Biochimica et Biophysica Acta 1853 (2015) 233–243

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

STIM1 phosphorylation triggered by epidermal growth factor mediates cell migration

Vanessa Casas-Rua [a], Patricia Tomas-Martin [a], Aida M. Lopez-Guerrero [a], Ignacio S. Alvarez [b], Eulalia Pozo-Guisado [a,*], Francisco Javier Martin-Romero [a,*]

[a] Department of Biochemistry and Molecular Biology, School of Life Sciences, University of Extremadura, Badajoz, Spain
[b] Department of Cell Biology, School of Life Sciences, University of Extremadura, Badajoz, Spain

ARTICLE INFO

Article history:
Received 2 August 2014
Received in revised form 27 October 2014
Accepted 29 October 2014
Available online 4 November 2014

Keywords:
Calcium
EGF
ERK1/2
Migration
Phosphorylation
STIM1

ABSTRACT

STIM1 is a key regulator of store-operated calcium entry (SOCE), and therefore a mediator of Ca^{2+} entry-dependent cellular events. Phosphorylation of STIM1 at ERK1/2 target sites has been described as enhancing STIM1 activation during intracellular Ca^{2+} emptying triggered by the inhibition of the sarco(endo)plasmic Ca^{2+}-ATPase with thapsigargin. However, no physiological function is known for this specific phosphorylation. The present study examined the role of STIM1 phosphorylation in cell signaling triggered by EGF. Using a human endometrial adenocarcinoma cell line (Ishikawa cells) EGF or H-Ras(G12V), an active mutant of H-Ras, was found to trigger STIM1 phosphorylation at residues Ser575, Ser608, and Ser621, and this process was sensitive to PD0325901, an inhibitor of ERK1/2. Both, ERK1/2 activation and STIM1 phosphorylation took place in the absence of extracellular Ca^{2+}, indicating that both events are upstream steps for Ca^{2+} entry activation. Also, EGF triggered the dissociation of STIM1 from EB1 (a regulator of microtubule plus-ends) in a manner similar to that reported for the activation of STIM1 by thapsigargin. Migration of the Ishikawa cells was impaired when STIM1 phosphorylation was targeted by Ser-to-Ala substitution mutation of ERK1/2 target sites. This effect was also observed with the Ca^{2+} channel blocker SKF96365. Phosphomimetic mutation of STIM1 restored the migration to levels similar to that found for STIM1-wild type. Finally, the increased vimentin expression and relocalization of E-cadherin triggered by EGF were largely inhibited by targeting STIM1 phosphorylation, while STIM1-S575E/S608E/S621E normalized the profiles of these two EMT markers.

1. Introduction

Stromal interaction molecule (STIM1) is a single transmembrane endoplasmic reticulum protein that activates plasma membrane Ca^{2+} channels on the occurrence of conditions that trigger Ca^{2+} depletion in the endoplasmic reticulum [1]. Transient reduction in the intraluminal Ca^{2+} levels leads to Ca^{2+} dissociation from the EF-hand domain of STIM1, triggering its multimerization. STIM1 clustering precedes its relocalization underneath the plasma membrane where it activates Ca^{2+} entry through store-operated Ca^{2+} channels [2,3]. Ca^{2+} channels activated by STIM1 include some members of the TRPC channel family, and ORAI channels [4,5], including ORAI1 (also called CRACM1), a highly selective Ca^{2+} channel responsible for the Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}) [6]. STIM1 is therefore a key regulator of Ca^{2+} entry (SOCE), and its role in physiological and pathological events that are mediated by Ca^{2+} mobilization is currently under investigation. In this regard, it is known that STIM1 participates in the signaling that regulates cell migration [7], and recent evidence supports a role for Ca^{2+} entry in non-tumor cell proliferation [8–10], as well as in cell adhesion, migration, and proliferation in a number of tumor cell types [11–14]. Indeed, the increase of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) activates the Ca^{2+}-regulated protease calpain and the Ca^{2+}-stimulated protein tyrosine kinase 2 beta (PTK2B or PYK2) [12], a known regulator of focal adhesion dynamics. Consequently, the use of Ca^{2+} channel blockers or the knockdown of STIM1 or ORAI channels reduces proliferation and promotes cell cycle arrest in different tumor cell lines [11–17], indicating that SOCE may be a potential target in cancer therapy. The mechanisms of STIM1 activation thus need to be addressed in order to define potential new cancer treatment strategies.

