Louis, MO) cytotoxic pressure screen. The living healthy cells containing pS/O-TAP and pCTAP empty vector were collected and cultured.

1.3 Mammalian cell tandem affinity purification and MS identification

HEK293 cells stably expressing bait proteins from ten 20 cm culture dishes (BD Biosciences, San Jose, CA) were treated with TG or not. And then the cells were collected and lysed by lysis buffer in InterPlay™ Mammalian TAP System kit (Stratagene, La Jolla, CA) with protein inhibitor cocktail (Sigma, St. Louis, MO) and PMSF (Sigma, St. Louis, MO) added. The whole tandem affinity purification followed the instruction manual of the TAP System kit and the final protein sample was concentrated with YM-3 concentration tube (Millipore, Billerica, MA). The concentrated sample was denatured and precipitated with tenfold volume of –20℃ acetone. Protein sample was then treated and analyzed with Finnigan™ LTQ™ LC-MS/MS (Thermo Finnigan, San Jose, CA) as instruction manual. Protein was automatically identified *via* peptide searching against an in-house human protein sequence database.

1.4 Co-immunoprecipitation

HEK293 cells in 9 cm petri dishes were used as one start material. Collected cells were lysed with RIPA buffer (PBS pH7.4 with 1% NP40, 0.1% DOC) on ice for 1 h and then were centrifuged for 10 min at 18 000 *g*. 15 μl anti-FLAG M2 resin (Sigma) was added into the supernatant and incubated in 4℃ for 1 h. The resin was then washed with PBS for 3 times and the binding proteins were eluted with 100 mmol/L glycine pH2.6.

1.5 Fluorescence microscopy imaging

Co-expression cells were observed under an Olympus FV500 confocal laser scanning microscope with a 60× (NA = 1.40) oil objective. Images were acquired using FLUOVIEW 2.0 (Olympus America Inc., Melville, NY) and treated with ImageJ 1.33u (NIH, USA). TIRFM was based on an evanescent wave microscope constructed as described previously [16]. A dual-view Micro-Imager (Optical-Insights, Santa Fe, NM) collected the green and red fluorescence which were excited by 488 and 561 nm lasers respectively. Images were collected with TILLVISION4.01 (TILL Photonics, Germany) and analyzed with ImageJ.

1.6 Single cell $[Ca^{2+}]_i$ measurements and CRAC current recording

Using Fura-2 as Ca^{2+} indicator, single cell $[Ca^{2+}]_i$ measurements was processed under Olympus IX71, a 340/380 nm laser excited inverted fluorescent microscope (Olympus, Tokyo, Japan). Dual-wavelength excitated fluorescence collected by TILL photometry system (TILL Photonics, Germany) was quantitated as fluorescence ratio, from which $[Ca^{2+}]_i$ could be calculated according to Grynkiewcz's formula: $[Ca^{2+}]_i = K_d \times (F_{min}/F_{max}) \times (R - R_{min}) / (R_{max} - R)$. CRAC current was recorded with patch-clamp performed in the standard whole-cell recording configuration. High-resolution current recordings were acquired using the EPC-10 (HEKA, Lambrecht, Germany). Experiment data was analyzed using IGOR Pro 5.01

(Wavemetrics, Portland, bOR).

2 Results

2.1 RCN2 can be detected from purified CRAC complex through mammalian TAP

Previous studies revealed that amplified CRAC currents were detected in cells co-expressing STIM1 and Orai1 in certain ratio[9]. We reconstructed plasmids based on plasmid pIRES2-EGFP [17] that expresses target gene and EGFP respectively. As Figure 1a shows, using plasmid pS/O-TAP, we can express the human STIM1 gene with TAP tag (3xFLAG-SBP-CBP) under the control of CMV promoter, and express human Orai1 gene with TAP tag under the control of IRES2 gene. While using plasmid pO/S-TAP, Orai1-TAP and STIM1-TAP can be expressed under the control of CMV promoter and IRES2 gene respectively. Western blot with anti-FLAG antibody revealed that HEK293 cells transfected with either of the two plasmids express STIM1 and Orai1 in different ratio (Figure 1b). Cells transfected with pS/O-TAP produced more STIM1 than Orai1, and shows amplified CRAC current in whole cell patch clamp recording as a previous study described[9] (Figure 1c). In the contrast, cells transfected with pO/S-TAP produced less STIM1 than Orai1 and failed to form reconstructed CRAC currents (data not shown).

