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βPAK-interacting exchange factor may regulate actin cytoskeleton through interaction with actin

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Abbreviations: GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; PAK, p21-activated kinase; βPIX, βPAK-interacting-exchange factor; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis

Abstract

p21-activated kinase (PAK)-interacting exchange factor (PIX) is known to be involved in regulation of Cdc42/Rac GTPases and PAK activity. PIX binds to the proline-rich region of PAK, and regulates biological events through activation of Cdc42/Rac GTPase. To further investigate the role of PIX we produced monoclonal antibodies (Mab) against βPIX. Three clones; N-C6 against N-terminal half and C-A3 and C-B7 against C-terminal half of βPIX were generated and characterized. N-C6 Mab detected βPIX as a major band in most cell lines. C-A3 Mab recognizes GIT-binding domain (GBD), but it does not interfere with GIT binding to βPIX. Using C-A3 Mab possible βPIX interaction with actin in PC12 cells was examined. βPIX Mab (C-A3) specifically precipitated actin of the PC12 cell lysates whereas actin Mab failed to immunoprecpitate βPIX. Co-sedimentation of PC12 cell lysates with the polymerized F-actin resulted in the recovery of most of βPIX in the cell lysates. These results suggest that βPIX may not interact with soluble actin but with polymerized F-actin and revealed that βPIX constitutes a functional complex with actin. These data indicate real usefulness of the βPIX Mab in the study of βPIX role(s) in regulation of actin cyoskeleton.

Keywords: actin; βPIX; monoclonal antibody; neurite outgrowth

Introduction

Small G proteins of the Rho family are critical regulators in a wide variety of cellular processes such as actin dynamics, gene transcription, cell-cycle progression and cell adhesion (Hall A, 1998). They convert between the inactive GDP-bound and the active GTP-bound state, which is tightly coordinated by a guanine nucleotide exchange factor (GEF), a GDP-dissociation inhibitor (GDI), and a GTPase activating protein (GAP). It therefore seems intriguing to understand how these upstream regulators of the Rho G proteins are activated and/or inactivated. Accumulating evidence indicates that RhoGDI is first dissociated from the Rho GTPase complex, and then GDP-GTP exchange by a GEF occurs. Later, the activity of the Rho G protein is turned off by a GAP. Many GEFs have been discovered initially as oncoproteins possessing transforming activity. Their activation mechanisms have thus been studied extensively. However, the detailed mechanism of the initial step, dissociation of RhoGDI from the Rho GTPase, has been elusive. DerMardirossian *et al.,* (2004) elegantly showed that this dissociation is mediated by p21-activated kinase (Pak) through phosphorylation of RhoGDI at Ser101 and Ser174.

PAK-interacting exchange factor (PIX) specifically catalyzes GDP-GTP exchange of Rac1/Cdc42 GTPase (Manser *et al.,* 1998). Two distinct genes encode PIX proteins, α- and βPIX. In their modular structure αPIX has an additional calponin homology (CH) domain at its N-terminus compared with βPIX. Since PIX belongs to the Dbl family GEF, it shows a tandem array of Dbl homology (DH) domain for its GEF activity and pleckstrin homology (PH) domain. At the N-terminus of these two domains lies a SH3 domain, which binds to the proline-rich region of PAK. Because of this interaction, when stimulated by growth factors such as basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), PAK is capable of phosphorylating βPIX at Ser525/Thr526 in the GIT1-binding do-

main (GBD) (Shin *et al.,* 2002). This results in activation of βPIX for its downstream Rac1/Cdc42 GTPases (Shin *et al.,* 2004). The leucine zipper (LZ) domain is at the C-terminus of PIX and mediates homodimerization of PIX, which seems to be essential for its biological activity (Kim *et al.,* 2001). Less well defined is a PXXP domain, which is located between the PH and GB domain.