One of the mechanisms that mediate the activation of STIM1 is the unfolding of the protein in response to store depletion into an open conformation that exposes a STIM1–ORAI1 activating region (SOAR) and allows it to interact with ORAI1 [18,19]. Another mechanism that

Abbreviations: EGF, epidermal growth factor; ER, endoplasmic reticulum; EMT, epithelial–mesenchymal transition; ERK, extracellular signal-regulated kinase; SOCE, store-operated Ca^{2+} entry

* Corresponding authors at: Department of Biochemistry and Molecular Biology, School of Life Sciences, University of Extremadura, Avenida de Elvas s/n. 06006-Badajoz, Spain. Fax: +34 924 289419.
E-mail addresses: epozo@unex.es (E. Pozo-Guisado), fjmartin@unex.es (F.J. Martin-Romero).

http://dx.doi.org/10.1016/j.bbamcr.2014.10.027
0167-4889/

has been shown to be involved in the regulation of STIM1 is the phosphorylation of the protein. The full set of phosphoresidues has already been listed [20–22], and we have previously described how STIM1 activity is modulated by ERK1/2-dependent phosphorylation at residues Ser575, Ser608, and Ser621 [22]. Constitutive dephosphorylation of these residues impairs SOCE [22], whereas simulation of constitutive phosphorylation of STIM1 by means of Ser-to-Glu mutation of the aforementioned residues enhances STIM1 multimerization and SOCE in response to Ca^{2+} store depletion [23]. In our previous reports, STIM1 phosphorylation was observed under treatment with thapsigargin, a specific sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor [24], that triggered ERK1/2 activation. Conversely, restoration of intraluminal Ca^{2+} levels led to STIM1 dephosphorylation [23], suggesting a role for as yet undescribed phosphatases in the regulation of STIM1 inactivation.

In addition to ORAI1 and other proteins such as TRPC1 and SERCA (reviewed in [25]), STIM1 directly binds to the microtubule plus-end tracking protein EB1 (end-binding protein 1) [26], a protein that regulates microtubule-growing ends [27,28]. Later, we demonstrated that STIM1 phosphorylation at ERK1/2 sites leads to the dissociation of STIM1 from EB1 [23], and that this dissociation enables the multimerization of STIM1 in microtubule-independent and immobile clusters that activate Ca^{2+} channels and Ca^{2+} entry [23]. However, little is known regarding the phosphorylation of STIM1 upon physiological stimuli. ERK1/2 becomes activated by the Ras–Raf–MEK–ERK pathway in response to a number of stimuli, including the activation of the epidermal growth factor (EGF) receptor [29,30]. Thus, the treatment of cells with EGF provides an opportunity to study the role of STIM1 phosphorylation in response to the activation of this specific signaling pathway. Furthermore, EGF triggers a chemotactic response in tumor cells [31], and is involved in the *in vitro* epithelial–mesenchymal transition (EMT) in other cell types [32,33].

Because constitutive dephosphorylation of STIM1 at ERK1/2 target sites impairs SOCE [22], we hypothesized that phosphorylation of STIM1 could regulate cell migration. To test this hypothesis, we investigated the phosphorylation profile of STIM1 in cells upon stimulation with EGF. Using Ishikawa cells, which derive from an endometrial adenocarcinoma [34], in the present report we demonstrate that EGF triggers a significant phosphorylation of STIM1 at residues Ser575, Ser608, and Ser621. We also show here the critical role of STIM1 phosphorylation at ERK1/2 target sites in cell migration. Ser-to-Ala mutation of target residues or inhibition of STIM1 phosphorylation by ERK1/2 was found to impair both EGF-dependent chemotaxis and the epithelial–mesenchymal transition (EMT) triggered by EGF. On the contrary, constitutive phosphorylation significantly promoted migration and EMT, indicating that STIM1 phosphorylation could be considered a potential therapeutic target against tumor progression.

