βPak-interacting Exchange Factor-mediated Rac1 Activation Requires smgGDS Guanine Nucleotide Exchange Factor in Basic Fibroblast Growth Factor-induced Neurite Outgrowth*

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Neuritogenesis requires active actin cytoskeleton rearrangement in which Rho GTPases play a pivotal role. In a previous study (Shin, E. Y., Woo, K. N., Lee, C. S., Koo, S. H., Kim, Y. G., Kim, W. J., Bae, C. D., Chang, S. I., and Kim, E. G. (2004) J. Biol. Chem. 279, 1994–2004), we demonstrated that βPak-interacting exchange factor (βPIX) guanine nucleotide exchange factor (GEF) mediates basic fibroblast growth factor (bFGF)-stimulated Rac1 activation through phosphorylation of Ser-525 and Thr-526 at the GIT-binding domain (GBD). However, the mechanism by which this phosphorylation event regulates the Rac1-GEF activity remained elusive. We show here that βPIX binds to Rac1 via the GBD and also activates the GTPase via an associated GEF, smgGDS, in a phosphorylation-dependent manner. Notably, the Rac1-GEF activity of βPIX persisted for an extended period of time following bFGF stimulation, unlike other Rho GEFs containing the Db1 homology domain. We demonstrate that C-PIX, containing proline-rich, GBD, and leucine zipper domains can interact with Rac1 via the GBD in vitro and in vivo and also mediated bFGF-stimulated Rac1 activation, as determined by a modified GEF assay and fluorescence resonance energy transfer analysis. However, nonphosphorylatable C-PIX (S525A/T526A) failed to generate Rac1-GTP. Finally, βPIX is shown to form a trimeric complex with smgGDS and Rac1; down-regulation of smgGDS expression by short interfering RNA causing significant inhibition of βPIX-mediated Rac1 activation and neurite outgrowth. These results provide evidence for a new and unexpected mechanism whereby βPIX can regulate Rac1 activity.

Rho GTPases regulate cytoskeletal dynamics and thus play a pivotal role in a wide range of biological responses, including cell polarity, cell adhesion and migration, and neuritogenesis (1–3). Of these GTPases Rac1, Cdc42, and RhoA have been well characterized to induce specific cytoskeletal structures as follows: membrane ruffles and lamellipodia by Rac1; filopodia by Cdc42; stress fibers by RhoA (4). Guanine nucleotide exchange factor (GEF), GTPase-activating protein, and guanine nucleotide dissociation stimulator coordinate the intracellular activities of these Rho GTPases by regulating their interconversion between the inactive GDP-bound and active GTP-bound forms. βPak-interacting exchange factor (βPIX) is a specific GEF for Rac1/Cdc42 (5). As a member of the Db1 family of GEFs, βPIX has a Db1 homology (DH) domain responsible for GDP-GTP exchange on Rho GTPases. Additionally, it has a number of other structural motifs as follows: SH3 domain for interaction with p21-activated kinase (Pak) (5); PH domain for protein/lipid interaction; proline-rich domain as yet uncharacterized; GB domain (GBD) for GIT binding (6); and leucine zipper (LZ) domain for homo- and heterodimerization of PIX (7, 8).

DH domain-mediated GEF activity is transient in principle, because it has a lower affinity toward the active GTP-bound form of Rho GTPases than inactive GDP-bound or nucleotide-depleted ones (9–11). Once Rho GTPase is activated by a DH domain, their interaction becomes weak and is followed by dissociation. Several laboratories have reported results consistent with the presence of a Rac/Cdc42-directed DH domain of βPIX (12–14). Platelet-derived growth factor-induced membrane ruffle was βPIX-dependent as determined 15 min after stimulation (12). βPIX is also involved in epidermal growth factor-induced reactive oxygen species generation as an intermediary.

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4 The abbreviations used are: GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; bFGF, basic fibroblast growth factor; GBD, GIT-binding domain; DH, Db1 homology; LZ, leucine zipper; GST, glutathione S-transferase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; siRNA, short interfering RNA; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; RSD, Rac1-binding domain; PBD, p21-binding domain; TRITC, tetramethylrhodamine isothiocyanate; βPIX, βPak-interacting exchange factor; GTPyS, guanosine 5'-3-O-(thio)triphosphate; ERK, extracellular signal-regulated kinase; SH3, Src homology 3; PH, pleckstrin homology; CRD, cysteine-rich domain; FL-PIX, full-length βPIX; C-PIX, C-terminal half of βPIX; N-PIX, N-terminal half of βPIX; Pak, p21-activated kinase.
step in sequential activation of phosphatidylinositol 3-kinase, PIX, Rac1, and Nox1 (13). Rac1 activation occurred in a transient manner, reaching a peak 15 min after epidermal growth factor stimulation. In mesangial cells through the protein kinase A-dependent pathway, endothelin-1 stimulates Cdc42 activation, which peaks at 2 min, decreases at 10 min, and returns to a basal level at 30 min after stimulation (14). Taken together, DH-mediated activation of Rac1/Cdc42 appears to return to a basal level at 30 min after stimulation (14). Taken together, DH-mediated activation of Rac1/Cdc42 appears to reach a peak and proceed to completion within 30 min after agonist stimulation. By contrast, we observed that βPIX stably interacts with Rac1, and more surprisingly, it exhibits prolonged activity toward Rac1 for up to 4 h following growth factor stimulation (15). This kinetic profile does not match the profile reported for other Rho GEFs (16–19).