Biological function and regulation of βPIX seem to be increasingly complex than thought before. Beta PIX is making a complex with various proteins; PAK (Manser *et al.,* 1998), Rac/Cdc42 (Aghazadeh *et al.,* 1998; Shin *et al.,* 2004), GIT (Zhao *et al.,* 2000) and Shank (Park *et al.,* 2003). Although it is important to understand that all these components make a unique complex or more than two heterogenous ones, it is not yet clear. Even more intriguing, it should be answered how this complex is formed or dissociated depending on the biological context. Through this interaction βPIX is involved in cell adhesion and migration, neurite outgrowth (Shin *et al.,* 2002; Albertinazzi *et al.,* 2003) and synapse formation (Parnas *et al.,* 2001). In the present study we therefore sought to produce monoclonal antibodies against βPIX to more carefully analyze βPIX-mediated biology and its regulation. We have generated three clones of monoclonal antibodies and characterized them. Using these antibodies we further showed that PIX interacts with actin.

Materials and Methods

Materials

Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, protein G Sepharose and glutathione Sepharose 4B were obtained from Amersham Biosciences (Uppsala, Sweden). Brilliant blue G 250, pristine (2, 6, 10, 14-tetramethyl pentadecane), polyethelene glycol (PEG), complete and incomplete Freund's adjuvant were purchased from Sigma-Aldrich (Louis, MO). Fetal bovine serum (FBS) and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Invitrogen (Carlsbad, CA). Cell culture dishes and ELISA plates were obtained from Nunc (Naperville, IL).

Purification of GST-PIX fusion proteins

Truncated cDNAs encoding the NH2-terminal 434 residues (N-PIX) or COOH-terminal 213 residues (C-PIX) of mouse βPIX were subcloned to the pGEX4T (Amersham Biosciences) and pQE70 (Qiagen), respectively. These proteins were expressed according to the manufacturer's recommendation. Briefly, they were grown at 37°C until the absorbance at 600 nm of the culture reached 0.5-0.6 and then induced by 100 μM IPTG for 3 h. Cells were collected, washed with phosphate-buffered saline (PBS) and lysed by sonication in PBS containing 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF). The GST-N-PIX and C-PIX-His were purified by using glutathione-Sepharose 4B and Ni^{2+} -Agarose, respectively.

Production of monoclonal antibody against PIX

The monoclonal antibodies against PIX were produced by procedures as described previously (Jin *et al.,* 2001). Briefly, 50 μg purified proteins (N-and C-PIX) in equal volume of complete (for the primary injection) or incomplete (for the subsequent injections) Freund's adjuvant were immunized three times at 3-weeks interval in 5-weeks old BALB/c mice. Hybridomas were prepared by fusing spleen cells with myeloma cell line SP2/0 as described previously (Lee *et al.,* 1996). Positive clones were characterized by enzyme linked immunosorbent assay (ELISA) using the N or C-PIX proteins.

Immunoprecipitation and Western blotting

Cells and tissues were lysed in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 200 μM sodium orthovanadate, 10 mM Na-pyruvate, 50 mM glycerophosphate, 1% triton X-100 and a protease inhibitor cocktail) and centrifuged at 12,000 rpm for 20 min. The lysates were immunoprecipitated with anti-βPIX antibody. Immunoprecipitates were washed five times with same lysis buffer and separated on SDS-polyacrylamide gel electrophoresis (PAGE). The proteins in the gel were then transferred onto PVDF membrane. For Western blotting, the transferred membranes were incubated with monoclonal antibodies to βPIX in 2% skim milk in PBS-T for 1 h and then with HRP-conjugated mouse IgG antibody. The signals were developed using enhanced chemiluminescence (ECL).

Cell culture

SP2/0 and PC12 cells were cultured in Dulbeccos modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U of penicillin/ml and 100 μg of streptomycin/ml at 37°C in a humidified, CO_2-controlled (5%) incubator.

Immunocytochemistry

For immunolocalization studies, PC12 cells were plated on poly-L-lysine coated glass coverslips in 6-well plate (1×10^5 cells/well) and were induced to differentiate in complete media containing 10 ng/ml of bFGF. Cells were serum starved for 16 h, stimulated

by bFGF for 24 h, and then fixed and permeabilized with 4% paraformaldehyde containing 0.2% triton X-100. Enodogenous PIX was detected by incubation with anti-C-PIX antibody for one and half hour followed by a fluorescein-conjugated anti-mouse IgG-specific secondary antibody (Molecular Probes, Eugene) for 1 h. For actin staining, cells were incubated with rhodamine phalloidin for 1 h. Cells were observed and photographed under a confocal microscope (Bio-Rad, MRC 1024, UK).

