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Leucine Zipper-mediated Homodimerization of the p21-activated Kinase-interacting Factor, βPix

IMPLICATION FOR A ROLE IN CYTOSKELETAL REORGANIZATION*

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Pix, a p21-activated kinase-interacting exchange factor, is known to be involved in the regulation of Cdc42/Rac GTPases. The 85-kDa βPix-a protein contains an Src homology 3 domain, the tandem Dbl homology and Pleckstrin homology domains, a proline-rich region, and a GIT1-binding domain. In addition to those domains, βPix-a also contains a putative leucine zipper domain at the C-terminal end. In this study, we demonstrate that the previously identified putative leucine zipper domain mediates the formation of βPix-a homodimers. Using *in vitro* and *in vivo* methodologies, we show that deletion of the leucine zipper domain is sufficient to abolish βPix-a homodimerization. In NIH3T3 fibroblast cells, expression of wild type βPix-a induces the formation of membrane ruffles. However, cells expressing the leucine zipper domain deletion mutant could not form membrane ruffle structures. Moreover, platelet-derived growth factor-mediated cytoskeletal changes were completely blocked by the leucine zipper domain deletion mutant. The results suggest that the leucine zipper domain enables βPix-a to homodimerize, and homodimerization is essential for βPix-a signaling functions leading to the cytoskeletal reorganization.

The Rho family GTPases, which include Rho, Rac, and Cdc42, function as molecular switches in a variety of cellular signaling pathways, many of which regulate the cell cytoskeletal organization and affect on physiological properties of cells such as cell motility (1–3). In fibroblasts, RhoA induces stress fibers associated with focal adhesions, Rac1 produces lamellipodia or membrane ruffles, and Cdc42 induces filopodia on the plasma membrane (4–6). Similar to all members of the Ras superfamily GTPase, the activity of Rho family proteins cycles between active GTP-bound and inactive GDP-bound conformational states, regulated by three kinds of proteins: GTPase activating proteins, guanine nucleotide dissociation inhibitors, and guanine nucleotide exchange factors (GEFs[1]) (7).

All members of the Dbl family possess a Dbl homology (DH) domain in tandem with a Pleckstrin homology (PH) domain. The DH domain typically represents the motif for binding the Rho family GTPases and stimulating GDP/GTP exchange, whereas the PH domain appears to be essential for mediating the appropriate cellular localization of the protein (8–10). Additionally, most members of the GEFs contain a number of other structural motifs that indicate a role in signal transduction. These domains presumably function to mediate protein/protein or protein/lipid interactions and serve to link GEFs to upstream regulators and downstream effectors (8).

Previously, we reported p85SPR (an SH3 domain-containing proline-rich protein) as a widely expressed focal protein (11). The same protein was identified as a p21-activated kinase (Pak)-interacting exchange factor (named βPix or p85Cool-1) and suggested as a putative GEF for Cdc42/Rac1 (12, 13). Two alternative spliced forms of βPix, named βPix-b and βPix-c, that are predominantly expressed in nervous tissue were also reported (14). βPix-a, which corresponds to βPix/p85Cool-1/p85SPR, is a multidomain protein with many potential binding sites that can mediate protein-protein interactions. In addition to the conventional tandem, a DH domain and a PH domain, βPix-a has an SH3 domain that directly binds to the proline-rich region of Pak, an important downstream effector in cellular signaling governed by Cdc42/Rac GTPases (12, 15). Other domains of βPix-a include a proline-rich region and a putative leucine zipper domain at the C terminus (11). Recently, βPix-a was reported to interact with ADP ribosylation factor-GTPase activating proteins such as GIT (G-protein-coupled receptor kinase-interacting targets), p95PKL (paxillin-kinase linker), and Cat (cool-associated, tyrosine-phosphorylated) through the GIT1-binding domain of the C-terminal end of βPix-a (16–18).

