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STIM1 tyrosine-phosphorylation is required for STIM1-Orai1 association in human platelets

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ABSTRACT

Stromal interaction molecule 1 (STIM1) is a key element of the store-operated Ca^{2+} entry mechanism (SOCE). Recently, regulation of STIM1 by glycosylation and phosphorylation on serine/threonine or proline residues has been described; however other modes of phosphorylation that are important for activating SOCE in platelets, such as tyrosine phosphorylation, have been poorly investigated. Here we investigate the latency of STIM1 phosphorylation on tyrosine residues during the first steps of SOCE activation.
Human platelets were stimulated and fixed at desired times using rapid kinetic assays instruments, and immunoprecipitation and western blotting techniques were then used to investigate the pattern of STIM1 tyrosine phosphorylation during the first steps of SOCE activation. We have found that maximal STIM1 tyrosine phosphorylation occurred 2.5 s after stimulation of human platelets with thapsigargin (Tg). STIM1 localized in the plasma membrane were also phosphorylated in platelets stimulated with Tg. By using chemical inhibitors that target different members of the Src family of tyrosine kinases (SKFs), two independent signaling pathways involved in STIM1 tyrosine phosphorylation during the first steps of SOCE activation were identified. We finally conclude that STIM1 tyrosine phosphorylation is a key event for the association of STIM1 with plasma membrane Ca^{2+} channels such as Orai1, hence it is required for conducting SOCE activation.

1. Introduction

Store-operated Ca $^{2+}$ entry (SOCE) is a mechanism by which cells regulate the opening of plasma membrane Ca^{2+} channels upon depletion of intracellular Ca^{2+} stores [1–3]. In human platelets, we have described a *de novo* conformational coupling model as a SOCE-activating mechanism where the movement of portions of the endoplasmic reticulum (ER) to the plasma membrane allows the contact between signaling molecules located in the ER, like the type 2 inositol 1,4,5-trisphosphate receptor (IP_3R) and stromal interaction molecule 1 (STIM1), and Ca^{2+} channels in the plasma membrane, hTRPCs and Orai1 [4–6]. Among these elements, STIM1 has been identified as a crucial factor sensing Ca^{2+} in the ER and communicating the intraluminal Ca^{2+} levels to channels located in the plasma membrane [7,8].

Homozygous nonsense mutation of *STIM1* gene was found in two patients suffering immunodeficiency and autoimmunity syndrome, thus revealing the significant contribution of STIM1 and, subsequently, SOCE to human healthy status [9]. Both patients presented, among other symptoms, thrombocytopenia [9]. STIM1 presents several functional domains: an N-terminal domain, facing the lumen of the Ca^{2+} stores or the extracellular medium depending on the location of STIM1 in the Ca^{2+} pools or in the plasma membrane, respectively, which contains the EF-hand domain (the Ca^{2+}-binding site) and a SAM domain; the N-terminal region is followed by the transmembrane region that lacks relevant function described nowadays; and finally, the cytosolic C-terminal domain, which contains coiled-coil regions (protein–protein interaction regions) required for the association with other proteins, such as Orai1 [10,11].

The regulation of STIM1 activity includes several events, but the major regulatory mechanism is Ca^{2+} dissociation from the EF-hand domain, which evokes STIM1 dimerization and subsequent activation [12]. Furthermore, STIM1 function has been reported to be influenced by post-translational modifications, such as glycosylation at the SAM domain and phosphorylation of several amino acidic residues in the

Abbreviations: STIM1, Stromal interaction molecule 1; SOCE, operated Ca^{2+} entry mechanism; SERCA2b, sarco-endoplasmic Ca^{2+}-ATPase isotype 2b; IP_3R II, inositol trisphosphate receptor; TRPC, transient receptor potential channel; SKFs, Src family of tyrosine kinases; Btk, Bruton's tyrosine kinase; ER, endoplasmic reticulum; $[Ca^{2+}]_c$, cytosolic calcium concentration; Tg, thapsigargin; SKI-606, bosutinib; DTT, dithiothreitol; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; BSA, bovine serum albumin; TBST, Tris-buffered saline with 0.1% Tween 20.

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C-terminal region [13–15]. The understanding of STIM1 regulatory mechanisms might provide important information concerning SOCE activation. Although phosphorylation of STIM1 at Ser/Thr or Pro residues, located in the C-terminal region, has been recently described, the pathways involving these post-translational modifications remain largely elusive. Taking into account that treatment of human platelets with either tyrosine kinase inhibitors or tyrosine phosphatase inhibitors dramatically impairs the activation of SOCE [16–19], we aimed the present study to elucidate whether STIM1 undergoes tyrosine phosphorylation during the activation of SOCE in human platelets.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (Fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, bovine serum albumin (BSA), dithiothreitol (DTT), thapsigargin (Tg), thrombin (Thr) and anti-Orai 1 antibody were from Sigma (Madrid, Spain). Bosutinib (SKI-606) was from LC Laboratories (Woburn, Massachusetts, USA). LFM-A13 was from Tocris Bioscience (Bristol, UK). Mouse monoclonal anti-GOK/STIM1 antibody was from BD Transduction Laboratories (Madrid, Spain). Mouse monoclonal anti-phosphotyrosine (Clone 4G10) antibody and agarose beads conjugated with protein A were from Millipore (California, USA). Anti-BTK (N-20) antibody and anti-c-Src antibody were from Santa Cruz (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, United Kingdom). All other reagents were of analytical grade.