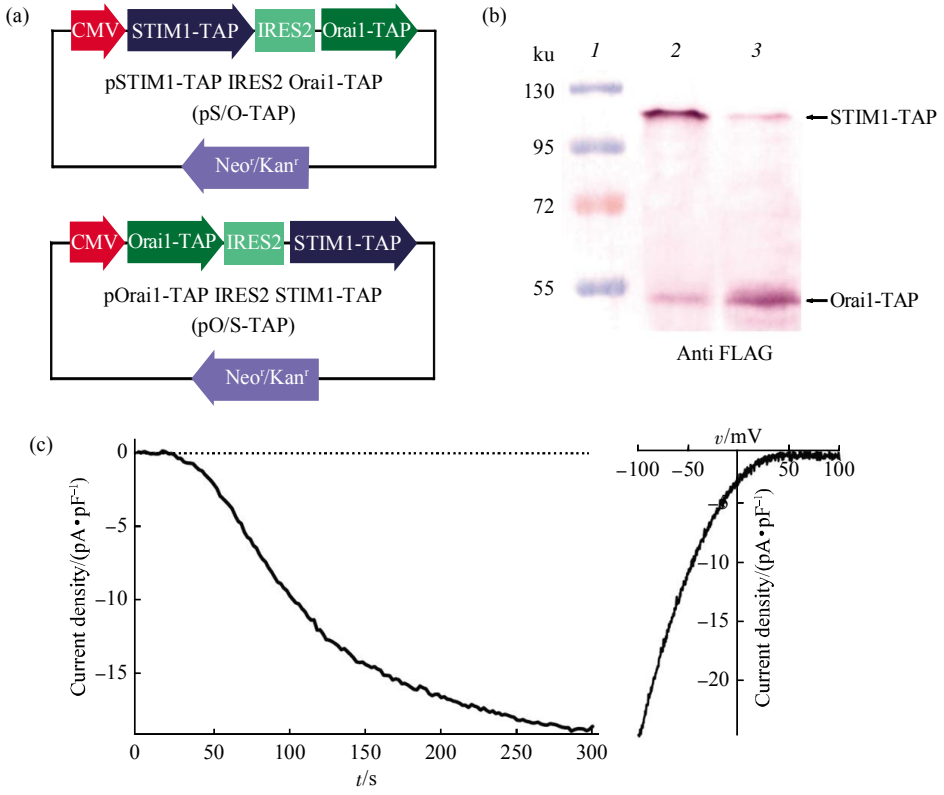

Fig. 1 The construction of STIM1-Orai1 complex TAP expression system

(a) The topology structure of plasmid pS/O-TAP and pO/S-TAP. STIM1-TAP and Orai1-TAP genes were expressed under the control of either CMV promoter or IRES2. (b) Anti-FLAG Western blot determination of the expression ratio between STIM1-TAP and Orai1-TAP produced by cells transfected with plasmid pS/O-TAP or pO/S-TAP. Total protein concentration loaded in both lanes was adjusted to same level by BSA method. *1*: Molecular mass marker; *2*: ST IRES Or; *3*: Or IRES ST.(c) The representative current-time and *I-V* curve of the CRAC channel produced by cell transfected with plasmid pS/O-TAP but not pO/S-TAP.

Based on the results, we chose plasmid pS/O-TAP to express STIM1-TAP and Orai1-TAP with ordinary physiological function as bait proteins for Tandem affinity purification. HEK293 cells transfected with pS/O-TAP or TAP negative control plasmid (Stratagene) were screened with G418 for stable expression. Before lysis, cells were incubated with 5 μmol/L TG for 30 min. Proteins were affinity purified with Streptavidin resin and Calmodulin resin following the instruction manual of mammalian TAP kit (Stratagene). The final purified protein solution samples were detected by LC-MS/MS. Both bait

proteins, RCN2 and some other proteins were detected in pS/O-TAP sample but not in TAP negative control sample.

This result was then verified by immunoprecipitation test. We inserted EGFP gene after the signal peptide sequence of RCN2 cDNA and subcloned it into pCDNA3.1 vector (Invitrogen) so that RCN2-EGFP with correct ER localization can be expressed. This plasmid RCN2-EGFP can be used not only in immunoprecipitation test, but also in co-localization test. Using anti-FLAG M2 resin (Sigma), RCN2-EGFP was co-precipitated by STIM1-Orai1 complex (Figure 2). Interestingly, as previous study described [13], RCN2 localizes in the lumen of ER and contains 6 Calcium binding EF hands. Considering the similar localization and EF hand functional domain of STIM1 [18,19], it is important to find out the subcellular localization of RCN2 with STIM1 in physiological condition.