F-actin co-sedimentation assay

PC12 (1×10^6) cells were plated on 60 mm dish. Three days after plating, the cells were washed with PBS and lysed with lysis buffer containing 100 mM NaCl, 50 mM HEPES (pH7.5), 5 mM EDTA, 50 mM β- glycerophosphate, 200 uM Na_3VO4, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 ug/ml leupeptin. The lysates were then centrifuged at 12,000 rpm at 4°C. The supernatant was centrifuged again at 160,000 *g* for 1 h at 4°C. F-actin co-sedimentation assay was done as described by the manufacturer's instruction (Cytoskeleton, CO). Briefly, the supernatant was mixed with 40 μg of polymerized F-actin and incubated for 1 h at room temperature. The mixture was then centrifuged at 160,000 *g* for 1 h at 25°C. After centrifugation, the supernatant and pellet fraction were analyzed by 9% SDS-PAGE, followed by immunoblotting with anti-βPIX and anti-actin antibodies and Ponceau S (Sigma, CA) staining.

Results

Production of monoclonal antibodies against βPIX

GST-fusion proteins of N-terminus (N-PIX) and His-C-terminus half (C-PIX) of bPIX were expressed in *E.coli* and purified from the resulting supernatant using glutathione-Sepharose 4B and Ni^{2+}-affinity chromatography, respectively. Even though contaminating bands were visualized, GST- or His-fusion proteins of βPIX appeared as the major bands. The purified GST- or His-fusion proteins were used as antigens to produce monoclonal antibody against bPIX. ELISA was set up to specifically select the clones of monoclonal antibody against GST-N-PIX/His-C-PIX. Finally, one clone for N-PIX (N-C6) and two clones for C-PIX (C-A3 and C-B7) were established.

Characterization of anti-βPIX monoclonal antibodies

To observe an expression pattern of βPIX in various cell lines, Western blot analysis was performed using N-C6 clone. βPIX is expressed in all the cell lines examined (Figure 1). Higher levels of expression were observed in Raji and Ramos cell lines than in the others (lanes 7 and 8). Different βPIX isoforms were also detected in several cell lines (lanes 2, 4, 5 and 8). To characterize the epitope of the clone C-A3, various deletion constructs of βPIX in GFP-fusion forms were generated as illustrated in Figure 2A. PC12 cells were transfected with each construct and the lysates were immunoprecipitated with anti-GFP antibody, followed by Western blotting with anti-GFP antibody or the C-A3 clone. This clone does not recognize βPIX when the part of the βPIX C-terminus (495-543) is deleted (Figure 2B). This result indicates that the epitope of this clone resides in the GB domain (495-543). We thus further examined whether this clone blocks the interaction of βPIX with GIT1. PC12 cell lysates were immunoprecipitated with anti-βPIX antibody, followed by immunoblotting with anti-GIT1 antibody. Apparently GIT1 was present in the βPIX immunoprecipitate (Figure 3A, left), indicating that this clone does not interfere with the interaction between βPIX and GIT1. To monitor subcellular localization of βPIX in PC12 cells, immunofluorescence microscopy was performed using C-A3. βPIX was stained as vacuole-like structures in the cytoplasm (Figure 2C, left). The protein was also concentrated as multiple spots at the periphery of cells. In the same area actin showed robust staining. In the merged picture it is more apparent that these structures are focal adhesions in which βPIX and actin co-localize (Figure 2C, right). This result is consistent with the previous data from NIH 3T3 cells (Oh *et al.,* 1997).

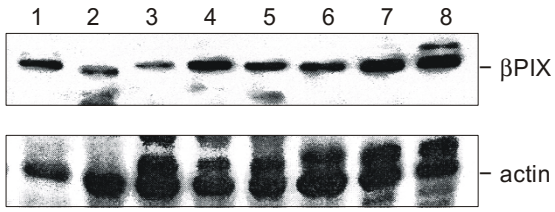

Figure 1. Expression of βPIX in various cell lines. Cells were lysed in the buffer as described in "Materials and Methods", and the lysates were then separated on an 8% SDS-polyacrylamide gel. Western blot analysis was performed with monoclonal antibody (N-C6) against N-terminus of βPIX (top) and actin antibody (bottom) as a control for loading the same amount of proteins. Lane 1, MDCK; 2, LLCPK11; 3, VSMC; 4, A10; 5, PC12; 6, U251; 7, Raji; 8, Ramos cells.

