Endothelin 1 Stimulates β_1Pix-Dependent Activation of Cdc42 Through the $G_{s\alpha}$ Pathway

AHMED CHAHDI AND ANDREY SOROKIN[1]

Division of Nephrology, Department of Medicine, Kidney Disease Center and Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

β_1Pix (PAK-interacting exchange factor) is a recently identified guanine nucleotide exchange factor (GEF) for the Rho family small G protein Cdc42/Rac. On stimulation with extracellular signals, GEFs induce the exchange of guanosine diphosphate to guanosine triphosphate, resulting in the activation of the small guanosine 5C-triphosphatases. This activation enables the signal to propagate to downstream effectors. Herein, we show that $G_{s\alpha}$ stimulation by cholera toxin increased Cdc42 activation by endothelin-1 (ET-1), whereas pertussis toxin had no effect. H-89, a protein kinase A (PKA) inhibitor, strongly inhibited Cdc42 activation by ET-1. Moreover, the overexpression of β_1Pix enhanced ET-1–induced Cdc42 activation. The essential role of β_1Pix in ET-1–induced Cdc42 activation was evidenced by the blocking of Cdc42 activation in cells expressing β_1Pix mutant lacking the ability to bind PAK (β_1Pix SH3m[W43K]) or mutant lacking GEF activity (β_1PixΔDH). The overexpression of mutant lacking the pleckstrin homology domain β_1PixΔPH, which is unable to bind phospholipids, had no effect on Cdc42 activation. These results demonstrate that β_1Pix, along with PKA, plays a crucial role in the regulation of Cdc42 activation by ET-1. Exp Biol Med 231:761–765, 2006

Key words: G proteins; Cdc42; Pix; endothelin; mesangial cells

Introduction

The Rho family small guanosine 5C-triphosphatases (GTPases) have emerged as key regulators that mediate extracellular signaling pathways, leading to the formation of polarized actin-containing structures such as stress fibers, membrane ruffles, lamellipodia, and filopodia. Besides changes in cytoskeletal architecture, these GTPases mediate diverse biologic events, including stimulation of DNA synthesis, cellular transformation, and signaling to the nucleus (1, 2).

Rho GTPases cycle between inactive guanosine diphosphate (GDP)–bound and active guanosine triphosphate (GTP)–bound forms. Interconversion between these two forms is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors. GEFs of the Dbl family stimulate activation of Rho GTPases by catalyzing GDP/GTP exchange of these G proteins (3–5). All members of this family contain the Dbl homology (DH) domain, which is responsible for catalytic activity. GEF proteins are activated in various ways, including phosphorylation by protein kinases (4–6). GEFs also contain a pleckstrin homology (PH) domain that is responsible for the interaction with phospholipids. PAK-interacting exchange factor (Pix) family proteins consist of two isoforms, αPix and βPix, and recently a new splice variant of βPix, designated as β_2Pix, has been identified (7). The human Pix family bind tightly through an N-terminal Src homology 3 (SH3) domain to a conserved proline-rich PAK sequence located at the C terminus and are colocalized with PAK to form activated Cdc42- and Rac1-driven focal complexes (8). Recently, Pix has been shown to form a trimolecular complex with PAK1 and p95PKL (also known as G protein–coupled receptor [GPCR] kinase-interacting target) (9). Furthermore, tyrosine-phosphorylated p95PKL can bind paxillin (10, 11) and therefore provides the link between Pix/PAK and focal complexes through this interaction. The presence of several domains allows Pix to interact with a variety of signaling proteins and suggests that Pix might have an important role in mediating the effects of extracellular signals (12–14).

In the present study, we demonstrated that the stimulation of a subunit of G_s protein by cholera toxin enhanced Cdc42 activation by endothelin-1 (ET-1). The overexpression of β_1Pix in mesangial cells enhanced Cdc42 activation by ET-1. We also showed that this activation is blocked by protein kinase A (PKA) inhibitor H-89.

This study was supported by a grant from the American Heart Association grant (A.C.) and grant HL22563 from the National Institutes of Health (A.S.)