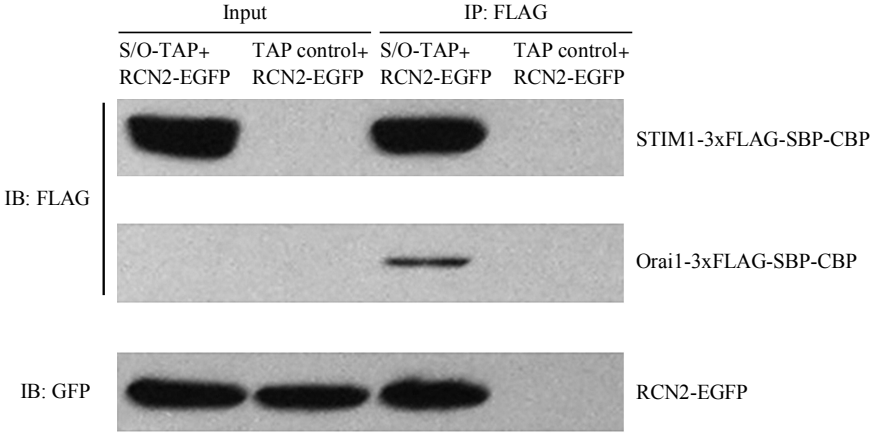

Fig. 2 The co-immunoprecipitation of STIM1-Orai1 complex and RCN2

HEK293 cells were co-transfected with pS/O-TAP and RCN2-EGFP or TAP control and RCN2-EGFP. Expressed recombinant STIM1-TAP and Orai1-TAP protein can be captured from cell lysis supernatant and RCN2-EGFP can be co-precipitated by STIM1-Orai1 complex but not by empty TAP control.

2.2 RCN2 co-localizes with STIM1 and slightly influences CRAC electrophysiological characters

In order to visualize both the proteins in single cell, we used the plasmid RCN2-EGFP and a plasmid STIM1-mOrange we constructed in previous study[20].

HEK293 cells were then co-transfected with STIM1-mOrange and RCN2-EGFP and were observed under confocal fluorescent microscope. As shown in Figure 3a, RCN2-EGFP nicely co-localized with STIM1-mOrange. Both proteins displayed diffuse ER distribution at resting state but formed aggregates after Ca^{2+} store depletion with TG. This co-localization result suggested that RCN2 may be involved in CRAC pathway.

Subsequently, store depletion evoked Ca^{2+} entry was tested by Ca^{2+} photometry in HEK293 cells expressing target proteins. A plasmid pHluorin-STIM1-IRES2-Orai1 (ppHl-S/O) was constructed in the similar way of pS/O-TAP construction. The cells transfected with ppHl-S/O produced large and rapid increase in SOC current after TG stimulation (Figure 3b), as co-transfection of STIM1 and Orai1 in previous reports[21, 22]. The EGFP gene in RCN2-EGFP was substituted by mCherry. The localization of RCN2-mCherry was the same with the localization of RCN2-EGFP (data not shown). HEK293 cells transfected with ppHl-S/O or co-transfected with RCN2-mCherry and ppHl-S/O were then incubated with Fura-2 solution and subsequently processed with single cell $[Ca^{2+}]_i$ measurements.

The representative enhancement of the $[Ca^{2+}]_i$ elevation evoked by SOC Ca^{2+} entry was detected in cells transfected alone with ppHl-S/O (Figure 3b) as reported previously[9, 21, 22]. However, the maximum $[Ca^{2+}]_i$ elevation in the cells co-transfected with RCN2-mCherry and ppHl-S/O was similar to that in the cells transfected alone with ppHl-S/O (Figure 3b). We then mutated all the six EF-hands in RCN2 at the same site as STIM1 EF-hand mutation to destroy its calcium binding function. The maximum $[Ca^{2+}]_i$ elevation of the cells transfected with ppHl-S/O + mutRCN2 showed slight decrease to the maximum

$[Ca^{2+}]_i$ elevation of cells transfected with ppHl-S/O alone or ppHl-S/O +RCN2. This result suggested that when the EF-hands of RCN2 are inactivated, the calcium response of CRAC complex will be slightly influenced.