Recent evidence indicates that a non-DH domain can participate in Rho GTPase binding and regulation of its activity as well. The Vav family of GEFs has a cysteine-rich domain (CRD), which is located C-terminal to the DH-PH bidomain and allows binding to GTPases (20). Isolated CRD associates with Rac1 and RhoA, but Vav-1 appears to be Rac1-specific. This interaction between Rac1/Rho (involving Ser-83 and Lys-116 of Rac1) may facilitate conformational changes and enhancement of Vav DH-mediated GEF activity toward Rac1. Interestingly, Vav-3 has a zinc finger domain, which can interact with RhoA in vitro and stimulate this GTPase (21). αPIX also has an extra Rac1-binding domain (RSID), which makes its dimeric form function as a unique Rac-specific GEF (22). We therefore hypothesized that βPIX might interact and activate Rac1 via a non-DH domain interaction and consequently promote Rac1 activation in a novel DH-independent mechanism. In this study, we identified GBD as a new stable Rac1-binding site. More importantly, this domain mediates Rac1 activation in collaboration with an associated GEF, smgGDS, in a phosphorylation (Ser-525 and Thr-526)-dependent manner.

**EXPERIMENTAL PROCEDURES**

*Materials*—Human recombinant bFGF, Lipofectamine 2000, G418, and hygromycin B were purchased from Invitrogen. Anti-Rac1 and anti-GFP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-smgGDS antibody and smgGDS cDNA (FLJ30470) were purchased from BD Biosciences and the National Institute of Technology and Evaluation (Chiba, Japan), respectively. pEGFP-C2 and pCMV-myc were purchased from Clontech. Nonspecific siRNAs and specific siRNAs for smgGDS were purchased from Invitrogen. Raichu-Rac1 probe for fluorescence resonance energy transfer (FRET) analysis was kindly provided by Dr. Matsuda Michiyuki (Osaka University, Osaka, Japan).

*Cell Culture and Differentiation*—PC12 cells overexpressing FGF receptor-1 in a tetracycline-dependent manner were cultured as described previously (23). Briefly, cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% Tet system approved fetal bovine serum (FBS), 2 mM glutamine, 1 × antibiotics (Invitrogen), 50 µg/ml hygromycin B, and 100 µg/ml G418 at 37 °C in 10% CO₂. Prior to differentiation, PC12 cells were induced to overexpress FGF receptor-1 in serum-free Dulbecco’s modified Eagle’s medium containing 1.5 µg/ml doxycycline for 24 h and replaced with Dulbecco’s modified Eagle’s medium supplemented with 20 ng/ml bFGF, 2% FBS and 1.5 µg/ml doxycycline for 24–48 h.

*Transient Transfection and siRNA Treatment*—PC12 cells were seeded on 60-mm culture dishes or 20 µg/ml poly-L-lysine-coated coverslips. A mixture of 5 µg of DNA and 5 µl of Lipofectamine 2000 was added to culture dishes according to the manufacturer’s instructions. For siRNA treatment, nonspecific siRNA and specific siRNA for smgGDS (5′-GAAGATGAATCCATGCAGAAATT-3′) at the indicated concentrations were transfected into cells with Lipofectamine 2000. After 3 days, expression of smgGDS was assessed by immunoblotting with anti-smgGDS antibody. The band intensity after exposure and development was digitized and analyzed by Quantity One software version 4.2.1 (Bio-Rad).

*Immunoprecipitation and Immunoblotting*—Cells were washed twice with PBS and lysed in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 10% glycerol, 1% Triton X-100, 200 µM orthovanadate, 1 µM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin) for 1 h at 4 °C. Proteins were immunoprecipitated with an appropriate antibody for 3 h at 4 °C. Immunoprecipitates were collected by adding protein G-Sepharose and washed five times with lysis buffer and twice with PBS. Samples were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane in a Tris-glycine/methanol buffer (25 mM Tris base, 200 mM glycine, 20% methanol). Membranes were blocked with 3% skimmed milk in phosphate-buffered saline (PBS) for 1 h, incubated with primary antibodies for 1 h at room temperature, and washed three times (10 min each) with PBS containing 0.1% Tween 20. Membranes were blotted with secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature. After five washes with PBS and 0.1% Tween 20, signals were detected using enhanced chemiluminescence reagent (Amersham Biosciences). In some cases, membranes were stripped and reprobed with different antibodies.

*In Vitro Binding Assay*—GST, GST-Rac1, and GST-smgGDS proteins were expressed in Escherichia coli (DH5α) and purified by glutathione-Sepharose affinity chromatography. C-PIX-His, GBD-His, and p21-binding domain (PBD)-His proteins were expressed in M15 and purified by Ni²⁺ affinity chromatography. Equal amounts of GST (GST-Rac1) glutathione-Sepharose beads were incubated with His-tagged proteins or lysates from cells expressing various domains of GFP-βPIX. Beads were washed five times with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 10% glycerol, 1% Triton X-100, 5 mM MgCl₂, 1 mM dithiothreitol, 200 µM orthovanadate, 1 µM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin), resolved on 9% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were immunoblotted with anti-His, GFP, or GST antibody.

