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ARTICLE ADDENDUM

Role of STIM1 in the surface expression of SARAF

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ABSTRACT

The store-operated Ca^{2+} entry-associated regulatory factor (SARAF), a protein expressed both in the endoplasmic reticulum and the plasma membrane, has been presented as a STIM1-interacting protein with the ability to modulate intracellular Ca^{2+} homeostasis. SARAF negatively modulates store-operated Ca^{2+} entry (SOCE) by preventing STIM1 spontaneous activation and regulating STIM1-Orai1 complex formation. In addition, SARAF is a negative regulator of Ca^{2+} entry through the arachidonate-regulated Ca^{2+} (ARC) channels. Here we explored the possible role of the surface expression of SARAF on the location of STIM1 in the plasma membrane. In NG115-401L cells, lacking a detectable expression of native STIM1, transfection with pHluorin-STIM1, which is able to translocate to the cell surface, enhances the plasma membrane location of SARAF as compared to cells transfected with YFP-STIM1, lacking the ability to translocate to the cell surface. These findings suggest that the surface location of SARAF is dependent on the expression of STIM1 in the plasma membrane.

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Introduction

The store-operated Ca^{2+} entry-associated regulatory factor (SARAF) is a 339 amino acid protein that has been presented as a novel regulator of STIM proteins. In human, this protein is encoded by the *SARAF* gene, formerly known as *TMEM66* (for transmembrane protein 66). SARAF was initially identified as a endoplasmic reticulum (ER)-resident protein that associates with the ER Ca^{2+} sensors STIM1 and STIM2[1,2] to modulate intracellular Ca^{2+} homeostasis.[3] SARAF negatively modulates store-operated Ca^{2+} entry (SOCE) in different ways, including the regulation of the STIM1-Orai1 complex to facilitate slow Ca^{2+}-dependent inactivation of the Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Furthermore, SARAF interacts with STIM1 under resting conditions thus preventing spontaneous activation of STIM1 and promotes STIM1 deoligomerization upon Ca^{2+} store refilling.[3] Consistent with the role of SARAF in the modulation of Ca^{2+} homeostasis, SARAF has been reported to interact with STIM2, a process that might be involved in the modulation of resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$).[3] Moreover, SARAF interacts with the arachidonate-regulated Ca^{2+} (ARC) channels and plays an important role in the regulation of ARC channel function.[4]

SARAF is a protein with a single putative transmembrane domain, a N-terminal region that is required for the activation of the protein, and a C-terminal domain that has been shown to be involved in the interaction with the C-terminal inhibitory domain of STIM1 (CTID), downstream the STIM1 Orai1 activation region (SOAR)).[5] Using homology modeling the STIM1 CTID region (amino acids 448–530) was identified, which contains 2 lobes: the STIM1 (448–490) lobe was found to restrict the interaction of SARAF with the SOAR region, while the STIM1 (490–530) lobe direct the SARAF-SOAR interaction. Deletion of the CTID region has been reported to induce spontaneous clustering of STIM1 and activation of CRAC channels independently of Ca^{2+} store depletion.[5]

Studies performed in Muallem's lab revealed that SARAF is associated to STIM1 at rest and Ca^{2+} store depletion results in a initial dissociation followed by re-interaction of both proteins.[5] Recently, we have reported that maximal dissociation occurs after 30 s of the initiation of store depletion by treatment with TG,

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and full re-association is achieved 30 s later.[6] In parallel, we have observed a reciprocal interaction of SARAF with Orai1 that might be aimed to enhanced Orai1 channel function as determined in NG115–401L cells lacking a significant expression of STIM1.[6]

As mentioned above, we have reported that SARAF negatively regulates ARC channel function.[4] These channels consist of a heteropentameric assembly of 3 Orai1 and 2 Orai3 subunits[7] with the participation of plasma membrane-resident STIM1.[8] As previous findings had presented SARAF as a modulator of ER-located STIM1,[3] the regulation of store-independent ARC channels by SARAF led us to explore the surface expression of this protein. Using biotinylation and impairing ER-plasma membrane interaction by treatment with jasplakinolide, a cell-permeant peptide that induces polymerization and stabilization of actin filaments,[9] we reported the presence of SARAF in the plasma membrane.[6] Extending our previous studies, here, we have investigated the possible dependence of the surface expression of SARAF on the location of STIM1 in the plasma membrane.