[1] To whom correspondence should be addressed at the Division of Nephrology, Department of Medicine, Kidney Disease Center and Cardiovascular Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. E-mail: sorokin@mcw.edu

Received October 3, 2005.
Accepted November 19, 2005.

1535-3702/06/2316-0761$15.00

Materials and Methods

Cell Culture and Transfection. All materials for cell culturing were purchased from Invitrogen (Carlsbad, CA). Previously characterized simian virus 40–transformed human mesangial cells (HMCs) (15) were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 37°C humidified incubator with 5% CO_2. Transient transfection of cells with mammalian expression vectors was performed using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions.

Pulldown Assays of Rho Family GTPases. Cells were transfected with empty vector, Myc-tagged β_1Pix, or its mutants for 24 hrs. After stimulation with ET-1 for 5 mins, cells were lysed in lysis/wash buffer (25 m*M* HEPES, pH 7.5, 150 m*M* NaCl, 1% Igepal CA-630, 10 m*M* $MgCl_2$, 1 m*M* EDTA, 1% glycerol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). To measure the active GTP-bound form of endogenous Cdc42 in the cell lysates, we performed pulldown assay (Cytoskeleton, Denver, CO) using recombinant glutathione S-transferase (GST)–tagged PAK1-PBD·PaK-binding domain (PDK). Aliquots (500 μg) of the supernatants mixed with glutathione agarose with 10 μg of GST·PAK1·PBD were precipitated by centrifugation. Complexes were boiled in a Laemmli sample buffer and then separated on 15% SDS polyacrylamide gels. The separated proteins were immunoblotted using specific anti-Cdc42 antibody.

Reverse Transcription Polymerase Chain Reaction (PCR) Analysis. Total RNA isolated from rat mesangial cells was reverse transcribed using Superscript reverse transcriptase (Invitrogen), oligo (dT) primers (Invitrogen), and deoxynucleotide triphosphate as specified by the manufacturer. β_1Pix was amplified by means of PCR using TITANIUM *Taq* polymerase (Clontech Laboratories, Inc., Palo Alto, CA) in the presence of deoxynucleotide triphosphate, the forward primer 5′-GGAATTCCATGACTGATAACGCCAACAGCCAA-3′, and the reverse primer 5′-GCTCTAGAGCTAGATTGGTCTCATCCCAAGCAGG-3′. The PCR products were subjected to electrophoresis in a 1% acrylamide gel, and the results were visualized using a bioimaging analyzer. The β_1Pix cDNA was cut with *Eco*RI and *Xba*I and inserted into the *Eco*RI-*Xba*I site of pcDNA3.1/Myc-His vector. β_1Pix mutants β_1Pix SH3m(W43K), β_1PixΔDH, and β_1PixΔPH were made using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) (16).

GDP/GTP Exchange Assays. The exchange assays were performed as previously described (17). For GTPγS binding, 2 μg of the recombinant GTPases were initially incubated for 5 mins in 60 μl of loading buffer (20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 2 m*M* EDTA, 0.2 m*M* dithiothreitol, 100 μ*M* adenosine monophosphate–purine nucleoside phosphorylase [AMP-PNP], and 10 μ*M* GDP) at room temperature. $MgCl_2$ was then added to a final concentration of 5 m*M*, and the incubation continued for an additional 15 mins. Finally, aliquots (20 μl) of GDP-loaded GTPases were mixed with 100 μg of lysates from cells overexpressing c-Myc-β_1Pix or c-Myc-β_1Pix(L238R, L239S) diluted in reaction buffer (20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 100 μ*M* AMP-PNP, 0.5 mg/ml bovine serum albumin, and 5 μ*M* [^{35}S]GTPγS) to initiate the exchange reaction (final volume, 100 μl) at room temperature. Aliquots (15 μl) of samples were taken at various time points from the reaction mixture and added to 10 ml of ice-cold phosphate-buffered saline. Bound and free nucleotides were separated by filtration through BA85 nitrocellulose filters. For the GDP dissociation assay, 10 μ*M* radiolabeled [^{3}H]GDP was used in the loading buffer instead of GDP, and 1 m*M* GTP was used in the reaction buffer instead of [^{35}S]GTPγS.

