Modulation of store-operated calcium entry and nascent adhesion by p21-activated kinase 1

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Abstract
Calcium mobilization is necessary for cell movement during embryonic development, lymphocyte synapse formation, wound healing, and cancer cell metastasis. Depletion of calcium in the lumen of the endoplasmic reticulum using inositol triphosphate (IP3) or thapsigargin (TG) is known to induce oligomerization and cytoskeleton-mediated translocation of stromal interaction molecule 1 (STIM1) to the plasma membrane, where it interacts with the calcium release-activated calcium channel Orai1 to mediate calcium influx; this process is referred to as store-operated calcium entry (SOCE). Furthermore, aberrant STIM1 or SOCE regulation is associated with cancer cell motility and metastasis. The p21-activated kinases (PAKs), which are downstream effectors of GTPases, reportedly regulate cytoskeletal organization, protrusive activity, and cell migration. Although cytoskeletal remodeling apparently contributes to calcium mobilization via SOCE, and vice versa, the mechanisms by which they regulate each other remain unclear. In this study, we aimed to characterize whether PAK1 modulates calcium mobilization and STIM1 localization. Our data demonstrate that PAK1 interacts with STIM1 in vitro and that this interaction was enhanced by treatment with a nascent adhesion inducer, such as phorbol 12,13-dibutyrate (PDBu). Under basal conditions, both proteins appeared to primarily colocalize in the cytosol, whereas treatment with PDBu induced their colocalization to vinculin-positive peripheral adhesions. Downregulation of PAK1 activity via chemical inhibitors or by PAK1 shDNA expression impaired STIM1-mediated calcium mobilization via SOCE. Based on these findings, we propose that PAK1 interacts with STIM1 to regulate calcium mobilization and the formation of cellular adhesions.

Introduction
Intracellular calcium (Ca\(^{2+}\)) levels are precisely controlled in non-neuronal cells by ligand-gated Ca\(^{2+}\) ion channels located on the plasma membrane (PM), endoplasmic reticulum (ER), and mitochondria. The activation of PM receptors by growth factors, neurotransmitters, or hormones results in the production of inositol triphosphate (IP3) and the subsequent activation of IP3 receptors in the ER, which triggers the release of Ca\(^{2+}\) ions from ER storage. Upon depletion of ER Ca\(^{2+}\) stores, the ER membrane protein stromal interaction molecule 1 (STIM1) undergoes a conformational change, resulting in self-oligomerization and translocation to the PM for interaction with the calcium release-activated calcium channel protein Orai. During the translocation process, STIM1 is known to bind to the microtubule (MT) tip attachment protein end-binding protein 1 (EB1), thus regulating the formation and extension of new ER tubules toward the PM. ER-PM junctions serve as platforms for lipid exchange between organelles and for cell
signaling, notably Ca\(^{2+}\) and cAMP signaling\(^{12}\). The stability of ER-PM junctions and the store dependence of junction abundance varies among different cell types, as the polymerization of cortical actin, which is thought to interfere with junction formation, inhibits store-operated calcium entry (SOCE) in some cells but not others\(^{8, 13, 14}\). In human disease models, STIM1 and SOCE have been shown to promote the migration or invasion of a diverse array of human cells, including prostate cancer cells\(^{15}\), colorectal cancer cells\(^{16}\), glioma cells\(^{17}\), clear cell renal cell carcinoma cells\(^{18}\), endometrial adenocarcinoma cells\(^{19}\), and cardiac c-kit\(^{+}\) progenitor cells\(^{20}\).

Rho family GTPases, which include Rac, Cdc42, and Rho, are small GDP/GTP-binding proteins that regulate several aspects of intracellular actin dynamics and cellular signaling\(^{21–23}\). In addition to GDP/GTP exchange, most Rho/Rac proteins must dock onto cell membranes to perform their biological functions\(^{24}\). A variety of effector proteins, including kinases, are known to be recruited during this process. Rho-associated serine/threonine kinases include p21-activated kinase (PAK), mixed-lineage kinase (MLK), Rho-associated kinase (ROK), myotonic-related Cdc42-binding kinase (MRCK), citron kinase (CRK), and protein kinase novel (PKN). Among these kinases, the regulation of PAK by Rac1 and Cdc42 is relatively well-characterized\(^{25–27}\).