Results and discussion

In order to investigate whether the surface expression of STIM1 plays a relevant role in the location of SARAF in the plasma membrane we have transfected NG115-401L cells, expressing a negligible amount of native STIM1,[10] with either pHluorin-STIM1 or YFP-STIM1. We have previously found the ability of pHluorin-STIM1 to locate at the plasma membrane, while YFP-STIM1 is unable to translocate to the cell surface. The surface expression of SARAF was assessed by biotinylation of plasma membrane proteins and collection with streptavidin-coated agarose beads. SDS-PAGE and Western blotting were used to identify the proteins in the biotinylated (plasma membrane) fraction and in the non-biotinylated fraction. As shown in Figure 1A (top panel), analysis of the biotinylated fraction of non-stimulated NG115–401L cells reveals a similar amount of the plasma membrane Ca^{2+}-ATPase (PMCA) in the lysates from cells transfected with pHluorin-STIM1, YFP-STIM1 or empty vector. STIM1 was found in the plasma membrane only in cells expressing pHluorin-STIM1 and not in cells transfected with YFP-STIM1 or mock-treated cells (Fig. 1A, middle panel), thus confirming the ability of these constructs to translocate to the cell surface. As depicted in Figure 1A, bottom panel, our results reveal a detectable amount of SARAF in the plasma membrane of cells transfected with YFP-STIM1 as well as in mock-treated cells; however, interestingly, the amount of SARAF detected in cells transfected with pHluorin-STIM1 was 2.8 ± 0.3-fold greater than in mock-treated cells. These findings indicate that, while a small pool of SARAF is located in the plasma membrane in the absence of detectable STIM1, the expression of STIM1 in the plasma membrane is sufficient to cluster a significant amount of SARAF in the cell surface.

We have further explored the expression of STIM1, SARAF and the PMCA in the non-biotinylated fraction and the whole cell lysate as control. As shown in Figure 1B, top panel, Western blotting of the non-biotinylated fraction with the anti-PMCA antibody reveals the absence of PMCA in this fraction, which confirms the eficacy of the biotinylation assay. By contrast, and consistent with the results presented in Figure 1A (top panel), a similar amount of PMCA was detected in the whole cell lysates under the different experimental conditions (Fig. 1C, top panel). pHluorin-STIM1 as well as YFP-STIM1 were detected in the whole cell lysates and in the non-biotinylated fraction of cells transfected with the corresponding plasmids, thus confirming successful expression of exogenous STIM1, while native STIM1 (expected at aprox. 85 kDa) was not detected in the different cell lysates (Fig. 1B and C, middle panel). A comparable amount of SARAF was detected at the different experimental conditions both in the non-biotinylated fraction, which is consistent with the ER location of this protein, and in the whole cell lysate (Fig. 1B and C, bottom panel).

Summarizing, our findings suggest for the first time that the location of SARAF in the plasma membrane is, at least partially, dependent on the surface expression of STIM1. SARAF, together with a number of SOCE regulatory proteins,[11] collaborate to fine tune the amount of Ca^{2+} entry upon cell stimulation with physiological agonists. In contrast to other SOCE modulators, including CRACR2A[12] or STIMATE,[13] SARAF seems to be an integral modulator of Ca^{2+} homeostasis, regulating from the resting $[Ca^{2+}]_c$ to Ca^{2+} entry via store-dependent and -independent mechanisms.