2 . Materials and methods

2.1. Materials

EGF was purchased from Sigma; DMEM, RPMI 1640, FBS, and NuPAGE Bis-Tris gels were from Life Technologies; PD0325901 from Axon Medchem BV (Groningen, The Netherlands); fura-2-acetoxymethyl ester (fura-2-AM) was from Calbiochem (a Merck brand, Darmstadt, Germany); thapsigargin (Tg) and SKF96365 were from AbCam Biochemicals (Cambridge, UK); SuperSignal substrate for chemiluminescence was from Thermo Scientific; GFP-Trap resin was from Chromotek GmbH (Planegg-Martinsried, Germany); protein G-sepharose was from Santa Cruz Biotechnology; polyethylenimine was purchased from Polysciences, Inc. (Eppelheim, Germany).

2.2. DNA constructs

DNA constructs for transient expression of STIM1, EB1, STIM1-S575A/S608A/S621A, and STIM1-S575E/S608E/S621E tagged with either Flag-, GFP-, or mCherry-, have been described elsewhere [23,35]. DNA constructs were verified by DNA sequencing using BigDye Terminator v3.1 cycle sequencing protocol (Life Technologies) at the DNA Sequencing Unit of STAB, University of Extremadura (Spain). The construct for the transient expression of active H-Ras (H-RasG12V, construct DU20700) was from the Division Signal Transduction and Therapy (DSTT), University of Dundee, UK.

2.3. Antibodies

Phospho-specific antibodies raised against phospho-Ser575-STIM1, phospho-Ser608-STIM1, and phospho-Ser621-STIM1 were produced in collaboration with the Division of Signal Transduction Therapy (DSTT), University of Dundee, UK. The specificity of these antibodies has been reported elsewhere [23,35]. Antibodies against phosphorylated (phospho-Thr202/Tyr204) and total forms of ERK1/2 (raised in rabbit), and the rabbit anti-GFP antibody were from Cell Signaling Technology (Danvers, MA, USA). The rabbit polyclonal anti-STIM1 antibody was from ProSci Inc. (Poway, CA, USA). The rabbit anti-EB1 antibody (H-70) was from Santa Cruz Biotechnology. Anti-vimentin (Clone V9) was from Sigma-Aldrich (St. Louis, MO, USA), and anti-E-cadherin was from BD Biosciences (San Jose, CA, USA).

2.4. Cell culture

Ishikawa cells were obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Transfection of cells was performed with 1–2 μg plasmid DNA per 10-cm dish and polyethylenimine in serum-containing medium, 24–36 h prior to the beginning of the experiments. During the last 12 h, cells were cultured in FBS-free, phenol red-free RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

2.5. Cell migration assays

In vitro wound-healing assays were performed in 35-mm dishes at 80% cell confluence. The cell monolayer was scratched with a pipette tip (~500 μm width). Cells were photographed under phase contrast microscopy (time 0) and cultured as described above for the following 24 h. Thereafter, cells were photographed and quantitative image analysis of the wound healing was performed with ImageJ software.

2.6. Cell lysis and immunoblot

Cells were cultured for 12 h in FBS-free and phenol red-free RPMI 1640 medium before performing the experiments. After every treatment, cells were immediately placed on ice, washed with ice-cold PBS, and lysed. The lysis buffer was 50 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Igepal, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, and 0.1 mM phenylmethanesulfonyl fluoride. Clarification was performed after lysis with 1 ml of ice-cold lysis buffer per 10 cm-diameter dish, and centrifugation at 4 °C for 15 min at 20 000 *g*. Protein concentration was determined using the Bradford reagent (Thermo) and measuring the absorbance at 595 nm. Samples were reduced by the addition of 10 mM DTT followed by heating at 90 °C for 4 min before subjecting them to electrophoresis on 8–10% polyacrylamide Bis-Tris gels. Protein samples were electroblotted onto nitrocellulose membranes, and assessed with the following antibodies: anti-STIM1 (1 μg/ml), anti-phosphoSer575-STIM1 (1 μg/ml), anti-phosphoSer608-STIM1 (1 μg/ml), anti-phosphoSer621-STIM1 (1 μg/ml), anti-phospho-ERK1/2 (1 μg/ml), anti-total-ERK1/2 (1 μg/ml),