Results

We first sought to determine whether β_1Pix regulates Cdc42 activation by ET-1. Pix family proteins are GEFs for the small GTPase proteins Cdc42/Rac (8) and have been shown to signal via these proteins. Therefore, we studied the effect of β_1Pix and its inactive mutants on ET-1–induced Cdc42 activation in HMCs. In our experiments, Cdc42 activation was measured after ET-1 treatment of HMC-overexpressing wild-type β_1Pix or its mutants β_1Pix SH3m(W43K), β_1PixΔDH, or β_1PixΔPH (8). ET-1 induced Cdc42 activation in cells expressing empty vector, and this activation was enhanced by β_1Pix overexpression. By contrast, β_1PixΔDH, which lacks GEF activity, and SH3 domain–mutated β_1Pix SH3m(W43K), which lacks the ability to bind to PAK, significantly decreased ET-1–induced Cdc42 activation (Fig. 1A). The expression of β_1PixΔPH had no effect on Cdc42 activity. This result indicates that PAK (or another SH3 domain) and GEF activity of β_1Pix are essential for the regulation of Cdc42 activation by ET-1, whereas the PH domain is not.

To demonstrate that Cdc42 is specifically activated by ET-1, we treated the cells with toxin B, which specifically inhibits Cdc42. Figure 1B shows that toxin B completely blocked Cdc42 activation by ET-1.

We wanted to confirm that β_1Pix is working as a GEF for Cdc42. *In vitro* assays measured the Cdc42 GEF activity of β_1Pix (Fig. 2). β_1Pix enhanced the incorporation of GTPγS into purified Cdc42, whereas β_1Pix had no effect on GTPγS incorporation into the dominant negative form of Cdc42 (Fig. 2A). In reciprocal experiments, [^{3}H]GDP dissociation from purified Cdc42 was enhanced by immunoprecipitated c-Myc–tagged β_1Pix but not by the β_1Pix(L238R, L239S) mutant, which has GEF activity (Fig. 2B). The GDP release assay confirmed that β_1Pix is a GDP/GTP exchange factor for Cdc42.

Specific small GTPases are activated by the phosphorylation of GEF (7, 8). Results of a previous study showed

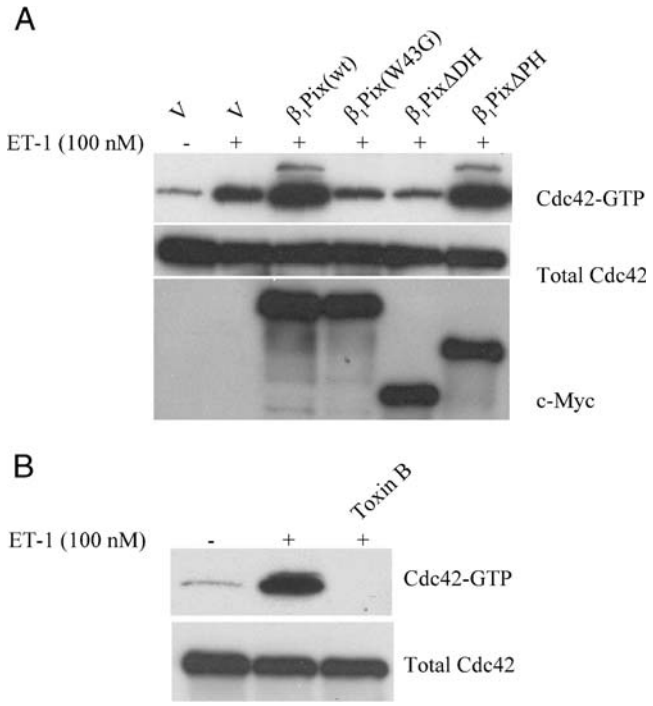

Figure 1. Role of β_1Pix in ET-1–induced Cdc42 activation. (A) HMCs were cotransfected with empty vector alone, Myc-tagged β_1Pix(WT), or the β_1Pix mutants β_1Pix SH3m(W43K), β_1PixΔDH, or β_1PixΔPH. After 24 hrs of transfection, cells were stimulated with ET-1 (100 n*M*) for 5 mins, and active Cdc42 was measured as described in the "Materials and Methods" section. (B) Cells were treated with toxin B (10 ng/ml) for 3 hrs before stimulation with ET-1 (100 n*M*) for 5 mins. The data are representative of three independent experiments.