*Modified GST-PBD Binding Assay*—GEF activities of βPIX/ truncated βPIX were measured as described previously (15). Briefly, GST-PBD was expressed in E. coli (DH5α) and purified with glutathione-Sepharose affinity chromatography. Cells were stimulated with or without 30 ng/ml bFGF for 1 h, lysed, and immunoprecipitated with anti-βPIX or anti-GFP antibody. Immunoprecipitates were further incubated with purified soluble GST-PBD (1 µg) at 4 °C for 2 h in binding.
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buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 30 mM MgCl₂, 40 mM NaCl, 0.5% Nonidet P-40) and washed five times with lysis buffer. Beads were boiled for 5 min, resolved by 12% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Membranes were immunoblotted with anti-GST antibody and then reprobed with anti-GFP, βPIX, Pak2, or Rac1 as described under “Transient Transfection and siRNA Treatment.”

**FRET Analysis**—PC12 cells were plated on 40-mm dishes containing poly-L-lysine-coated 18-mm glass coverslips. One day after plating, Myc-tagged PIX constructs were co-transfected with Raichu-Rac1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Thirty six hours after transfection, cells were starved for 16 h and then treated with 30 ng/ml bFGF for 1 h. For Myc-tagged PIX staining, cells were fixed in 3.7% paraformaldehyde/PBS for 10 min at room temperature, permeabilized with ice-cold methanol for 15 min, and then blocked with 10% FBS for 1 h. Cells were incubated with anti-Myc antibody in 2% FBS/PBS for 1 h at 37 °C, followed by incubation with Alexa Fluor 633-conjugated goat anti-mouse IgG antibody (Molecular Probes, OR) for 1 h at 37 °C. After staining, coverslips were mounted onto a glass slide with mounting medium (DakoCytomation). FRET was analyzed using a Leica TCS SP2 confocal microscope system with HCX PL APO ×63 objective (Leica, Wetzlar, Germany). Excitation was provided by 20-milliwatt multimode argon ion laser lines. Donor (CFP) was excited at 458 nm, and fluorescence was detected in a bandwidth of 470–500 nm (CFP channel), whereas for acceptor (YFP), excitation was at 514 nm and emission at a bandwidth of 535–565 nm (YFP channel). For FRET, the excitation was at 458 nm and emission at a bandwidth 535–565 nm (FRET channel). Donor and FRET images were acquired from the respective CFP and FRET channels under the similar conditions. After background subtraction, FRET: CFP ratio images were generated by dividing the FRET image by the CFP image using MetaMorph software version 6.01 (Universal Imaging), and these ratios were used to represent FRET efficiency.

**Statistical Analysis**—Paired t test was applied for statistical analysis of neurite outgrowth assay and FRET using SPSS version 10.0 for windows and the statistical significance was set at \( p < 0.05 \).

**RESULTS**

**PIX Binds to Rac1 via GIT-binding Domain**—To assess which domain of βPIX might be involved in a non-DH domain interaction, we generated two truncated βPIX constructs as follows: the first contains the N-terminal half of βPIX (N-PIX), including the SH3-DH-PH domains in sequence, and the second contains the C-terminal half of βPIX (C-PIX) (as illustrated in Fig. 1A). These constructs were introduced into PC12 cells and assessed for GST–Rac1 binding. As expected, both DH-containing full-length βPIX (FL-PIX) and N-PIX interacted with GST–Rac1 but not with GST or SH3 domain of βPIX (Fig. 1B, left). Interestingly, GST–Rac1 also bound C-PIX to the same extent as the N-terminal domain, although there did not appear to be any cooperativity of binding in the case FL-PIX. These results, however, did not exclude the possibility that the C-PIX:Rac1 association occurs indirectly through heterodimerization of C-PIX with endogenous FL-PIX, via the LZ dimerization (7, 8).

We therefore tested the ability of bacterially expressed His-tagged βPIX to bind GST–Rac1 in vitro. βPIX interacted specifically with GST–Rac1 (Fig. 1B, right, lane 2), but not with GST alone (lane 1). These results indicate that βPIX binds Rac1 through a region independent of the DH domain. To further determine which part of C-PIX is involved in this binding, C-PIX was divided into three parts, namely a proline-rich region (PXXP), the GBD, and the LZ domains (Fig. 1A). Each domain was expressed in PC12 cells as GFP fusion proteins, and lysates were incubated with GST (control) or GST–Rac1 immobilized on glutathione-Sepharose beads (Fig. 1C, left). C-PIX consistently showed a specific interaction with GST–Rac1, and thus was employed as a positive control (Fig. 1C, left, lanes 1 and 5). Only GBD exhibited a strong interaction comparable with that of the parental C-PIX (Fig. 1C, left, lanes 3 and 7). Further binding study with bacterially expressed His-tagged GBD revealed that direct interaction occurs between Rac1 and GBD (Fig. 1C, right). These results indicate that GBD plays a role in binding Rac1, independent of classical DH interactions. The related region (amino acid 546–779) derived from αPIX, which has 56% homology to βC-PIX (amino acids 401–646), also bound to GST–Rac1 (but not GST) (Fig. 1D, lanes 2 and 5). We thus determined whether the GBD of αPIX plays a similar role in binding Rac1. It also interacted with wild-type Rac1 and Cdc42 (Fig. 1E, lanes 1–6). Finally, we tested whether interaction between the C-PIX (GBD) and Rac1 is altered depending on the activation status of Rac1. It is well known that the p21-binding domain (PBD) of p21-activated kinase (Pak) specifically binds to Rac1-GTP but not Rac1-GDP. This interaction was therefore presented as a positive control (Fig. 1F, lanes 1 and 2). Unlike PBD–Rac1 interaction, GDP or GTP loading did not affect interaction of both C-PIX-Rac1 and GBD–Rac1 (Fig. 1F, lanes 3–6). This unexpected interaction between C-PIX (GBD) and Rac1 would make it clear how the modified GST-PBD assay as described under “Experimental Procedures” works (15). More importantly, these results suggest that active Rac1-GTP can remain stably bound to βPIX via the GBD, providing a rationale to persistent βPIX-mediated Rac1 activity.