PAK1 has been implicated in signaling pathways associated with cytoskeletal changes, cell motility\(^{28, 29}\), and focal adhesion\(^{30–34}\). Additionally, PAK activity reportedly plays a role in Ca\(^{2+}\) mobilization. Bone marrow mononuclear cells lacking PAK1 exhibit reduced Ca\(^{2+}\) mobilization and altered depolymerization of cortical filamentous actin (F-actin) in response to stimulation of the FcRn IgE receptor\(^{35}\). Conversely, PAK2 does not appear to regulate Ca\(^{2+}\) influx and is not enzymatically controlled by Ca\(^{2+}\) influx\(^{36, 37}\). In a murine cardiac model, PAK1 appears to be an important regulator of sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) expression in cardiomyocytes for ventricular Ca\(^{2+}\) homeostasis\(^{38}\).

Because members of the PAK family of proteins are key players in regulating the intracellular cytoskeleton, and because STIM1/SoCE are involved in cell migration, we sought to investigate whether PAK1 modulated STIM1-mediated Ca\(^{2+}\) mobilization and STIM1 localization. Biochemical and confocal imaging assays were used to study the interaction between PAK1 and STIM1, as well as their colocalization. A Ca\(^{2+}\) mobilization assay involving thapsigargin (TG) was used to determine whether PAK1 activity correlated with SOCE activity level. Finally, we investigated changes in cellular adhesion following phorbol 12,13-dibutyrate (PDBu) treatment in cells, using vinculin as a marker to identify the nature and extent of PAK1 and STIM1 localization.

**Materials and methods**

**Plasmids and reagents**

Full-length human STIM1 cDNA was PCR-amplified using gene-specific primers and cloned into the cytomegalovirus (CMV) promoter-derived mammalian expression vector pCS4-3xFlag, followed by DNA sequence verification. The cytoplasmic region (amino acids 235–685) and the C1 (amino acids 235–350), C2 (amino acids 341–490), and C3 (amino acids 491–685) STIM1 cDNA regions were cloned into the pGEX-4T-1 plasmid to express and purify cytoplasmic STIM1 GST fusion proteins of varying lengths. Full-length human Orai1 cDNA was cloned into the pmCherry-N1 vector, followed by DNA sequence verification. Wild type (WT), dominant active (DA), and dominant-negative (DN) PAK1 cDNAs were cloned into pCMV-myc-tagged plasmids as described in Kim et al.\(^{39}\). The DA and DN forms of PAK1 in RFP (pDsRed2-N1) plasmids were provided by Eun-Young Shin at Chungbuk National University, Korea.

PAK1 inhibitors [1, 4-naphthohydroquinone (1, 4-NHQ) and PF3758309] were purchased from Sigma-Aldrich (St Louis, MO, USA) and Calbiochem (San Diego, CA, USA), respectively. PDBu was purchased from Tocris Bioscience (Bristol, UK) and used at a 100 nM concentration for western blot analysis and confocal imaging.

Antibodies against the Myc (9E10; Santa Cruz Biotechnology) and Flag (M2; Sigma) epitopes and vinculin (Abcam, Cambridge, UK) were used for western blot analysis and confocal imaging. Secondary antibodies against Myc and Flag epitopes (goat anti-mouse Alexa Fluor® 488 and goat anti-rabbit Alexa Fluor® 594 and 633; Invitrogen) were also used. Rabbit monoclonal PAK1 (Abcam, ab40852) and mouse monoclonal STIM1 (Santa Cruz, sc-68897) antibodies were used to detect endogenous PAK1 and STIM1, respectively.