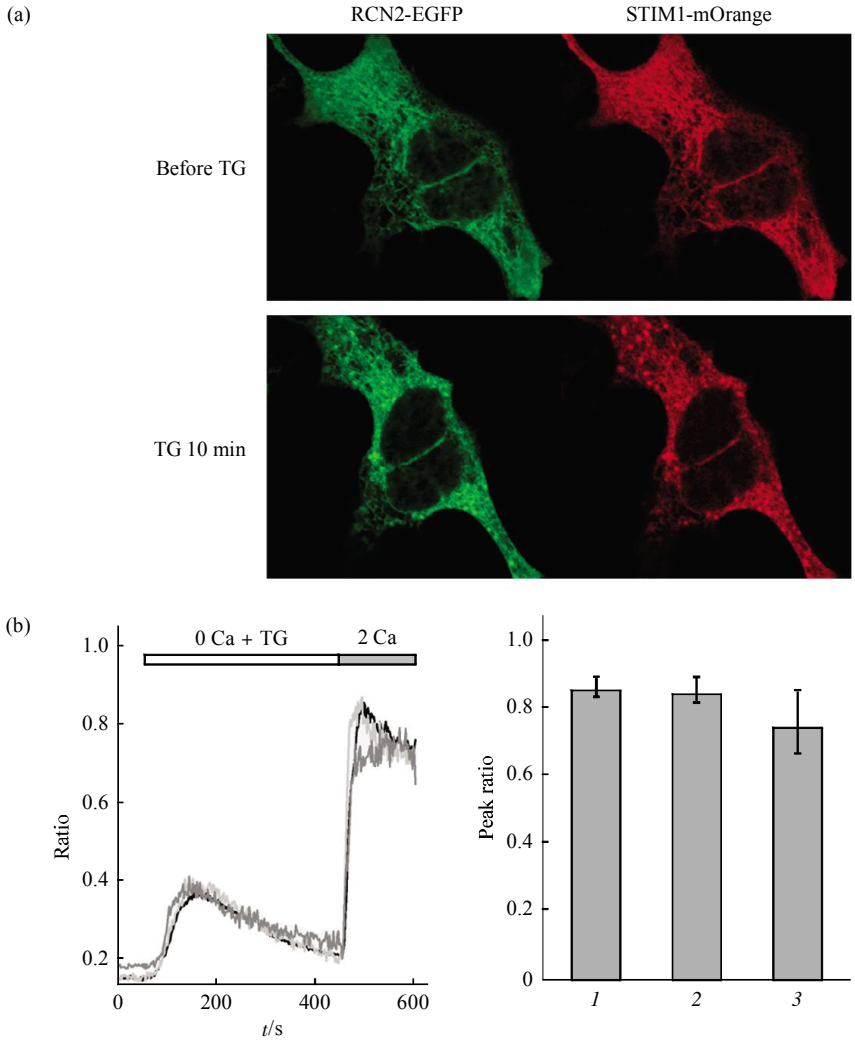

Fig. 3 RCN2 co-localizes with STIM1 and slightly influences CRAC electrophysiological characters

(a) A representative cell co-expressing RCN2-EGFP and STIM1-mOrange was observed under a confocal microscope before and after TG stimulation. The green and red emission was excitated and detected in sequence. (b) Single cell $[Ca^{2+}]_i$ measurements results of cells expressing pHluorin-STIM1 IRES2 Orai1 (ppHl-S/O) (light grey, n=10), ppHl-S/O with RCN2-mCherry (Black, n=12) and ppHl-S/O with mutRCN2-mCherry (dark grey, n=7). 10 μmol/L TG buffer was substituted with 2 mmol/L $CaCl_2$ buffer at the time of ~420 s. *1*: ppHI-S/O; *2*: ppHI-S/O+RCN2; *3*: ppHI-S/O+mutRCN2.

2.3 A novel collar form aggregation of RCN2 surrounding STIM1 cluster

The slight influence of RCN2 on SOC current suggested that there may be some difference between the localization of RCN2 and STIM1. Therefore, we then checked the basal plane near plasma membrane of the cells co-transfected with RCN2-EGFP and STIM1-mOrange through TIRF microscopy.

As shown in Figure 4a, at resting state, RCN2-EGFP and STIM1-mOrange co-localized nicely in ER tubular distribution. Interestingly, after the Ca^{2+} store was depleted by TG, STIM1 aggregated in clustering form while RCN2 formed collar-like aggregation surrounding STIM1 clusters (Figure 4b). Besides, EF-hands mutated RCN2-EGFP can also form the collar-like aggregation (Figure 4c). These results revealed that the co-localization relationship of RCN2 and STIM1 before Ca^{2+} store depletion changed into an inclusion relationship in response to TG stimulation. This distribution appeared to be independent on the calcium binding EF-hands of RCN2.