**C-PIX Mediates Activation of Rac1 but Not Cdc42 in Response to bFGF**—Given that C-PIX stably interacts with Rac1, we wished to test whether this binding influences the regulation of Rac1. We conducted two different assays to measure Rac1 activation. One uses the ability of active GTP-bound, but not inactive GDP-bound Rac1/Cdc42, to bind GST-PBD (the modified GST-PBD binding assay) (15). The conventional GST-PBD binding assay has been used to detect active forms of Rac1/Cdc42 bound, but this method can not identify the GEF responsible for their activation. To overcome this handicap, the modified protocol we have been using involves immunoprecipitation of PIX constructs followed by the addition of soluble GST-PBD to bind βPIX-associated GTP-Rac1 or Cdc42. Active Rac1/Cdc42 in the immunoprecipitates of βPIX is able to bind added GST-PBD as detected by anti-GST. An underlying principle of this assay is that βPIX can stably hold the active GTP-bound form of Rac1/Cdc42 via a non-DH domain, GBD interaction (Fig. 1F). The other assay analyzes...
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**FIGURE 1. PIX binds to Rac1 via the GBD.** A, schematic diagram of the domain structures of βPIX and various βPIX constructs. B, left panel, the βPIX constructs were expressed as GFP-tagged proteins, and 24 h later, cells were lysed and equal amounts of proteins were incubated with immobilized GST (lanes 1–4) or GST-Rac1 (lanes 5–8) at 20 °C for 2 h. Beads were washed five times with binding buffer and subjected to Western blot analysis with anti-GFP (top) or anti-GST (bottom panel) antibody. Expression levels of each construct were evaluated with anti-GFP using lysates from the transfected cells (middle panel). B, right panel, His-tagged C-PIX was expressed in E. coli and was incubated with immobilized GST (lane 1) or GST-Rac1 (lane 2). Bound and applied C-PIX was visualized with anti-His (top and middle panels), whereas GST and GST-Rac1 were probed with anti-GST antibody (bottom panel). C, left panel, C-PIX and its fragments, including PX, GB, and LZ domains, were expressed as GFP fusion proteins and were incubated with immobilized GST (lanes 1–4) or GST-Rac1 (lanes 5–8) followed by the same procedure as described above. Proteins in each column were visualized with the following antibodies: anti-GFP (top and middle panels) and anti-GST (bottom panel). C, right panel, His-tagged GBDS was expressed in E. coli and was incubated with immobilized GST (lane 1) or GST-Rac1 (lane 2). Bound and applied C-PIX was visualized with anti-His (top and middle panels). GST and GST-Rac1 were probed with anti-GST antibody (bottom panel). E, GBDs derived from βPIX (lanes 1–3 and 7) and βPIX (lanes 4–6 and 8) were expressed as GFP fusion proteins. They were incubated with immobilized GST (lanes 1 and 4), GST-Rac1 (lanes 2 and 5), or GST-Cdc42 (lanes 3 and 6). GST pulldown was performed as described above, and proteins were visualized with anti-GFP (top panel) and anti-GST (bottom panel). Expression levels of GBDs in lysates were probed with anti-GFP antibody (lane 7 and 8). F, GST pull-down of GBDs (lanes 1, 3, and 5) or GST-βPIX (lanes 2 and 4) and GST-βPIX (lanes 5 and 6) were expressed as His-tagged proteins in E. coli and were incubated with immobilized GST-Rac1 loaded with GDP-βS (lanes 1, 2, and 5) or GTP-βS (lanes 3, 4, and 6). Bound and applied proteins were detected by Western blotting with anti-His (top and middle panels) and anti-GST antibody (bottom panel). For detection of bound GBD (lanes 5 and 6) only one-third of the reaction beads were used.
fluorescence resonance energy transfer (FRET) images using a Raichu-probe for Rac1 in PC12 cells (24, 25).

As shown in Fig. 2A, left, in both FL-PIX- and C-PIX-expressing cells, bFGF increased levels of associated active Rac1/Cdc42, as determined by the appearance of a GST-PBD band (lanes 4 and 8). Only Rac1 (not Cdc42) was detectable in the immunoprecipitates retrieved by anti-GFP. Note C-PIX but not N-PIX had a stimulatory effect on associated Rac1-GTP levels that is comparable with that of FL-PIX (Fig. 2A, left, lanes 7 and 8). Surprisingly, neither GFP nor N-PIX activated Rac1 by these criteria, indicating that the DH domain in N-PIX by itself is not sufficient to mediate GEF activity (Fig. 2A, left, lanes 1, 2, 5, and 6); the N-PIX is likely compromised by a failure to undergo dimerization. Shin et al. (23) previously showed that bFGF treatment resulted in phosphorylation of Ser-525 and Thr-526 within the GBD of βPIX, and this phosphorylation is critical for βPIX-mediated Rac1 activation (15). Interestingly, the new Rac1-binding domain falls within the same GBD region; such phosphorylation may therefore affect Rac1 binding/activation. To test this, cells were transfected with plasmids encoding C-PIX or mutant C-PIX (SS25A/T526A), and their activity was measured by the modified GST-PBD binding assay (Fig. 2A, right). Wild-type C-PIX mediated the expected bFGF-stimulated Rac1 activation (Fig. 2A, right, lanes 1 and 2). However, cells expressing the nonphosphorylatable C-PIX did not generate Rac1-GTP (Fig. 2A, right lanes 3 and 4). Inability of mutant C-PIX to activate Rac1 was not because of disruption of its interaction with Rac1. Similarly, FL-PIX- and C-PIX-mediated Rac1 activation were dependent on phosphorylation of Ser-525/Thr-526 (15). The C-PIX GBD-mediated Rac1 activation in response to bFGF suggested that this domain might associate with another GEF.