Material and methods

Materials

Bovine serum albumin (BSA) was from Sigma (Madrid, Spain). Rabbit polyclonal anti-TMEM66 (SARAF)

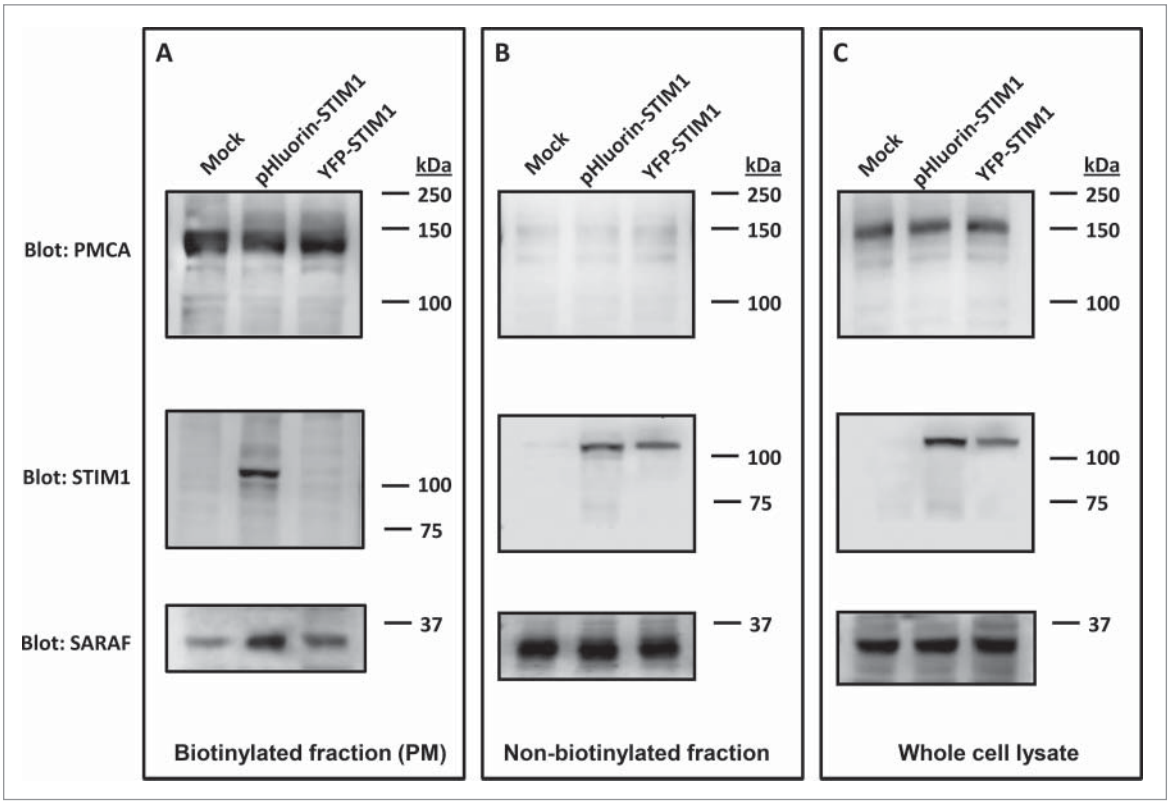

Figure 1. STIM1-dependent expression of SARAF in the plasma membrane. A and B, NG115–401L cells were transfected with pHluorin-STIM1, YFP-STIM1 or empty vector (mock). Forty eight hours later cells were mixed with biotinylation buffer containing 100 μg/mL EZ-Link sulfo-NHS-LC-biotin, and cell surface proteins were labeled by biotinylation, as described in Material and Methods. Labeled proteins were pulled down with streptavidin-coated agarose beads. The pellet (containing the plasma membrane fraction; A) and the supernatant (B) were analyzed by SDS-PAGE and Western blotting using anti-PMCA, anti-STIM1 or anti-SARAF antibody, as indicated. These results are representative of 5 separate experiments. C, NG115–401L cells were transfected with pHluorin-STIM1, YFP-STIM1 or empty vector (mock). Forty eight hours later cells were lysed. Whole cell lysates were analyzed by Western blotting using anti-PMCA, anti-STIM1 or anti-SARAF antibody, as indicated. Positions of molecular mass markers are shown on the right. These results are representative of 5 separate experiments.