2.2. Platelet preparation

Platelet-rich plasma (PRP) was obtained by centrifugation (5 min at 700 ×*g*) of blood samples drawn from healthy volunteer (according to Helsinki Declaration and University of Extremadura Ethical Committee) and mixed with acid/citrate dextrose anticoagulant. Platelet-rich plasma was supplemented with aspirin (100 μM) and apyrase (40 μg/ml). Platelets were then collected from PRP by centrifugation at 350 ×*g* for 20 min and finally, isolated platelets were resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and (40 μg/ml) apyrase. Fura-2-loaded platelets were prepared by incubating PRP for 45 min at 37 °C with 2 μM fura2-AM, centrifuged as previously described, and resuspended in HBS.

2.3. Stopped-flow kinetic measurements

The kinetics of fluorescence change from fura-2-loaded platelets was investigated by stopped-flow fluorimetry at 37 °C using a Hi-Tech Scientific SF-61SX2 Single-Mixing Stopped-Flow System (Hi-Tech Ltd., Salisbury, Wilts., U.K.), with an excitation wavelength of 340 or 360 nm and emission at 500 nm, depending whether Ca^{2+}-evoked fura-2 fluorescence changes (to monitor calcium release) or manganese-evoked fura-2 fluorescence quenching (to monitor Mn^{2+} entry) were determined, respectively. Platelets (100 μl) and agonist solution (100 μl, 400 nM Tg) were introduced into the sample flow circuit via separate reservoirs at the top of the sample-handling unit. Fluorescence changes were expressed as the increase in fluorescence after mixing (F_n) divided by the average of values of fluorescence of the cell suspension under resting conditions (F_0). Mn^{2+}-induced quenching of fura-2 fluorescence excited at 360 nm was used as a surrogate for monitoring Ca^{2+} entry, since Mn^{2+} and Ca^{2+} share the same channels and mechanisms for entering platelets, as previously described [20].

2.4. Protein sample collection and immunoprecipitation

Cells were stimulated at 37 °C, fixed and subsequently collected using a Hi-Tech Scientific RQF-63, Dimension D1 Rapid Quench-Flow System (Hi-Tech Ltd., Salisbury, Wilts., U.K.). Briefly, the cell suspension (100 μl) and agonist solution (100 μl) were introduced into the sample flow circuit, via separate reservoirs at the top of the sample-handling unit, and mixed at the times indicated with RIPA (3×, supplemented with protease cocktail inhibitor and Na_3VO_4) for 10 min. Previously STIM1 was immunoprecipitated, the protein concentration in the platelet cytosolic samples was standardized using Bradford's technique [21]. STIM1 was isolated from the cell lysates by incubating with protein A-conjugated agarose beads and a specific anti-Gok/STIM1 antibody (2 μg/ml) that recognized the EF-hand domain of STIM1. Samples were incubated at 4 °C overnight and then immunoprecipitated STIM1 was collected by centrifugation of the beads after washing five times in PBS freshly supplemented with Na_3VO_4. In order to determine the phosphorylation state of STIM1, immunoprecipitated samples were denatured in Laemmli's buffer containing 5% DTT and heated for 10 min at 70 °C.

2.5. Western blotting

One-dimensional SDS-electrophoresis of protein samples extracted from platelets was performed on 10% sodium dodecyl sulfate-polyacrylamide gels and separated proteins were electrophoretically transferred for 2 h at 0.8 mA/cm^2 in a semi-dry chamber onto nitrocellulose for subsequence 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. Blocked membranes were then incubated with the anti-phospho-tyrosine (4G10) antibody diluted 1:1000 in blocking buffer for 1 h. Additionally, membranes were incubated for 2 h either with an anti-$pp60^{src}$ antibody or an anti-Btk (N20) antibody, both diluted 1:1000 in blocking buffer. In the experiments where STIM1/Orai1 coupling was investigated, Western blotting was performed with a specific anti-Orai1 antibody incubated overnight at 4 °C and diluted in TBST (1:1000). Excess of primary antibody was removed from the blots by washing six times for 5 min with TBST. In order to detect the primary antibody, blots were incubated for 1 h with an appropriate horseradish peroxidase-conjugated IgG antibody diluted 1:5000 to 1:10000 in TBST. After exposure to the secondary antibodies membranes were washed six times in TBST, and exposed to an enhanced chemiluminescence reagents for 1–5 min. Blots were then exposed to photographic film and the optical density was estimated using scanning densitometry. Subsequently, membrane reproving using the antibody against the immunoprecipitated proteins was done in order to asses that similar amounts of proteins were loaded in each gel lanes.

2.6. STIM1 surface membrane protein isolation

Dimethyl-BAPTA-loaded platelets were stimulated as required with Tg in the presence of EGTA and subsequently fixed with ice-cold para-formaldehyde (3%) for 10 min. Fixed-platelets were then washed twice with PBS prior to incubation with anti-STIM1 antibody for 2 h at room temperature. Excess antibody was removed by washing twice in PBS (supplemented with Na_3VO_4), and cell were lysed with RIPA (2×) supplemented with protease inhibitor cocktail and Na_3VO_4. Samples were then incubated overnight with agarose beads to isolate STIM1 bound to anti-STIM1 antibody. Samples were then washed 5 times in PBS before proteins were denatured by mixing with Laemmli's buffer containing 5% DTT and heated to 70 °C for 10 min. Subsequent Western blotting was performed to analyze the tyrosine phosphorylation state of STIM1, as previously described.