Earlier studies have focused on the interactions of βPix with other signaling proteins but have not considered the possibility of its self-association. The leucine zipper domain is known to mediate the formation of homo- or heterodimers in various kinds of proteins such as transcription factor, adaptor, and kinase and regulate the functions of the proteins (19). In this study, we demonstrated that βPix homodimerizes through its leucine zipper domain *in vitro* and *in vivo*. This ability of βPix to homodimerize was found to be necessary for the βPix-mediated membrane ruffle formation in NIH3T3 fibroblast. Our results suggest that βPix-a homodimerization plays an essential role in βPix-a signaling leading to the cytoskeletal reorganization.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Schematic diagrams of the various βPix-a expression constructs used in this study are shown in Fig. 1*A*. Expression plasmids for hemagglutinin (HA)-tagged βPix-a, FLAG-tagged

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[1] The abbreviations used are: GEF(s), guanine nucleotide exchange factor(s); DH, Dbl homology domain; PH, Pleckstrin homology domain; SH3, Src homology 3 domain; Pak, p21-activated kinase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; LZ, leucine zipper; PDGF, platelet-derived growth factor; HIV, human immunodeficiency virus; C-ter, C terminus.

βPix-a, and Myc-tagged βPix-a were constructed by subcloning the polymerase chain reaction-amplified cDNA fragments from βPix-a.

In Vitro Transcription and Translation—In vitro transcription and translation were performed with the TNT reticulocyte lysate system (Promega). 1 μg of supercoiled plasmid was used according to the manufacturer's protocol. The 50-μl reaction mixture contained 25 μl of TNT rabbit reticulocyte lysate, TNT reaction buffer, T7 RNA polymerase, 20 units of RNasin, and amino acid mixture without methionine, supplemented with [^{35}S]methionine as the radioactive precursor (1000 Ci/mmol; PerkinElmer Life Sciences). After a 90-min incubation at 30 °C, the reaction mixture was used for co-immunoprecipitation assay.

GST Fusion Proteins and Pulldown Assay—GST-SH3 domain and GST-C-terminal fusion proteins were prepared for this study. Primers were taken directly from the published cDNA sequence of βPix-a. The recombinant vector was transformed into *Escherichia coli* DH5α, and the fusion proteins were induced with isopropyl-1-thio-β-D-galactopyranoside and affinity-purified according to the standard protocol of Amersham Pharmacia Biotech. 2 μg of GST fusion proteins was incubated with 1 mg of cell lysates in a volume of 1 ml. To immobilize the GST protein, glutathione-agarose beads equilibrated in binding buffer were added to the reaction mixture and incubated for 1 h at 4 °C under constant rotation. After washing the resin with the same buffer, the bound proteins were analyzed by immunoblotting.

Cell Lysis and Immunoprecipitation—Transfected cells were lysed on ice with 1 ml per 100-mm dish of ice-cold lysis buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 15 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 10 μg/ml aprotinin). After 20 min, lysates were precleared by centrifugation at 20,000 × *g* for 20 min at 4 °C and immunoprecipitated with a primary antibody for 2 h followed by incubation with protein A-Sepharose for 1 h. Immunoprecipitates were washed three times with the same lysis buffer, and samples were resolved by SDS-PAGE. Proteins were transferred to Immobilon P membranes (Millipore) and subjected to immunoblotting analysis. All immunoblots were developed using enhanced chemiluminescence.

Cell Culture, Transfection, and Immunofluorescence Microscopy—COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and maintained in 10% CO_2 at 37 °C. Mouse NIH3T3 fibroblast cells were grown in medium consisting of DMEM containing 10% bovine calf serum, 100 IU/ml penicillin, and 50 μg/ml streptomycin and maintained at 37 °C in an atmosphere of 5% CO_2. The cells were transfected using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer's protocol. For morphological studies, cells were serum-starved for 16 h, fixed with 3.7% formaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min. Actin cytoskeleton was stained by incubation with rhodamine-conjugated phalloidin (Molecular Probes) for 1 h followed by three washes with phosphate-buffered saline. For immunolocalization studies, cells were fixed and permeabilized as above. Myc-tagged βPix was detected by incubating with anti-Myc antibody, 9E10, for 1 h followed by a fluorescein-conjugated anti-IgG-specific secondary antibody for 1 h. Cells were observed and photographed under a Zeiss fluorescence microscope.