To assess the effect of C-PIX on Rac1 in vivo, we used an established FRET probe designated Raichu-Rac1 (24). PC12 cells were co-transfected with Raichu-Rac1 plasmid and various βPIX constructs, starved for 24 h, and then stimulated with bFGF for 1 h prior to fixation. Representative YFP:CFP ratio images and their emission ratio for each construct were shown (Fig. 2B, upper panel). The FRET images for cells expressing FL-PIX or C-PIX generated positive signals at the periphery where they stimulated activation of Rac1 (Fig. 2B, upper panel, represented by red color in the intensity bar). Emission ratios for FL-PIX and C-PIX reached maxima of 1.96 and 2.03, respectively. FL-PIX exhibited relatively higher levels of background activity, although they caused activation of Rac1 in a bFGF-dependent manner. Control Myc-transfected cells also showed a limited amount of activation at the cell periphery, as indicated by an increase in the emission ratio from 1.13 to 1.47. However, in cells expressing N-PIX, no significant activation was observed (Fig. 2B, upper panel, blue color in the whole area of cell body with the average ratio of 0.95). Collectively, these results indicated that C-PIX alone like FL-PIX is sufficient to mediate responsiveness to bFGF-stimulated Rac1 activation.

C-PIX Induces Actin Cytoskeletal Changes During Neurite Outgrowth in PC12 Cells—Rat PC12 cells can be induced to differentiate to neuron-like cells that harbor extended neurites by both bFGF and nerve growth factor. Activation of Rho GTPases, such as Rac1, is crucial in all neuronal systems (2, 3). To further assess the role of βPIX in this process, we tested whether C-PIX is biologically active with respect to its effects on neurite outgrowth (versus FL-PIX). As depicted in Fig. 3, FL-PIX-expressing cells responded normally to bFGF consistent with previous observations (23). In cells expressing N-PIX, neurite-like structures were rarely observed in less than 20% of the population. Diffuse actin staining in the cytoplasm overlapped with N-PIX. Similarly, most of GFP-expressing cells exhibited an undifferentiated round shape. By contrast, cells expressing C-PIX showed strong actin staining and prominent co-localization of these proteins in the tips of extending neurites, a pattern also seen with FL-PIX. The accumulation of C-PIX in large lamellipodial structures at the cell periphery suggested that C-PIX may induce Rac1-driven cytoskeletal changes required for neurite outgrowth.

**βPIX-mediated Rac1 Activation Is smgGDS-dependent**—Recently, Lanning et al. (26) reported that the polybasic region (KKRRKK) at the C terminus of Rac1 functions as a nuclear localization signal. They also suggested Rac1 requires small GTP-binding protein GDP dissociation stimulator (smgGDS) for this function; smgGDS is an unusual GEF for Ras and Rho family GTPases (26). Its sequence indicates a member of the Armadillo (ARM) family of proteins, containing 11 ARM domains, which may interact with the polybasic domain in Rac1 (27, 28). The βPIX-Rac1 complex could bind smgGDS, and this, in turn, raised the possibility that βPIX could regulate the GEF activity of smgGDS. To test this idea, we conducted co-immunoprecipitation and modified GEF assays. Myc-tagged smgGDS was overexpressed and tested for βPIX binding. The Myc-tagged smgGDS indeed contained βPIX and Rac1 (Fig. 4A, left). Conversely, endogenous βPIX co-precipitated Rac1 and Myc-smgGDS (Fig. 4A, right) but not controls (lanes 1 and 3). These results indicated that these proteins form a tricomplex. To determine whether smgGDS interacts directly with C-PIX or indirectly through Rac1, we conducted an in vitro binding assay (Fig. 4B). Consistently, Rac1 showed a strong interaction with bacterially expressed C-PIX (Fig. 4B, lane 2). SmgGDS also displayed a positive binding to C-PIX but a relatively weak binding compared with Rac1 (Fig. 4B, lane 3). Furthermore, GST pulldown assay revealed that GBD is an interaction site of smgGDS (lanes 4 and 5). These results suggest that the GBD of βPIX anchors both Rac1 and smgGDS, providing a clue that two GEFs, βPIX and smgGDS, might regulate a G protein, Rac1, in a cooperative manner (Fig. 7).