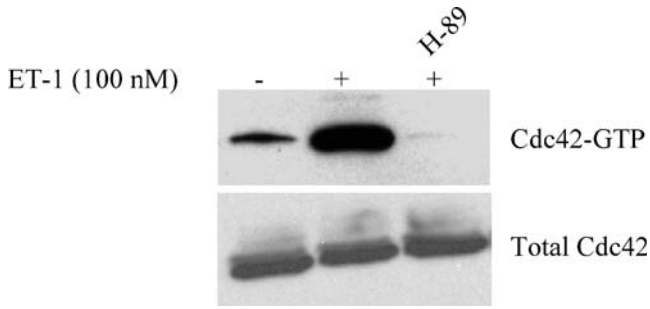

Figure 3. Effect of PKA inhibitor on ET-1–induced Cdc42 activation. HMCs were treated with H-89 (10 μ*M*) for 45 mins and then stimulated with ET-1 (100 n*M*) for 15 mins. Activated Cdc42 was measured as described in the "Materials and Methods" section. The data are representative of three independent experiments.

that PKA phosphorylates β_1Pix on Ser516 and Thr526 (16). Accordingly, we examined whether this kinase regulates ET-1–induced Cdc42 activation. For this purpose, we used H-89, a PKA inhibitor. Cells were starved for 24 hrs and stimulated with ET-1 for 15 mins. Preincubation with H-89 abolished ET-1–induced Cdc42 activation (Fig. 3). This result strongly indicates that PKA acts upstream to regulate Cdc42 activation. This finding is in agreement with previous data showing that the overexpression of a nonphosphorylatable form of β_1Pix, β_1Pix(S516A, T526A), prevents ET-1–induced Cdc42 activation (16). Collectively, the results herein strongly support the theory that β_1Pix phosphorylation by PKA occurs upstream of Cdc42 activation. This phosphorylation results in β_1Pix activation and its translocation to focal adhesion complexes where it activates Cdc42. Indeed, H-89 treatment of the cells overexpressing β_1Pix or the expression of β_1Pix(S516A, T526A) blocked β_1Pix translocation to focal adhesion complexes (16).

It appears that activation of Cdc42 can be mediated by different GPCRs. ET-1 receptors are coupled to different G proteins depending on the cell type. In an attempt to determine which G protein is involved in ET-1–induced