RESULTS AND DISCUSSION

To address whether βPix-a forms dimers (or oligomers), we first tested the ability of full-length βPix-a to form oligomers by a co-immunoprecipitation approach. For this study, two different epitope-tagged (FLAG and Myc) βPix-a expression vectors were transfected either alone or together into COS7 cells. Anti-Myc antibody, 9E10, was used to immunoprecipitate the putative βPix-a complexes. As shown in Fig. 1*B*, the FLAG-tagged form of βPix-a was detected only when co-expressed with Myc-tagged βPix-a, indicating that these different epitope-tagged forms of βPix-a do, in fact, form oligomers *in vivo* when overexpressed in COS7 cells. We next examined whether the domain of βPix-a that is responsible for the oligomerization resides in the N or C terminus of the βPix-a. For this purpose, the GST-SH3 domain and the GST-C-terminal half of βPix-a were used for GST pulldown assay using Myc-tagged βPix-a overexpressed in COS7 cell lysates. As shown in Fig. 1*C*, the GST-C-terminal half could form a complex with Myc-tagged βPix-a, whereas the GST-SH3 domain failed to associate with the βPix-a. These results indicate that the possible intermolecular

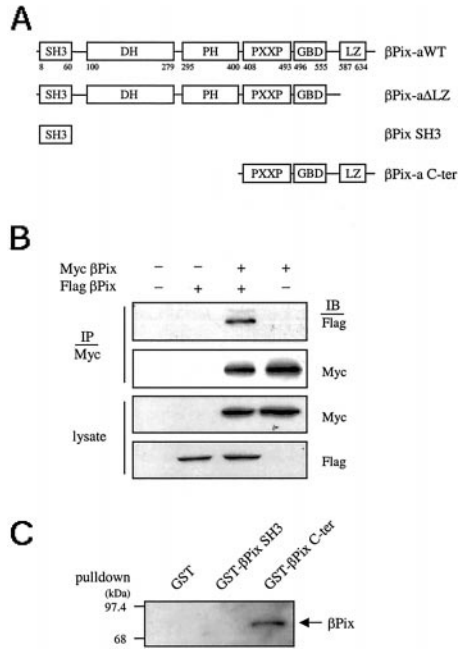

FIG. 1. **Oligomerization of βPix-a is mediated by the C terminus of βPix-a.** *A*, schematic representation of mouse βPix-a constructs (*SH3*, *DH*, *PH*, *PXXP* (proline-rich region), *GBD* (GIT1-binding domain), and *LZ*). *B*, βPix-a forms oligomers when overexpressed in COS7 cells. FLAG- and Myc-tagged βPix-a were transiently expressed in COS7 cells either alone or together as indicated. Anti-Myc antibody was used for immunoprecipitation (*IP*), and anti-FLAG antibody was used for immunoblotting (*IB*). *C*, oligomerization of βPix-a requires the GST-C terminus of βPix-a *in vitro*. COS7 cells were transiently transfected with Myc-tagged βPix-a, and the cell lysates were incubated with 2 μg of immobilized GST fusion proteins for 2 h at 4 °C, and samples were then washed and subjected to SDS-PAGE and immunoblotting with anti-Myc antibody.

interaction of the βPix-a SH3 domain with its own proline-rich region is not involved in βPix-a oligomerization. Thus, we focused on the involvement of the C-terminal leucine zipper domain in the oligomerization of βPix-a.

The region between amino acid 587 and 634 of mouse βPix-a contains several leucines that are spaced seven residues apart, a feature characteristic of leucine-zippered coiled-coil domains. Thus, we hypothesized that βPix-a could dimerize/oligomerize through this putative leucine zipper sequence. To better characterize the leucine zipper domain, we analyzed the sequences of mouse βPix-a by using the program Multicoil (20). As shown in Fig. 2*A*, the Multicoil program predicted the existence of a coiled-coil domain capable of forming a parallel side by side homodimer in βPix-a. Although the dimer probability is very high and considered significant, the trimer peaks at the shoulders of the dimer are due to artifacts of the algorithm and are not significant. Although we cannot exclude formation of high order oligomers, we assume and will refer to this interaction as dimerization. The alignment of βPix of other species and αPix leucine zipper domains is shown in Fig. 2*B*. The key features of the leucine zipper sequence are well conserved in all Pix proteins. In the search using the BLAST network, a number of other GEFs such as p115Rho-GEF and GEF-H1 did not show any homology with βPix the LZ domain.