**Cell culture and cell lines**

HEK293 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin and streptomycin. For starvation, the cells were transfected with plasmids, the growth media was discarded after 12 h, and the cells were washed once with 1× phosphate-buffered saline (PBS) and replenished with DMEM containing penicillin and streptomycin but no FBS for the indicated time periods. A PAK1 knockdown (K/D) cell line was established by transfecting HeLa cells with a PAK1 shRNA vector (Origene, pRFP-C-RS), followed by selection for puromycin resistance and western blotting analysis of reduced PAK1 expression using a polyclonal anti-PAK1 antibody (Cell Signaling Technology, 2602S).
Fig. 1 (See legend on next page.)
Transfection, western blotting, and immunoprecipitation

HEK293 and HeLa cells were seeded in 6-well plates one day prior to transfection using polyethylenimine (PEI; Sigma-Aldrich) and treated as previously described.

In vitro kinase assay

GST-STIM1 fusion proteins (GST-STIM1-C-term, -C1, -C2, and -C3) or GST control proteins were purified from E. coli, and an equal amount (2 µg) of each GST-chimeric protein was mixed with 1 µl of kinase-active PAK1 (PV3820, Thermo Fisher Scientific, MA, USA) in the presence of [γ-32P] ATP and kinase buffer (20 mM HEPES pH 7.4, 1 mM DTT, 0.1 mM Na3VO4, 2 mM EGTA, 20 mM MgCl2, and protease inhibitor) for 30 min at 30 °C before subjection to SDS-PAGE and autoradiography. GST was included as a negative control (g). EF Hand motif (Ca2+ binding), SAM STIM1 oligomerization, TM trans-membrane region, C-C coiled coil domain (Orai1 interaction), P proline rich region, K polybasic cluster, GST glutathione S-transferase

Immuno fluorescence microscopy

The cells were plated in a 4-well dish (NUNC) at a density of 5 x 103 cells/well and incubated overnight. The cells were then transfected as described above, washed with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature (RT), permeabilized in 0.1% Triton X-100 in 1 x PBS for 10 min, washed three times with PBS, and blocked with 10% FBS. To detect STIM1 and PAK1, the transfected cells were incubated with anti-flag and anti-myc antibodies, respectively, for 1 h at RT, washed with PBS, and incubated for 30 min with fluorochrome-conjugated secondary antibodies (Alexa Fluor® 488, 594, and 633 conjugated antibodies). After mounting, the cells were analyzed using a laser-scanning confocal microscope (Zeiss LSM710). The localization of endogenous STIM1 and PAK1 was analyzed as described above using monoclonal PAK1 and polyclonal STIM1 antibodies.

Assay for calcium mobilization

HeLa cells were transfected with DA or DN PAKi cDNA (PAK1 in pDsRed2-N1 plasmid) and incubated for 12 h. Then, the cells were incubated with 1 µM Fluo-4 (Invitrogen, USA), a fluorescent Ca2+ indicator dye, for 30 min before washing once with Krebs-Ringer-HEPES buffer (125 mM NaCl, 2.6 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 5.5 mM glucose, and 10 mM HEPES, pH 7.4) and replenishing with Krebs-Ringer-HEPES high K+ buffer (40.6 mM NaCl, 87 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7H2O, 5.5 mM glucose, and 10 mM HEPES, pH 7.4). Under these conditions, EGTA (final [1.25 mM]), TG (final [1 µM]), and Ca2+ (final [2 mM]) were added to the media, and the fluctuation of intracellular Ca2+ mobilization was imaged using confocal microscopy (Carl Zeiss LSM710). Ca2+ mobilization in the PAK1 stable K/D cells was also analyzed as described above.