PIX interacts with actin

As specific localization of PIX at the actin structures (Figure 2C) and its enzymatic activity of guanine nucleotide exchange against Rac1/Cdc42, critical regulators of actin rearrangement, suggest that βPIX

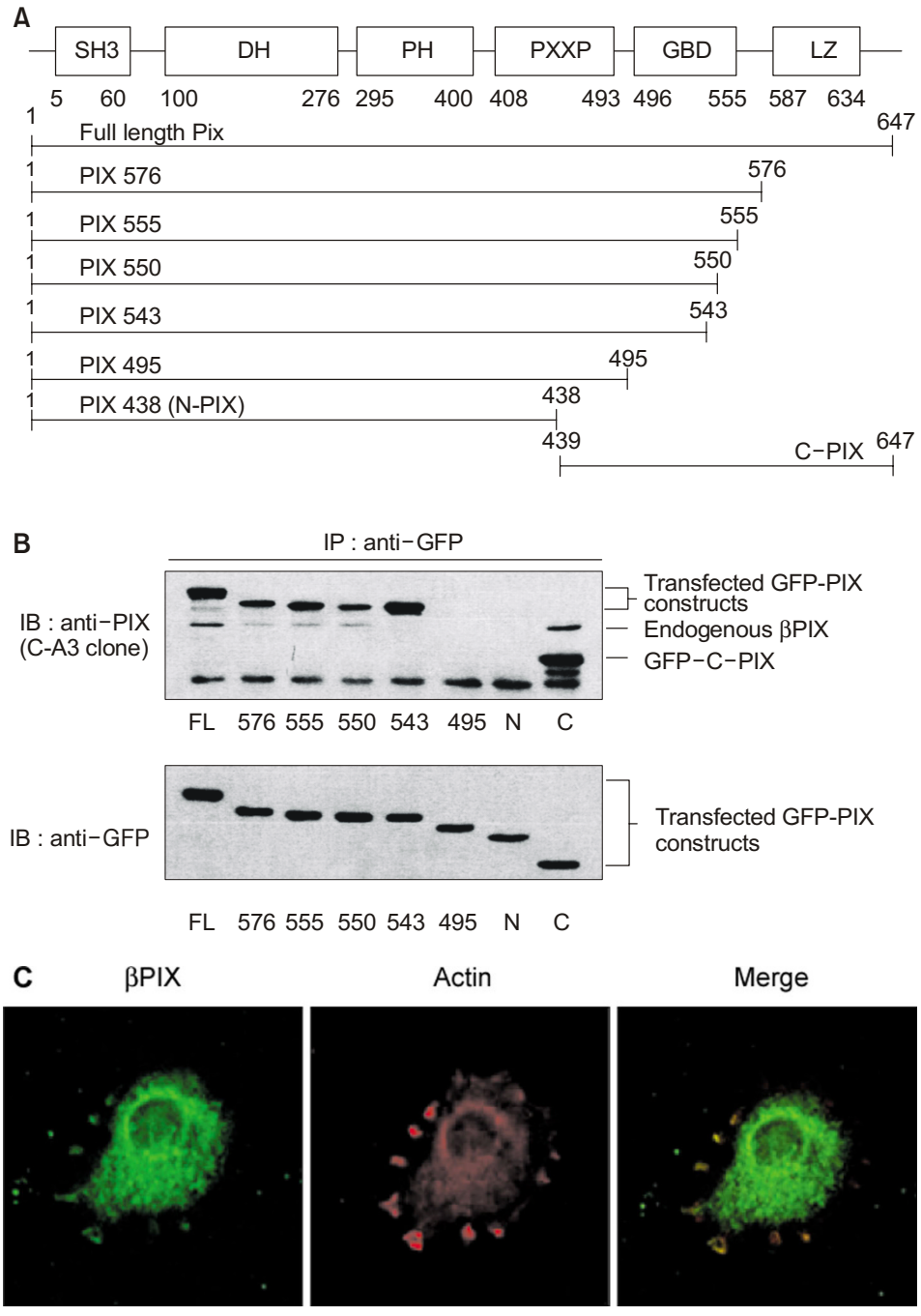

Figure 2. Characterization of an anti-βPIX monoclonal antibody (C-A3 clone). (A) Schematic diagram of βPIX constructs. To determine the specific epitope for C-A3 clone, each deletion construct for βPIX was cloned to pEGFP vector and expressed in PC12 cells. (B) Determination of the specific epitope for C-A3 clone. PC12 cells were transfected with the βPIX constructs described above. Cell lysates were separated on 10% SDS- PAGE and transferred to a PVDF membrane. The membrane was immunoblotted with C-A3 clone (top) and anti-GFP antibody (bottom) as a control for expression of each construct as GFP-fusion proteins. (C) Immunocytochemistry with C-A3 clone. PC12 cells were serum-starved for 20 h and cultured in the presence of bFGF (10 ng/ml) for 24 h. For immunocytochemistry, differentiated cells were fixed and permeabilized with 4% paraformaldehyde containing 0.02% Triton X-100. Cells were then stained with anti-βPIX antibody (C-A3 clone) and fluorescien-conjugated mouse IgG as a secondary antibody. To visualize actin, cells were incubated with rhodamine phalloidin for 1 h. Fluorescence images were observed and photographed under a confocal microscope.