To confirm a direct interaction between the βPix-a leucine zipper domain and itself, an HA-tagged C-terminal half of βPix-a was transcribed and translated *in vitro* either alone or in combination with Myc-tagged full-length βPix-a (βPix-aWT) or leucine zipper domain-deleted βPix-a (βPix-aΔLZ). As shown in Fig. 3*A*, βPix-aWT was co-immunoprecipitated with the HA-tagged C-terminal region, but βPix-aΔLZ failed to co-immunoprecipitate. To determine whether the leucine zipper domain is

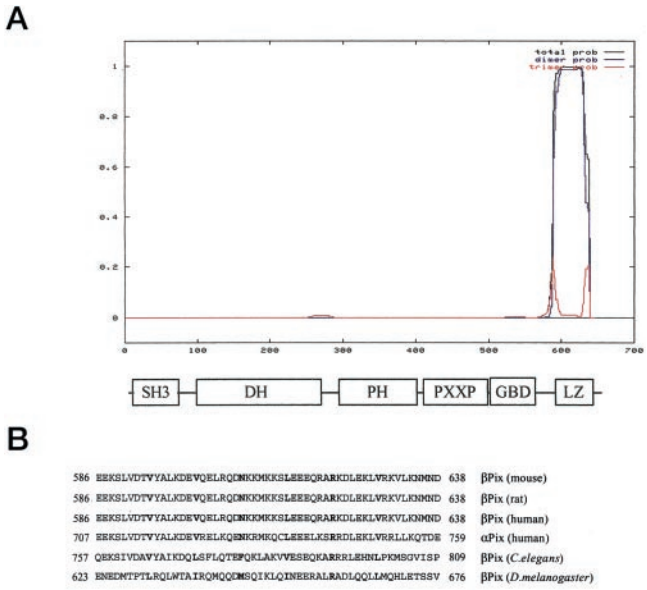

FIG. 2. **The leucine zipper domain of βPix-a is predicted to mediate dimer formation.** *A*, βPix-a amino acid sequences were analyzed using the Multicoil program, which predicts the presence of coiled-coils and the probability for dimer and trimer formation based on pairwise residue interactions. The probabilities for dimer (*blue*) and trimer (*red*), as well as overall score (*black*), are shown. *B*, alignment of the predicted dimerization regions of βPix-a proteins shows heptad repeats with characteristically placed hydrophobic residues. The critical amino acid residues forming the heptad repeat are shown in *bold*.

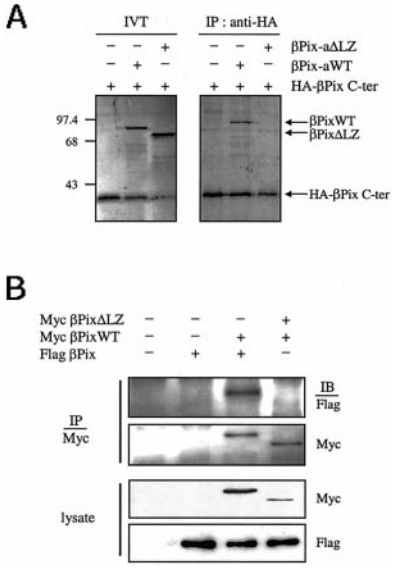

FIG. 3. **Homodimerization of βPix-a is mediated by the leucine zipper domain.** *A*, *in vitro* dimerization of βPix-a. HA-tagged βPix C-terminal cDNA was translated *in vitro* alone or together with βPix-aWT or βPix-aΔLZ as indicated in the presence of [^{35}S]methionine (*left panel*, *IVT*). Immunoprecipitates of each translated products with anti-HA antibody were shown in the *right panel* (*IP*). Each sample was run on a 10% SDS-PAGE and exposed to autoradiography. *B*, βPix-a homodimerization via LZ domain *in vivo*. FLAG-tagged βPix-aWT was transiently expressed in COS7 cells either alone or together with the Myc-tagged βPix-aWT or βPix-aΔLZ as indicated. Anti-Myc antibody was used for immunoprecipitation (*IP*) and anti-FLAG antibody for immunoblotting (*IB*).

required for βPix-a homodimerization *in vivo*, Myc-tagged βPix-aWT and βPix-aΔLZ were transiently transfected in COS7 cells with FLAG-tagged βPix-aWT. As shown in Fig. 3*B*, FLAG-tagged βPix-aWT co-immunoprecipitated with Myc-tagged βPix-aWT but not with Myc-tagged βPix-aΔLZ. These data demonstrate that βPix-a homodimerizes in cells in a