Accordingly, the next question we addressed was whether association of βPIX and smgGDS cooperates in Rac1 activation. For this purpose, we checked whether βPIX-associated Rac1-GEF activity is affected by treatment with smgGDS-specific siRNA. The modified GST-PBD binding assay was then performed as described above (Fig. 2A), where βPIX was immunoprecipitated (Fig. 5A), and monitored for the ability to bind GST-PBD. In control cells (Fig. 5A, lanes 1 and 2) or nonspecific siRNA-treated cells (Fig. 5A, lanes 3 and 4), βPIX responded to the bFGF stimulus, resulting in generation of Rac1-GTP. When smgGDS was reduced to 20% of its endogenous level, βPIX activity was barely detectable, with little PBD recovered (Fig. 5A, lanes 5 and 6). These surprising results clearly indicated that smgGDS participates in βPIX-mediated Rac1 activation;
FIGURE 2. C-PIX activates Rac1 in response to bFGF stimulation. **A**, modified GST-PBD binding assay for Rac1/Cdc42 activation. *Left panel*, PC12 cells were transfected with plasmids encoding GFP-tagged FL-PIX, N-PIX, or C-PIX and maintained for 24 h. They were then stimulated with 30 ng/ml bFGF for 1 h. Equal amounts of protein from each lysate were immunoprecipitated (IP) with anti-GFP and incubated with soluble GST-PBD to trace active GTP-bound Rac1/Cdc42 in the immunoprecipitate. Bound GST-PBD was probed with anti-GST antibody (top panel). Expression of each construct as GFP fusion proteins was evaluated by probing with anti-GFP (middle panel) and anti-Cdc42 and Rac1 (bottom panel), respectively. In the right panels, cells were transfected with pEGFP plasmids encoding wild-type C-PIX (*lanes 1 and 2* ) or mutant C-PIX (S525A/T526A) (*lanes 3 and 4* ), and then the above procedure was applied.

**B**, FRET analysis was performed to investigate Rac1 activation using a Raichu-Rac1 probe. Various Myc-tagged/His252 PIX constructs were co-transfected with a Raichu-Rac1 probe into PC12 cells. Cells were starved for 16 h and then treated with bFGF or left untreated. Confocal images were obtained 1 h following bFGF stimulation as described under “Experimental Procedures.” Representative ratio images of FRET:CFP after bFGF stimulation are shown in the intensity-modulated display mode. In the intensity-modulated display mode, eight colors from red to blue were used to represent the FRET:CFP ratio, with the intensity of each color indicating the mean intensity of FRET and CFP image. The upper and lower limits of the ratio image are shown on the right. Bar graphs represent the relative emission ratio (FRET:CFP) of the whole cell area. The number of cells examined for each sample was as follows: Myc (*n = 10* ), FL-PIX (*n = 20* ), N-PIX (*n = 15* ), C-PIX (*n = 20* ). *, p < 0.05.
Involvement of smgGDS in βPIX-mediated Rac1 Activation

![Image](image1.png)

**FIGURE 3. C-PIX promotes neurite outgrowth in PC12 cells.** PC12 cells were transfected with pEGFP plasmids encoding GFP (control), FL-PIX, N-PIX, and C-PIX. Twenty-four hours after transfection, cells were incubated in complete medium containing bFGF (20 ng/ml) for 48 h. Representative confocal images are shown here (top panel). To visualize actin, cells were stained with TRITC-phalloidin (middle panel). Merged pictures are shown at the bottom. Neurite outgrowth was determined in cells expressing the above constructs. Green cells bearing neurites greater than 15 μm were counted positive using the MetaMorph software. They were counted under at least 10 different microscopic fields. Values represent mean ± S.D. from three independent experiments. *, p < 0.05; n = 100.

![Image](image2.png)

**FIGURE 4. smgGDS is associated with βPIX.** A. PC12 cells were transfected with pMyc-smgGDS and lysed, and equal amounts of proteins were incubated without any antibody (lanes 1 and 3) or with anti-Myc (left, lane 2) or anti-βPIX (right, lane 4) at 20 °C for 2 h. Beads were washed five times with binding buffer and subjected to Western blot analysis with anti-βPIX (top panel), anti-Myc (middle panel), or anti-Rac1 (bottom panel), B, as described above, GST pulldown assay was performed to detect interaction between smgGDS and C-PIX or GBD. Proteins were expressed as His-tagged (C-PIX and GBD) or as GST-fused (Rac1 and smgGDS) in E. coli. C-PIX was incubated with GST (lane 1), GST-Rac1 (lane 2), or smgGDS (lane 3), whereas GBD was incubated with GST (lane 4) or GST-smgGDS (lane 5). Bound and applied proteins were Western-blotted with anti-His (top and middle panels) and anti-GST antibody (bottom panel). IP, immunoprecipitation.

this GEF activity of C-PIX was actually comparable with that seen with FL-PIX (Fig. 2). These results also suggest that DH-mediated GEF activity is not important downstream of bFGF. Indeed when the modified GEF assay was performed with lysates of cells expressing wild-type or catalytically inactive βPIX (L238R/L239S) (5), both βPIX constructs generated similar GEF activity toward Rac1 (Fig. 5A). Taken together, these results highlight the DH domain-independent, smgGDS-coupled Rac1-GTP generation in bFGF-stimulated cells. Previously, we showed that phosphorylation-defective full-length βPIX (S525A/T526A) is not capable of activating Rac1 (15). As shown in Fig. 2A, phosphorylation-defective C-PIX can not mediate Rac1 activation either. Then how does phosphorylation of Ser-525/Thr-526 at the GBD regulate the GEF activity of smgGDS? C-PIX (GBD) makes a weak but direct contact with smgGDS (Fig. 4B). Thus phosphorylation of βPIX may play a role in enhancing recruitment of smgGDS. To test this idea, we specifically interacted with smgGDS (Fig. 5D, lanes 1 and 3). However, phospho-mimetic E mutants showed a weak signal for interaction with smgGDS (Fig. 5D, lane 4). Taken together, these results are consistent with the idea that phosphorylation of βPIX at Ser-525/Thr-526 destabilizes association between βPIX and smgGDS.