Results

PAK1 interacts with and phosphorylates STIM1

To investigate a potential interaction with STIM1, HEK293 cells were transfected with PAK1 cDNAs alone or in combination with the STIM1 expression plasmid. The expression of myc-tagged PAK1 proteins, which included the WT, DA, and DN forms, was evaluated using an anti-myc antibody (Fig. 1), and STIM1 expression was evaluated using an anti-flag antibody. Among the three PAK1 protein forms, DN PAK1 appeared to exhibit the highest expression level regardless of STIM1 co-expression (Fig. 1a, middle row). Conversely, STIM1 expression levels increased in the presence of PAK1 (Fig. 1a, bottom row). A pull-down assay with an anti-flag (STIM1) antibody revealed that WT, DA, and DN PAK1 expression levels increased in the presence of STIM1 and PAK1 proteins immunoprecipitated with STIM1, indicating that STIM1 can interact with PAK1 proteins independent of its kinase activity (Fig. 1a, top row). Then, we tested the interaction between endogenous STIM1 and PAK1 using STIM1 and PAK1 antibodies, and found that both endogenous proteins interacted with each other under basal conditions. Interestingly, their interaction appeared to increase upon treatment with PDBu (Fig. 1b). To rule out the possibility of an indirect or non-specific protein–protein interaction in a human cancer cell background, such as with HeLa cells, the cytosolic region

(see figure on previous page)

Fig. 1 PAK1 interacts with and phosphorylates STIM1. HEK293 cells were transfected with the PAK1 plasmid (c-myc epitope tagged) and/or with STIM1 plasmid (flag epitope tagged). After determining the expression of the STIM1 and/or PAK1 proteins (a, middle and bottom rows), the cleared cell lysates were immunoprecipitated with an anti-flag (STIM1) antibody, followed by Western blotting with an anti-c-myc (PAK1) antibody (a, top row). HeLa cells were treated with 100 nM PDBu for 30 min, and the cell lysates were cleared for immunoprecipitation with an anti-STIM1 antibody and then analyzed by western blotting with an anti-PAK1 antibody (b). The GST-STIM1 and GST proteins were expressed in E. coli and, after purification, were incubated with purified commercial PAK1 protein followed by western blotting with an anti-PAK1 antibody (c, d). The C-terminal cytosolic region of STIM1 or three sub regions of STIM1 were expressed as GST-STIM1 fusion proteins in E. coli (e, f). Radioactive in vitro kinase assays were performed by incubating 1 µg of the PAK1 protein and 2 µg of purified GST-STIM1 fusion proteins in kinase buffer containing [γ-32P] ATP for 30 min at 30 °C before subjection to SDS-PAGE and autoradiography. GST was included as a negative control (g). EF Hand motif (Ca2+ binding), SAM STIM1 oligomerization, TM trans-membrane region, C-C coiled coil domain (Orai1 interaction), P proline rich region, K polybasic cluster, GST glutathione S-transferase.
Fig. 2 (See legend on next page.)
of STIM1 was expressed as a GST fusion protein in *E. coli* and incubated with purified PAK1 protein overnight at 4°C after purification, followed by washing and immunoblotting with anti-PAK1 antibody. The results of this assay revealed that STIM1 interacted with purified PAK1 as a GST fusion protein, whereas the GST protein did not pull down the PAK1 protein (Fig. 1c, d).

To further define the cytosolic region in STIM1 necessary for the interaction with PAK1, the C-terminal region of STIM1 was divided into three regions (C1: spanning amino acids 235–350, C2: 341–490, and C3: 491–685) (Fig. 1e) and expressed as GST-STIM1 fusion proteins in *E. coli* (Fig. 1f). An in vitro kinase assay was performed to identify which of the three STIM1 cytosolic regions was phosphorylated by PAK1 kinase; this revealed that the C1 region of STIM1 may be the primary phosphorylation site and potential binding site of the PAK1 kinase (Fig. 1g). However, we were not able to further analyze the specific phosphorylation site in the C1 and C2 regions by LC-MS/MS due to a low signal-to-background noise ratio (data not shown).