antibody (catalog number PA5-24237. epitope: amino acids 33–62 of the N-terminal region of human TMEM66), mouse monoclonal anti-PMCA antibody (Clone 5F10, epitope: amino acids 724–783 of of human PMCA, catalog number MA3-914), EZ-Link Sulfo-NHS-LC-Biotin, streptavidin–conjugated agarose beads and Turbofect transfection reagent were from Thermo Fisher (Madrid, Spain). Mouse monoclonal anti-STIM1 antibody (Clone 44/GOK, epitope: amino acids 25–139 of human STIM1, catalog number 610954) was from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Horseradish peroxidase-conjugated anti-mouse IgG antibody and anti-rabbit IgG antibody were from Jackson ImmunoResearch (West Baltimore Pike, PA, USA). Complete EDTA-free protease inhibitor tablets were from Roche (Madrid, Spain). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, U. K.). All other reagents were of analytical grade.

Cell culture and transfection

NG115-401L cell lines were obtained from Sigma (Madrid, Spain) and cultured at 37°C with a 5% CO_2 in DMEM supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 100 U/mL penicillin and streptomycin.

Cells were transfected with expression plasmids for YFP-STIM1, provided by Dr. Romanin (Johannes Kepler University, Linz, Austria), as well as with pHluorin-STIM1, provided by Dr Pingyong Xu (Chinese Academy of Sciences Beijing, China), or scramble plasmid as described previously[4] using Turbofect transfection reagent.

Biotinylation protocol

Cells were washed 3 times with phosphate-buffered saline (PBS, NaCl 137 mM, KCl 2.7 mM, KH_2PO_4

1.5 mM, $Na_2HPO_4 \bullet 2H_2O$ 8 mM, pH 7,4), subsequently resuspended in biotinylation buffer (50 mM $NaHCO_3$ and 0.9% NaCl) and surface labeled with 100 μg/mL sulfo-NHS-LC biotin at RT. Labeling was stopped 1 h after reaction with 1% NH_4Cl and PBS supplemented with 50 μM EDTA and washed 2 times in PBS/EDTA. Biotinylated cells were subsequently lysed in Nonidet P40 buffer (NP40), and protein lysates were incubated with streptavidin-conjugated agarose beads overnight at 4°C on a rocking platform. Biotinylated proteins bound to streptavidin-conjugated agarose beads were isolated by centrifugation and washed 3 times in NP40 buffer. Biotinylated and non biotinylated fraction as well as total cell lysates were loaded and separated in 8% SDS-PAGE and analyzed by Western blotting.[14]

Western blotting

Proteins were resolved by 8% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose membranes for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of SARAF, STIM1 and PMCA was achieved by incubation overnight at 4 °C with anti-SARAF, anti-STIM1 or anti-PMCA antibody diluted 1:1000 in TBST. The primary antibody was removed and blots were washed 6 times for 5 min each with TBST. To detect the primary antibody, blots were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibody diluted 1:10000 in TBST and then exposed to enhanced chemiluminiscence reagents for 4 min. The density of bands was measured using C-DiGit Chemiluminescent Western Blot Scanner (LI-COR, Cambridge, UK). Data were normalized to the amount of protein recovered by the antibody used for the immunoprecipitation.

Abbreviations

ARC channel	Arachidonate-regulated Ca^{2+} channel
$[Ca^{2+}]_c$	Cytosolic free Ca^{2+} concentration
CRAC channel	Ca^{2+} release-activated Ca^{2+} channel
CTID	C-terminal inhibitory domain of STIM1
SARAF	Store-Operated Calcium Entry-Associated Regulatory Factor
SOAR	STIM1 Orai1 activation region
SOCE	Store-operated Ca^{2+} entry

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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