may be linked somehow to actin. A co-immunoprecipitation study was performed. Anti-βPIX antibody (C-A3) specifically precipitated actin (Figure 3A, left). However, in the actin immunoprecpitate no βPIX was detectible (Figure 3A, right). Since these results suggest that βPIX may not interact with soluble actin but with polymerized F-actin, we checked this possibility with co-sedimentation analysis. PC12 cell lysates were mixed with polymerized F-actin, followed by ultracentrifugation. The precipitates were then Western blotted with anti-βPIX (Figure 3B, top) or anti-actin (Figure 3B, middle) antibody. Addition of polymerized F-actin was verified by staining with Ponceau S solution (Figure 3B, bottom). In the precipitate most of βPIX was found (Figure 3B, top). Relatively small amount of βPIX was also observed in the soluble fraction. These results suggest that βPIX may somehow interact with polymerized F-actin and regulate

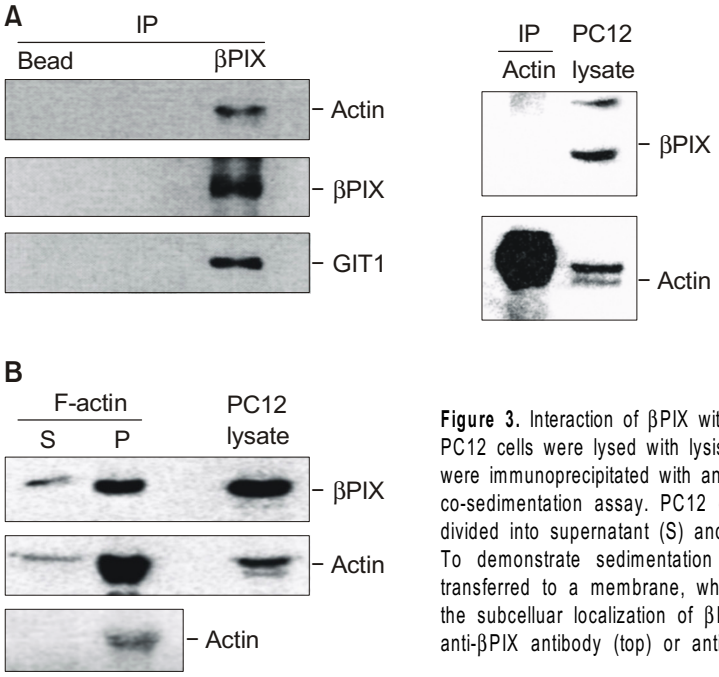

Figure 3. Interaction of βPIX with actin. (A) Co-immunoprecipitation analysis of βPIX and actin. PC12 cells were lysed with lysis buffer as described in "Materials and Methods". The lyastes were immunoprecipitated with anti-βPIX C-A3 clone (left) or anti-actin (right) antibody. (B) Actin co-sedimentation assay. PC12 cell lysate was incubated with polymerized F-actin and then divided into supernatant (S) and pellet (P) fraction as described in "Materials and Methods". To demonstrate sedimentation of F-actin, following SDS-PAGE proteins in the gel was transferred to a membrane, which was stained with Ponceau S solution (bottom). To trace the subcelluar localization of βPIX and actin, the same membrane was immunoblotted with anti-βPIX antibody (top) or anti-actin antibody (middle).