smgGDS Is Indispensable for βPIX-mediated Neurite Outgrowth—Because smgGDS appears to activate Rac1 coupled with βPIX, we tested whether this biochemical activity of smgGDS correlates with βPIX-mediated neurite outgrowth in PC12 cells. To this end, cells were treated with nonspecific or smgGDS-specific siRNA, followed by Western blot analysis and immunocytochemistry to confirm that endogenous smgGDS was down-regulated (Fig. 6, A and B). Neurite outgrowth was then measured in these siRNA-treated cells (Fig. 6C). Nonspecific siRNA-treated cells showed staining of endogenous smgGDS (Fig. 6B, upper two rows) and responded to exogenous βPIX in a GFP fusion form, resulting in a 3-fold increase in determined which form of endogenous βPIX, dephospho- or phospho-βPIX, has a binding preference toward smgGDS. To obtain maximum amounts of dephospho- or phospho-βPIX, cells were starved or stimulated with bFGF (30 ng/ml), and these lysates were incubated with GST-smgGDS immobilized on glutathione-Sepharose beads. Identity of smgGDS-bound βPIX, whether it is a dephospho- or phospho-form, can be easily recognized by using mobility shift on electrophoresis (23). Only dephospho-βPIX showed a significant binding to smgGDS (Fig. 5C, lanes 3 and 4). In particular, on lane 4 of Fig. 5C, where most of βPIX exists as phosphorylated, if phospho-βPIX had a higher affinity to smgGDS than dephospho-βPIX, one can expect that smgGDS-bound βPIX would be the phosphorylated ones. However, a negligible amount of phospho-βPIX was detected. Contrary to our initial hypothesis, these results suggest that phosphorylation of βPIX dissociates smgGDS from βPIX rather than recruiting smgGDS. To confirm that, GST pulldown assay was performed using S525A/T526A (A mutant) and S525E/T526E (E mutant) mutants of C-PIX. To minimize the effect of dimerization between these mutants and endogenous βPIX, A mutant-transfected cells were starved, whereas E mutant-transfected cells were stimulated with bFGF for 1 h prior to lysis. Both A and E mutants did not bind GST (Fig. 5D, lanes 1 and 2). Dephospho-mimetic A mutants have a binding preference toward smgGDS, whereas E mutant-transfected cells showed staining of endogenous βPIX (Fig. 6B, upper two rows) and responded to exogenous βPIX in a GFP fusion form, resulting in a 3-fold increase in
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A striking feature of smgGDS−/− mice is that their neonatal cardiomyocytes, thymocytes, and neuronal cells underwent apoptotic death (29). The major physiological function of smgGDS is thus considered to protect cells from apoptosis. However, no apparent apoptosis was observed in smgGDS-deficient PC12 cells under these culture conditions. Depletion of smgGDS significantly inhibited βPIX-mediated neurite outgrowth (Fig. 6C). These results strongly suggest that βPIX coupling to smgGDS correlates with its biological function.

DISCUSSION

The conserved DH domains found in Dbl-like GEFs are responsible for GTase binding and GDP-GTP exchange activity on Rho proteins (11, 30, 31). In a previous study, we provided data that the DH domain of βPIX appears to exhibit the same properties. However, further investigation uncovered a previously unrecognized Rac1-binding site in βPIX, which is within the known GBD. The GBD ofPIX is 80% identical and exhibits specific interaction with Rac1. The specificity of this interaction may be strengthened by cooperation of smgGDS with Rac1 but not Cdc42 (Fig. 2A), resulting in formation of a ternary complex of βPIX-Rac1-smgGDS. Thus PIX proteins may achieve its Rac1 activation through both DH and this new domain. Recent evidence provides several examples of a GTase cascade (activated GTase/GEF/downstream GTase) in which a downstream GTase is regulated in a positive or negative feedback (33–35). In the activated Ras/Sos GEF/Ras cascade, activated Ras binds to a distinct noncatalytic site on the SOS molecule (33). However, in the signaling cascade in which PIX functions as an intermediate GEF, the activated forms of Rac1/Cdc42 are suggested to bind to the DH domain of PIX but slightly distinct from the site where the GDP-bound or nucleotide-depleted forms of Rac1/Cdc42 interact (34). Feng et al. (22) demonstrated that dimerization of αPIX is essential for regulation of the downstream Rac1, because only in a dimeric state can an empty DH domain in trans be available for its binding. To the complexity of βPIX-Rac1 interaction, recently ten Klooster et al. (36) reported that βPIX binds to a proline stretch in the C terminus of Rac1 via its SH3 domain. This interaction is regulated by Pak1; knock-out of Pak1 induced the stronger interaction, resulting in a higher Rac1 activity with more efficient spreading of Pak1−/− cells compared with Pak1+/+ cells. These findings suggest that Pak1 plays a negative regulatory role in formation of focal adhesion, which is consistent with the previous result (37). Our observation suggests another possibility that the GBD might be the one to hold Rac1. Particularly in βPIX that has no RSID seen in αPIX, the GBD may take the place of RSID. Furthermore, because GBD in a monomeric state can interact with Rac1, it may not necessitate PIX dimerization.