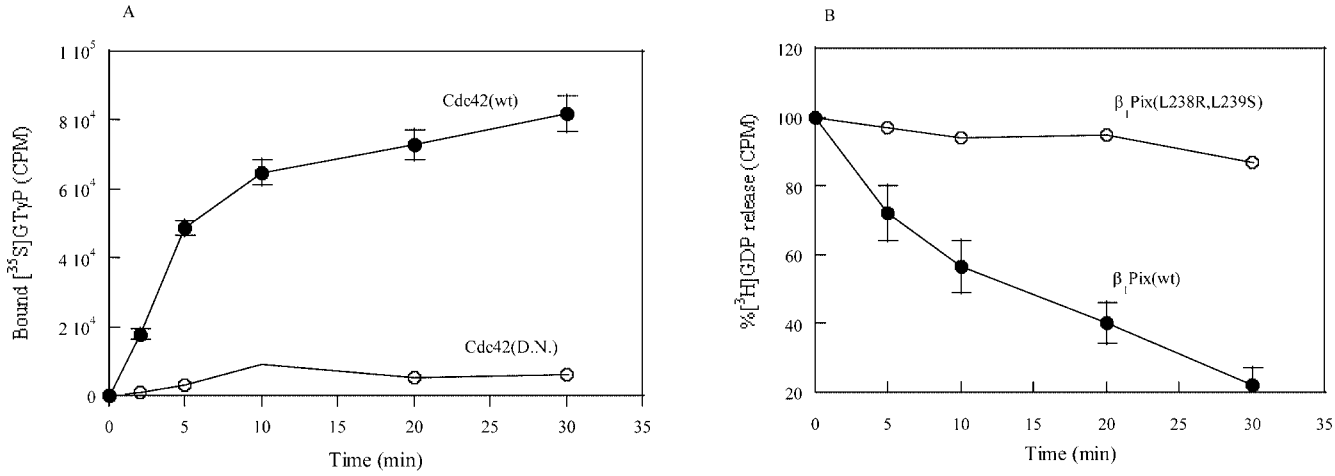

Figure 2. β_1Pix is a specific guanine nucleotide exchange factor for Cdc42. (A) Time course for the binding of [^{35}S]GTPγS to purified Cdc42(wt) or dominant negative Cdc42. Purified Cdc42 (solid circles) or its dominant negative form (open circles) were preloaded with GDP and then added to reaction incubations containing [^{35}S]GTPγS together with aliquots from HMC cell lysates overexpressing c-Myc–tagged β_1Pix. (B) Time course of the dissociation of [^{3}H]GDP from purified Cdc42. Purified Cdc42 was preloaded with [^{3}H]GDP and then added to reaction incubations containing 1 m*M* GTP together with aliquots from HMC cell lysates overexpressing β_1Pix (solid circles) or the β_1Pix(L238R, L239S) mutant (open circles). The data are expressed as means ± SE of three independent experiments.