**PAK1 colocalizes with STIM1**

PAK1 and STIM1 have been reported to exhibit different cellular localization patterns near the cell membrane depending on their activation status in the cytosol or ER, respectively. Based on our findings, we subsequently examined the localization of PAK1 and STIM1 using confocal microscopy. When overexpressed individually in HeLa cells, WT PAK1, and STIM1 each appeared primarily in the cytosol or ER, but not in the cell membrane compartment (Fig. 2b). However, the co-expression of both proteins resulted in a dramatic change in their localization patterns from the cytosol to the cell periphery or protrusions (Fig. 2a). The DA form of PAK1 primarily localized in the cytosol with some expression in the cell periphery, whereas co-expression with STIM1 resulted in colocalization of both proteins in the cytosol; the overall cell morphology appeared similar to cells expressing only the PAK1 mutant. In contrast, the DN form of PAK1 induced a cell shape with lamellipodia, whereas the co-expression of PAK1 and STIM1 resulted in colocalization of both proteins at cell adhesions. Under basal conditions, endogenous PAK1 and STIM1 colocalized in the cytosol, but treatment with PDBu caused a shift in the localization patterns of both proteins to include the cell periphery and protrusions (Fig. 2c).

**The level of intracellular Ca$^{2+}$ mobilization correlates with PAK1 activity**

Next, we attempted to determine whether PAK1 modulates SOCE activity. Accordingly, HeLa cells were transfected with kinase-active and -inactive forms of PAK1 cDNAs in a RFP plasmid and loaded with Fluo-4 for Ca$^{2+}$ imaging using confocal microscopy. The depletion of ER Ca$^{2+}$ was induced with TG, and SOCE activation was analyzed after the addition of 2 mM Ca$^{2+}$. As shown in Figure 3a, the kinase-active form of PAK1 exhibited increased Ca$^{2+}$ mobilization after both TG stimulation and SOCE activation compared to the RFP vector control, whereas the kinase inactive form of PAK1 appeared to inhibit Ca$^{2+}$ mobilization. To confirm this finding, we used chemical inhibitors of PAK1, including 1,4-naphthoquinone (1,4-NHQ) and PF3758309, in the Ca$^{2+}$ mobilization assay. As shown in Figure 3b, c, treatment with both PAK1 inhibitors prevented TG-induced Ca$^{2+}$ mobilization in a dose-dependent manner. Additionally, the localization of WT STIM1 and WT PAK1 changed following treatment with TG from cell protrusions or puncta in the cytosol to stress fiber-like structures (Fig. 3d).

We also established a PAK1 K/D HeLa cell line expressing PAK1 siRNA and analyzed whether PAK1 expression levels were correlated with SOCE-mediated Ca$^{2+}$ mobilization levels. As shown in Figure 3e, the PAK1 K/D cell line exhibited decreased PAK1 levels and reduced SOCE activity compared to the control (scrambled) HeLa cell line (Fig. 3e), clearly suggesting the significance of optimal PAK1 levels for the SOCE process.

**PAK1 and STIM1 localize at vinculin-positive cell protrusions and nascent adhesions**

To characterize the nature of PAK1 and STIM1 localization at cell protrusions and the cell periphery, HeLa cells were transfected with *PAK1* cDNAs (c-Myc-tagged) and/or eGFP-STIM1. The transfected cells were treated with PDBu to elicit a change in cell adhesion before processing the cells using anti-c-myc (PAK1) and anti-
Fig. 3 (See legend on next page.)
vinculin antibodies for confocal imaging. In control cells, vinculin localized to stable focal adhesions, whereas STIM1 and PAK1 did not appear to colocalize with vinculin. However, after modulating the focal adhesions with PDBu treatment, vinculin colocalized with PAK1 and STIM1 at nascent adhesions on the cell periphery and cell protrusions (Fig. 4a). Conversely, when PAK1 was over-expressed alone, it did not colocalize with the vinculin marker protein at the focal adhesions, whereas PDBu treatment induced numerous nascent adhesions to which PAK1 and vinculin colocalized (Fig. 4b control). Under control basal conditions, eGFP-STIM1 appeared to localize primarily in the cytoplasm and rarely colocalized with vinculin in the cell periphery. In PDBu-treated cells, some degree of eGFP-STIM1 colocalizations with vinculin was found in the cell periphery.