The GBD appears to couple βPIX to smgGDS (containing a different GEF domain), which is known to act on both Ras and Rho family GTases. Given that this new domain stably interacts with Rac1, three possible mechanisms can be considered for C-PIX-mediated Rac1 activation. First, C-PIX might have intrinsic GEF activity; however, this domain is small and is not

neurite outgrowth compared with GFP-transfected cells (Fig. 6C), which is consistent with the previous result (23). Localization of βPIX is also seen at the growth cone-like structure (Fig. 6B, arrowhead). Administration of smgGDS-specific siRNA caused a marked decrease in the expression levels of smgGDS (Fig. 6A, lanes 3 and 4), which can be proved at the cellular level by immunocytochemical staining (Fig. 6B, lower two rows).
found elsewhere. Second, because βPIX forms a dimer through its LZ domain (7, 8), C-PIX might recruit endogenous βPIX. In this scenario, we could envisage that a heterodimer of C-PIX: endogenous βPIX might be functionally active in stimulating GDP-GTP exchange. This dimeric state may provide an interactive environment by pulling C-PIX and the DH domain of endogenous βPIX together in close proximity. Third, based on our unexpected observation that C-PIX possesses GEF activity (Fig. 2), association of C-PIX with another GEF must be considered as a potential mechanism for Rac1 activation. The recent report (26, 38) that Rac1 can be transported to the nucleus through interaction with smgGDS suggested that the PIX-Rac1-smgGDS complex might exist. Indeed, co-immunoprecipitation studies revealed the existence of this trimeric complex in cells (Fig. 4A). Subsequent investigation with specific siRNA directed against smgGDS clearly showed that smgGDS, but not βPIX, is predominantly responsible for Rac1-GEF activity (Fig. 5A). Furthermore, when we tested the DH-inactive βPIX (L238R/L239S), we could still detect Rac1 activation (Fig. 5B). The molecular mechanism of smgGDS activation has been poorly understood to date. In this study, we showed for the first time how this GEF is regulated by extracellular signals such as bFGF. The role for smgGDS in βPIX-mediated Rac1 activation is analogous to that for the DOCK180-Elmo complex, a functional unit of unconventional GEF, in ARNO-mediated activation of Rac1 (39). ARNO is a GEF for ARF6 GTPase; however, its expression in migrating Madin-Darby canine kidney cells induced the formation of large lamellipodia at the leading edge, suggesting the robust activation of Rac1. Santy et al. (39) resolved this puzzle by demonstrating that the DOCK180-Elmo complex functionally links ARNO-mediated ARF6 activation and the downstream activation of Rac1. These two cascades illustrate the following interesting feature: two distinct GEFs, PIX/smgGDS and ARNO/DOCK180, collaborate to regulate the downstream Rac1.

Based on our observations and those from other laboratories, we propose a model in which βPIX mediates Rac1 activation in both a transient and persistent manner (Fig. 7). In this model transiently activated Rac1 via the A pathway may play a vital role in dynamic responses such as membrane ruffling (12) and reactive oxygen species production (13). On the other hand, the B pathway in which βPIX cooperates with smgGDS, a GEF of the Armadillo protein family, can mediate persistent activation of Rac1 for long term responses such as neurite outgrowth.
βPIX-mediated Rac1 activation in PC12 cells shows unusual kinetic properties compared with other Dbl family GEFs; the activity is detectable even at 4 h following bFGF stimulation (15). In contrast, Rac1 activation mediated by other Dbl family members is not sustained beyond 30 min (16–19). Persistent activation of the ERK cascade is critical for various aspects of PC12 differentiation, including neurite outgrowth (40, 41).

In bFGF signaling (23), Phosphatidylinositol 3-kinase (PI3K) can regulate the GEF activity of the DH domain in reactive oxygen species (ROS) production via this signaling pathway (13). In contrast, in the B pathway Rac1 is persistently activated, where it functions downstream of an ERK mitogen-activated protein kinase cascade. GBD and Rac1 interact stably (filled arrow) but similarly to an interaction between DH domain and Rac1; smgGDS is considered to dissociate from Rac1 following activation of Rac1.

Finally, this model can explain the relatively weak inhibitory effect on biological responses when catalytically inactive DH mutants (L238R/L239S) were introduced into cells. Obermeier et al. (32) reported that they did not observe a significant dominant negative effect on lamellipodia formation in growth cones or along the neurite shafts in PC12 transfectants stably expressing the DH mutant (L238R/L239S) of βPIX. Consistent with this, βPIX DH mutants (L238R/L239S) retained their GEF activity on Rac1 (Fig. 5B). Our model can therefore provide an explanation as to how βPIX regulates Rac1 activity in a manner that depends on the biological responses mediated by DH domain GEF activity or the interplay of GDP/Rac1/smgGDS.

In summary, in this study we have provided evidence that GBD in βPIX acts as an independent Rac1-binding site, which also serves a platform to recruit smgGDS for regulation of this GTPase. These results imply that a complex regulatory mechanism of βPIX exists, which involves both protein/protein interaction and DH-mediated GEF activity. Further information from site-directed mutagenesis as well as resolution of the three-dimensional structure of C-PIX (GBD):Rac1 in a crystallographic/NMR study may shed light on the characteristics of this binding. It also remains to be resolved whether GIT, a well-known partner of GBD, is involved in this interaction.

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