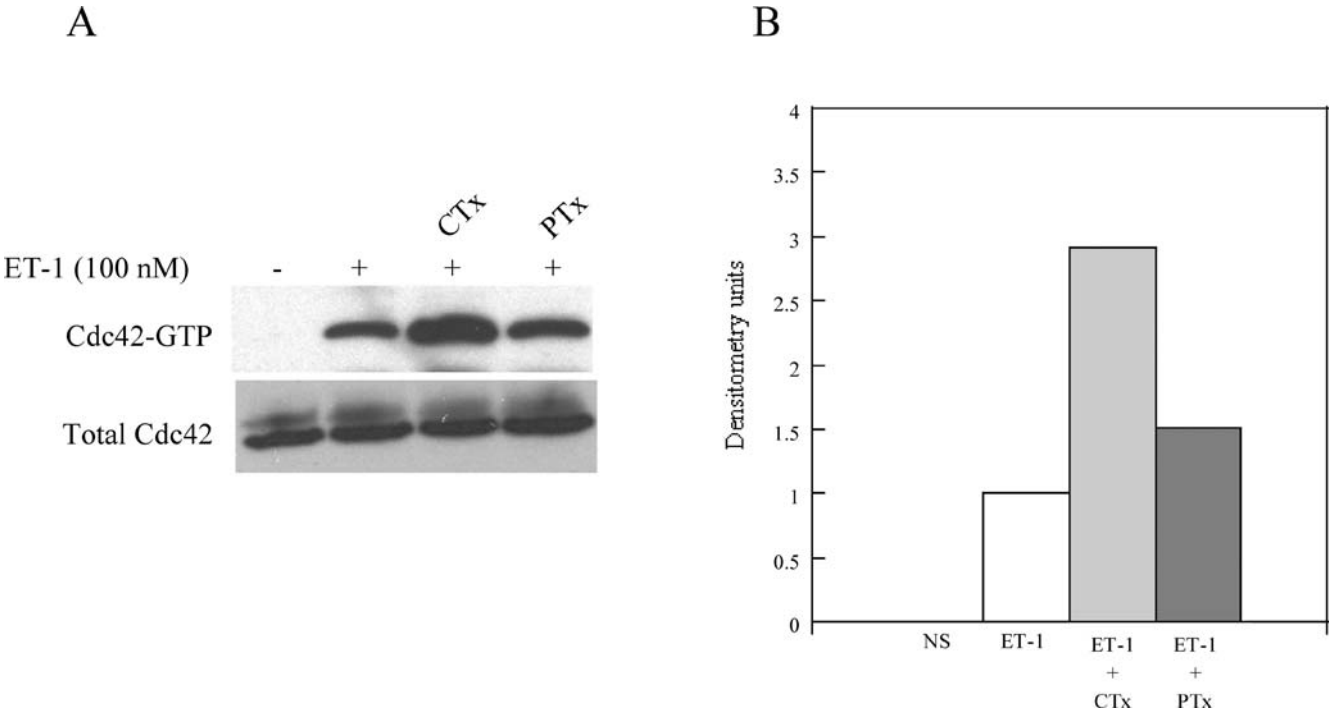

Figure 4. Effect of cholera and pertussis toxins on Cdc42 activation by ET-1. (A) HMCs were treated with cholera toxin (200 ng/ml) or pertussis toxin (100 ng/ml) for 4 hrs before ET-1 stimulation. Active Cdc42 was measured using pulldown assay. (B) Quantitative analysis of the results obtained in (A) by densitometry.

Cdc42 activation, we used different toxins. Cholera toxin, which adenosine diphosphate-ribosylates and permanently activates the α subunit of G_s protein, enhanced Cdc42 activation by ET-1 (Fig. 4, lane 3). Inhibition of $G_{i/o}$ proteins by pertussis toxin had no effect on ET-1–induced Cdc42 activation (Fig. 4, lane 4), indicating that $G_{s\alpha}$ protein is transducing the signal from ET-1 receptors downstream to Cdc42. This result corroborates our findings that PKA acts upstream of Cdc42 activation by ET-1. It is well established that the $G_{s\alpha}$ signaling pathway induces the activation of PKA through the production of cyclic adenosine monophosphate (cAMP) by adenylate cyclase.

Discussion

There is accumulating evidence that GPCR agonists activate small GTPases that, in turn, modulate a variety of biologic responses, including cell differentiation and growth (12). It has been shown that the cAMP analogue 8-Br-cAMP can stimulate Cdc42 (17), and in this study we tested the hypothesis that $G_{s\alpha}$ may mediate ET-1–induced Cdc42 activation. Treatment of HMCs by cholera toxin, which permanently activates $G_{s\alpha}$ proteins, enhanced ET-1–induced Cdc42 activation, whereas pertussis toxin, which inhibits $G_{i/o}$ proteins, had no effect. This result suggests that $G_{s\alpha}$ mediates the activation of Cdc42 by ET-1. However, we cannot rule out the existence of other mechanisms that may activate adenylate cyclase that do not directly involve the $G_{s\alpha}$ subunit.

We showed that ET-1 stimulates Cdc42 by a PKA-dependent mechanism, as ET-1–induced Cdc42 activation was inhibited in the presence of the selective PKA inhibitor H-89. It has been proposed that PKA can directly modulate activity of some small GTPases. Activation of Rap1 and inhibition of RhoA were ascribed to their phosphorylation by PKA (18, 19). In line with these studies, we show herein that β_1Pix overexpression enhanced ET-1–induced Cdc42 activation, whereas deletion of the DH domain or the mutated SH3 domain strongly inhibited Cdc42 activation by ET-1. Moreover, it has been previously shown that β_1Pix can be phosphorylated by PKA in vitro on Ser516 and Thr526 (16). The overexpression of β_1Pix(S516A, T526A) inhibited Cdc42 activation and β_1Pix translocation to focal adhesion complexes (16).

The discovery of GEFs, GTPase-activating proteins, and GDP dissociation inhibitors has improved our understanding of how small G proteins are regulated. It is possible that subunits of activated heterotrimeric proteins coupled to GPCR can directly bind to GEFs, as recently demonstrated for $G_{\alpha 12}/G_{\alpha 13}$ and PDZ-RhoGEF (20–22). In addition, GPCR can regulate small G proteins through other pathways triggered by heterotrimeric G proteins, including tyrosine kinases, protein kinase C, and cAMP (23, 24).

1. Kaibuch K, Kuroda S, Fukata M, Nakagawa M. Regulation of cadherin-mediated cell-cell adhesion by the Rho family GTPases. Curr Opin Cell Biol 11:591–596, 1999.
2. Aspenström P. The Rho GTPases have multiple effects on the actin cytoskeleton. Exp Cell Res 246:20–25, 1999.