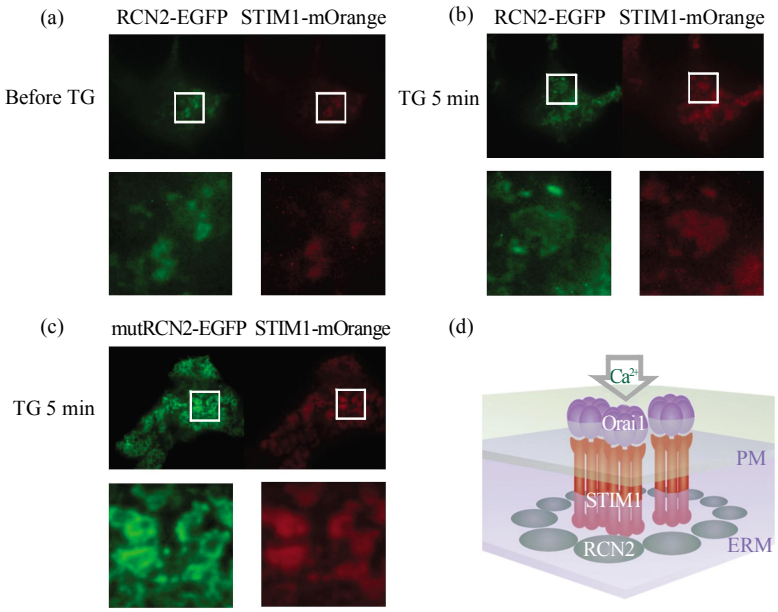

Fig. 4 Representative cells co-expressing RCN2-EGFP and STIM1-mOrange were observed under TIRF microscope before and after TG stimulation

The green and red emission was excitated and detected in sequence. (a) RCN2 nicely co-localized with STIM1 before TG stimulation. (b) RCN2 formed independent collar-like aggregation surrounding STIM1 clusters in response to Ca^{2+} store depletion. (c) EF-hands mutated RCN2 still formed the collar-like aggregation. (d) A model of the space relationship of RCN2, STIM1 and Orai1 in response to Ca^{2+} store depletion.

3 Discussion

Based on immunofluorescence and surface biotinylation experiment results in previous study, some believed that STIM1 migrates from ER-like sites and inserts into plasma membrane after Ca^{2+} store depletion[3]. Although this possibility can not be completely ruled out, other groups and our previous research showed experimental evidences that STIM1 localized on ER membrane and aggregated right underneath the plasma membrane upon Ca^{2+} store depletion[20, 21]. However, it is still unknown that what enables STIM1 to find the position on ER for aggregation.

Using mammalian TAP method, RCN2 was detected from purified STIM1-Orai1 complex[10]. RCN2 belongs to CREC family that consists of a plurality of proteins with multiple EF-hands. Most of these proteins localize in the secretion pathway of mammalian cells[14]. We only detected slight influence of RCN2 on CRAC electrophysiological characters. However, the independent collar-form aggregation of RCN2 surrounding STIM1 clusters suggested a hypothesis model of STIM1 aggregation controller: in resting condition, RCN2 co-localizes with STIM1. While Ca^{2+} store is depleted, RCN2 proteins aggregate in fence form to restrain STIM1 within them. Two functions of RCN2 in this pathway were inferred from this hypothesis: recruiting STIM1 to aggregate, and maintaining the structure of STIM1 clusters to enable STIM1 to interact with Orai1. Based on the clustering and co-localization results of Orai1 with STIM1 we observed before[20], a model of space relationship of RCN2, STIM1 and Orai1 after Ca^{2+} store depletion was shown as Figure 4d.

In summary, our research suggested a clustering maintaining function of RCN2 involved in CRAC complex. Therefore, besides STIM1 and Orai1, some other Ca^{2+} binding proteins may also play their roles in the complicated SOC regulatory system. Considering the important putative functional properties of CREC family proteins and their relationships with many diseases, more CREC family proteins may be characterized in the SOC pathway in future.

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内质网上的蛋白质 RCN2 与 STIM1-Orai1 复合体相互作用 *

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摘要 膜蛋白质 Orai1 组成了一类被称为钙释放激活钙通道(CRAC)的离子通道，并且由相互作用的蛋白质 STIM1 作为其在内质网上的钙感受器．但是这类通道的调节机制还未研究透彻．通过串连亲和纯化 STIM1-Orai1 复合体，发现与之相互作用的内质网蛋白质 RCN2．共聚焦显微术显示 RCN2 与 STIM1 在钙库排空前后完全共定位．对 RCN2 的 EF hands 结构突变体所作单细胞测钙，结果显示其对钙库操控通道电流特性有微弱影响．全内反射荧光显微术显示，RCN2 以花环状围绕包围 STIM1 聚集堆，这提示 RCN2 在 STIM1 聚集中起到一种结构约束作用．

关键词 RCN2，STIM1，Orai1，串连亲和纯化
学科分类号 Q735

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