Discussion

PAK1 is known to control cellular focal adhesion strength by regulating the distribution of myosin IIA and actin dynamics to optimize cell migration \(^ {33}\) and cell adhesion \(^ {40}\). Furthermore, these biological roles are known to require precise spatial and kinetic control of PAK1 localization and activity \(^ {28, 41}\).

Our study demonstrated that PAK1 interacts with STIM1 in vitro under endogenous and overexpression conditions and that PAK1 activity does not appear to be important in the interaction. The results of the in vitro kinase assay suggested that the C1 and C2 domains of STIM1 might be overlapping or separate sites for the interaction with and/or the phosphorylation by PAK1. However, the C3 C-terminal end domain, which is 195 amino acids long and contains the site of ERK1/2 phosphorylation for EB1 dissociation and SOCE activation \(^ {42}\), was not phosphorylated by PAK1. This could suggest that the PAK1 and MAPK signaling pathways regulate STIM1 differently by targeting different domains of STIM1 \(^ {43}\). Hence, it will be interesting to determine whether PAK1 can phosphorylate STIM1 irrespective of the Ca\(^{2+}\) influx due to the activation of SOCE, as seen with ERK1 \(^ {43}\).

PAK1 reportedly localizes primarily in the cytosol in an inactive form; the activation of PAK1, via interaction with the activators Cdc42-GTP and Rac1-GTP, promotes its localization at cellular protrusions \(^ {41}\). As noted previously, STIM1 translocates from the cytosol near the PM by binding to EB1, thus connecting the tubular ER directly to growing microtubules toward the ER-PM junction \(^ {9, 10}\). However, these GTPases and/or their downstream effector PAK1 regulate Ca\(^{2+}\) mobilization has been unclear.

Our confocal data revealed that STIM1 and PAK1 primarily localized in the cytoplasm, whereas PDBu treatment distinctly induced their colocalization at nascent adhesions, suggesting that both proteins were activated. How these two proteins exhibit such a colocalization pattern remains unclear, as well as whether a reciprocal control mechanism exists between the two proteins or unilateral regulation by one protein over the other. Precise PAK1 kinase activity might be important, as only WT PAK1, but not the DA or DN PAK1 mutants, induced nascent adhesions similar in size and number to the nascent adhesions of PDBu-treated cells.

We were interested in characterizing the nature of the cellular protrusions to which PAK1 and STIM1 colocalized. Under basal conditions, neither protein colocalized with vinculin at the cell periphery, and both were found in the cytosol. However, when the cells were treated with PDBu to modulate stable focal adhesions for nascent adhesions, both proteins appeared to colocalize at vinculin-positive nascent adhesions at the cell periphery but not in the stable focal adhesions (Fig. 4). This result indicated that PAK1 and STIM1 might transiently interact and colocalize in nascent adhesions before the formation of stable focal adhesions.

Taken together, our data demonstrate that PAK1 interacts with STIM1 and phosphorylates specific STIM1 cytosolic domains. Based on the Ca\(^{2+}\) assay data, PAK1 appears to play a role in modulating the STIM1-mediated SOCE process. The PDBu confocal data suggest that PAK1 and STIM1 transiently migrate from the cytosol to the cell periphery in nascent adhesions. However, further studies are required to determine the exact role PAK1 and STIM1 play in the formation of nascent adhesions with respect to Rho family GTPases and whether there is an optimal Ca\(^{2+}\) concentration window for the formation of these transient adhesion structures. Additional studies are also needed to elucidate the role of PAK1 in cancer cell migration and metastasis.
Fig. 4 PAK1 and STIM1 localize at vinculin-positive cell protrusions and nascent adhesions. PAK1 (c-myc-tagged) and/or eGFP-STIM1 plasmids were transfected into HeLa cells; after an overnight incubation, the cells were treated for 30 min with (a, b PDBu) or without 100 nM PDBu (a, b control) before processing with anti-PAK1 (c-myc) and anti-Vinculin antibodies for confocal imaging. The area where both proteins distinctly localize is marked with either boxes or circles.
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Conflict of interest
The authors declare that they have no conflict of interest.

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