3. Schmidt A, Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 16:1587–1609, 2002.
4. Zheng Y. Dbl family guanine nucleotide exchange factors. Trends Biochem Sci 26:724–732, 2001.
5. Whitehead IP, Campbell S, Rossman KL, Der CJ. Dbl family proteins. Biochim Biophys Acta 1332:F1–F23, 1997.
6. Hoffman GR, Cerione RA. Signaling to the Rho GTPases: networking with the DH domain. FEBS Lett 513:85–91, 2002.
7. Koh CG, Manser E, Zhao ZS, Ng CP, Lim L. β1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. J Cell Sci 114(pt 23):4239–4251, 2001.
8. Manser E, Loo TH, Koh CG, Zhao ZS, Chen XQ, Tan L, Tan I, Leung T, Lim L. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol Cell 1:183–192, 1998.
9. Ku GM, Yablonski D, Manser E, Lim L, Weiss A. A PAK1-PIX-PKL complex is activated by the T-cell receptor independent of Nck, Slp-76 and LAT. EMBO J 20:457–465, 2001.
10. Bagrodia S, Bailey D, Lenard Z, Hart M, Guan JL, Premont RT, Taylor SJ, Cerione RA. A tyrosine-phosphorylated protein that binds to an important regulatory region on the cool family of p21-activated kinase-binding proteins. J Biol Chem 274:22393–22400, 1999.
11. Turner CE, Brown MC, Perrotta JA, Riedy MC, Nikolopoulos SN, McDonald A, Bagrodia S, Thomas S, Leventhal PS. Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. J Cell Biol 145:851–863, 1999.
12. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348: 730–732, 1990.
13. Zegers MM, Forget MA, Chernoff J, Mostov KE, Martin BA, Beest T, Hansen SH. Pak1 and PIX regulate contact inhibition during epithelial wound healing. EMBO J 22:4155–4165, 2003.
14. Chahdi A, Sorokin A, Dunn MJ, Landry Y. The Rac/Cdc42 guanine nucleotide exchange factor β_1Pix enhances mastoparan-activated G_i-dependent pathway in mast cells. Biochem Biophys Res Commun 317: 384–389, 2004.
15. Sraer JD, Delarue F, Hagege J, Feunteun J, Pinet F, Nguyen J, Rondeau E. Stable cell lines of T-SV40 immortalized human glomerular mesangial cells. Kidney Int 49:267–270, 1996.
16. Chahdi A, Miller B, Sorokin A. Endothelin 1 induces β_1Pix translocation and Cdc42 activation via protein kinase A–dependent pathway. J Biol Chem 280:578–584, 2005.
17. Feoktistov I, Goldstein AE, Biaggioni I. Cyclic AMP and protein kinase A stimulate Cdc42: role of A(2) adenosine receptors in human mast cells. Mol Pharmacol 58:903–910, 2000.
18. Lang P, Gesbert F, Delspine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J 15:510–519, 1996.
19. Vossler MR, Yao H, York RD, Pan MG, Rim CS, Storck PJ. cAMP activates MAP kinase and Elk1 through a B-Raf– and Rap 1–dependent pathway. Cell 89:73–82, 1997.
20. Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, Sternweis PC. p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. Science 280:2109–2111, 1998.
21. Fukuhara S, Murga C, Zohar M, Igishi T, Gutkind JS. A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J Biol Chem 274:5868–5879, 1999.
22. Dutt P, Jaffe AB, Merdek KD, Hall A, Toksoz D. Gαz inhibits serum response factor–dependent transcription by inhibiting Rho signaling. Mol Pharmacol 66:1508–1516, 2004.
23. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477, 1998.
24. Kawasaki H, Springett GM, Toki S, Canales JJ, Harlan P, Blumenstiel JP, Chen EJ, Bany IA, Mochizuki N, Ashbacher A, Matsuda M, Housman DE, Graybiel AM. A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia [published correction appears in Proc Natl Acad Sci U S A 96:318, 1999]. Proc Natl Acad Sci U S A 95: 13278–13283, 1998.