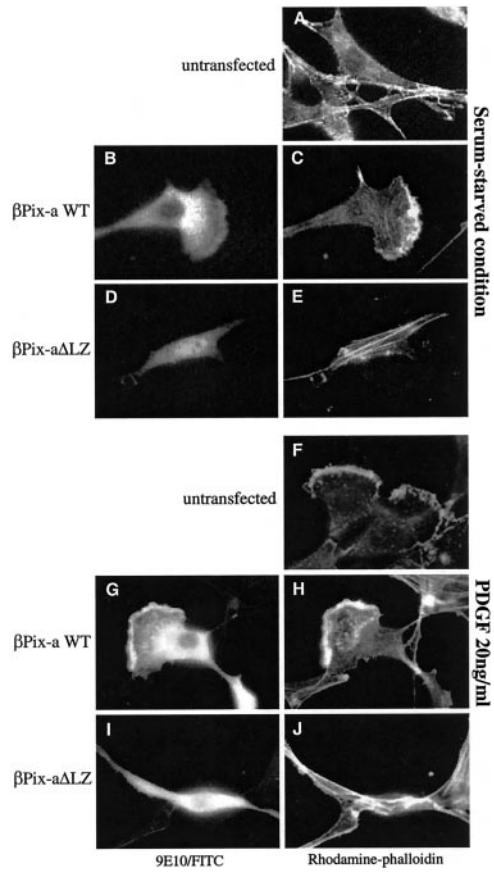

FIG. 4. **Overexpression of a βPix-aΔLZ mutant in NIH3T3 fibroblast cells inhibits membrane ruffle formation in response to PDGF.** NIH3T3 fibroblast cells untransfected or transfected with Myc-tagged βPix-aWT or βPix-aΔLZ were serum-starved for 16 h and fixed before (*A–E*) or after (*F–J*) stimulation with 20 ng/ml PDGF for 10 min. βPix distribution was visualized using an anti-Myc antibody, 9E10, and fluorescine-conjugated secondary antibody. Actin distribution was visualized with rhodamine-conjugated phalloidin.

leucine zipper domain-dependent manner.

Studies in transfected cells have shown that βPix-a has the potential to localize to focal adhesions and can promote the formation of membrane ruffles that co-localize with focal adhesions (12). To examine a role for the leucine zipper domain in βPix-a-mediated cytoskeletal reorganization such as membrane ruffle formation, NIH3T3 fibroblast cells were transfected with either Myc-tagged βPix-aWT or βPix-aΔLZ cDNAs. Expression of βPix-aWT caused formation of membrane ruffle at cell periphery, and βPix-aWT is concentrated at F-actin-rich membrane ruffle structures (Fig. 4, *B* and *C*). However, in cells expressing βPix-aΔLZ, morphological changes were not exhibited, and βPix-aΔLZ showed a diffuse cytoplasmic localization (Fig. 4, *D* and *E*). Next, serum-starved cells were stimulated with platelet-derived growth factor (PDGF) for 10 min to induce membrane ruffles. Cells expressing βPix-aWT exhibited polarized membrane ruffles in response to PDGF (Fig. 4, *G* and *H*). The extent of membrane ruffling in cells expressing βPix-aWT was comparable with that in PDGF-stimulated nontransfected cells (Fig. 4, *A* and *F*). However, surprisingly, cells expressing the leucine zipper domain deletion mutant did not show any apparent ruffle-like structures (Fig. 4, *I* and *J*). Blocking of PDGF-induced membrane ruffling in cells expressing βPix-aΔLZ indicates that βPix-aΔLZ could function as a dominant inhibitory mutant. Diffuse cytoplasmic localization of βPix-aΔLZ suggests that it might sequester the downstream effector molecules, such as Pak and/or other βPix-a interacting proteins, essential for membrane ruffling at the cytoplasm. To examine the importance of the C terminus in βPix-a function, the effect of co-expression of βPix-aWT with βPix-a

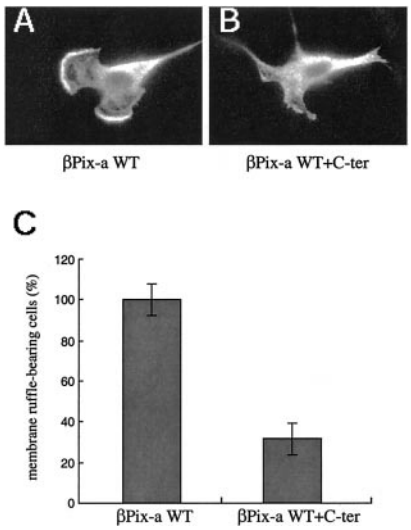

FIG. 5. **Co-expression of βPix-a C-ter blocked βPix-a-induced membrane ruffling in NIH3T3 fibroblast cells.** Cells were transfected with Myc-tagged βPix-aWT alone (*A*) or together with HA-tagged βPix-a C-ter (*B*), serum-starved for 16 h, and fixed. Double immunofluorescence staining for Myc-βPix-a and HA-C-ter were carried out using anti-βPix-a antiserum (30,000:1 diluted) or anti-HA antibody (data not shown), respectively. Quantitation of a typical result from three independent experiments is shown in *C*. Values are expressed as the percentage of membrane ruffle-bearing cells over all transfected cells. Data represent means ± S.E. from three different fields.

C-ter was investigated. Cells expressing βPix-aWT alone exhibited membrane ruffles as expected (Fig. 5*A*). However, βPix-aWT-induced membrane ruffling was inhibited in cells co-expressing βPix-aWT with βPix-a C-ter (Fig. 5*B*). Quantitation of membrane ruffle-bearing cells showed that co-expression of βPix-a C-ter inhibits membrane ruffling in 60–70% of cells expressing βPix-aWT (Fig. 5*C*).

In this study, we demonstrated that the leucine zipper domain at the C-terminal end of βPix-a mediates the formation of βPix-a homodimers *in vitro* and *in vivo*. The leucine zipper domain is an α-helical structure formed by several heptad repeats of hydrophobic residues, usually leucine and isoleucine, that are commonly found in nuclear transcription factors, and its role in promoting the homo- and heterodimerization of these proteins has been well characterized (21, 22). Leucine zipper domains have also been identified in many other proteins such as protein kinases, adaptors, and cytoskeletal proteins, but their function in these proteins has been less extensively studied. Recently, it has been reported that the leucine zipper domain-dependent homodimerization of a ZIP kinase, a serine/threonine kinase, is necessary for their activity (23).

In the functional study of the leucine zipper domain in βPix-a-mediated cytoskeletal reorganization, we found that the deletion of the leucine zipper domain and the resulting loss of homodimerization made βPix-a fail to induce the formation of membrane ruffles in NIH3T3 fibroblasts. These results suggest that the leucine zipper domain of βPix-a plays an important role in the regulation of the βPix-a function. Recently, Yoshii *et al*. (24) reported that Pix could form a complex with PDGF receptor and mediate Cdc42/Rac signaling by PDGF stimulation. Therefore, considering our results that the leucine zipper domain deletion mutant blocked completely the morphological changes of NIH3T3 cells in response to PDGF, βPix-a homodimerization is required for the PDGF receptor-mediated signaling cascade leading to the morphological changes.

βPix is, to our knowledge, the first GEF protein that is demonstrated to have the ability to homodimerize through a leucine zipper domain-dependent mechanism. The roles of the coiled-coil domain of other GEFs have been reported previously. The leucine zipper domain-mediated interaction between human immunodeficiency virus (HIV)-1 transmembrane protein gp41 and p115-RhoGEF is essential for the HIV replication cycle and p115-RhoGEF-mediated stress fiber formation (25). In the case of GEF-H1, the coiled-coil domain is essential for the GEF-H1 microtubule co-localization (26). Dimeric βPix proteins could function as molecular bridges to recruit and juxtapose other βPix-binding proteins such as Pak and Nck within the signaling complex. Dimer formation could also mask or expose functional domains or residues of βPix-a as a result of conformational changes that might be induced by dimerization. For example, the deletion of the leucine zipper domain of c-Cbl, a multidomain adaptor protein, caused a decrease in both the tyrosine phosphorylation of Cbl and its association with the epidermal growth factor receptor (27). Finally, it is also possible that βPix heterodimerizes with other leucine zipper domain-containing proteins or other βPix isoforms such as βPix-b and βPix-c (14). In our preliminary data, βPix-a could interact with βPix-b and βPix-c.[2] Because three βPix isoforms differ in the primary structure at the C terminus, these differences may affect the abilities of βPix isoforms to interact with each other or form distinct signaling complexes, and such heterodimerization could be crucial to understand the function of βPix isoforms.

In conclusion, we have shown that the putative leucine zipper domain at the C terminus of βPix-a mediates the formation of homodimers and that the homodimerization of βPix is required for the cytoskeletal changes by βPix-a overexpression. Moreover, βPix-a homodimerization plays essential roles in PDGF-mediated signaling pathway leading to morphological changes. Further studies on the role of the leucine zipper domain and βPix-a homodimerization will provide additional important insights in the regulation of βPix activities and functions.

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[2] Unpublished data.