actin dynamics.

Discussion

βPIX was identified as a component of focal adhesion in fibroblast cells (Oh *et al.,* 1997). Later, Manser *et al.* (1998) demonstrated that βPIX has a GEF activity toward Rac1/Cdc42 *in vitro* and is a binding partner of PAK. βPIX also binds to GIT1 through GIT binding domain (Zhao *et al.,* 2000). Therefore, biological function of βPIX in formation or dissociation of focal complex has been intensively studied in relation with these binding partners. However, its precise role has remained elusive. It has been further shown that its downstream G protein Rac1 constitutes this complex (Aghazadeh *et al.,* 1998; Shin *et al.,* 2004). It thus seemed rather complicated to determine how this PAK-βPIX-GIT1-Rac1 complex mediates the signals from the extracellular matrix or the agonists that activate G-protein coupled receptor or receptor tyrosine kinases. Because anti-βPIX monoclonal antibodies of good quality seemed to be essential to elucidate the pending issue, we set out this project. They are commercially available from BD Biosciences Pharmingen (San Diego, CA) and Chemicon International, Inc (Temecula, CA). However, application of these antibodies includes Western blot and immunocytochemistry, but not immunoprecipitation. In this study we have successfully generated anti-PIX monoclonal antibodies, which can be used for most of immunological analysis such as Western blot, immunoprecipitation and immunocytochemistry (Figures 1-3). One of the initial aims was to establish several clones, which can inhibit the binding between βPIX and PAK or GIT1. The epitope of C-A3 clone has been determined as the C-terminal portion of GB domain. However, this clone proved to be unsuccessful to block βPIX binding to GIT1.

Previously we characterized βPIX-a, which is a major form in PC12 cells (Shin *et al.,* 2002). This isoform is expressed as a major one in the most cell lines examined except in LLCPK cells. A slower migrating form in Ramos cells corresponds to αPIX, whose size is approximately 90 kD. Our monoclonal antibodies, however, cannot discriminate between αPIX and βPIX, since these two proteins share most of domain structures except unique calponin homology (CH) domain residing in the N-terminus of αPIX. It is desirable to raise specific monoclonal antibodies to recognize αPIX. Particularly for immunohistochemistry of brain where diverse βPIX isoforms and αPIX are co-expressed these antibodies are indispensable.

Immunohistochemistry in Figure 2A shows that βPIX is concentrated at the cell periphery where actin is strongly stained, suggesting that βPIX is in the vicinity of actin to regulate actin polymerization. This idea is supported by co-immunoprecipitation and actin co-sedimentation study (Figure 3). In PC12 cells the co-localization pattern later extends to the growth cones at the neurite tips where actin structures of lamellipodia and filopodia take an active participation in neurite outgrowth (Shin *et al.,* 2004). It has been demonstrated that βPIX activates Rac1/Cdc42 fol-

lowing growth factor stimulation and its phosphorylation is an activation mechanism of Rac1 (Shin *et al.,* 2002). Kinases such as PAK, LIM kinase, Rho kinase and myosin light chain kinase and other actin binding proteins act downstream of these G proteins (Bishop *et al.,* 2000; Luo L, 2000; Kim *et al.,* 2004). It does seem to be unlikely that βPIX and actin interact directly. Our speculation is that actin binding proteins or non-muscle myosin may bridge between βPIX and actin.

In conclusion, we have made several clones of anti-βPIX monoclonal antibodies, which might be valuable in elucidating the biological function of βPIX. Identification of new βPIX binding proteins, which can provide a clue to unravel the puzzle of the βPIX-mediated acin regulation, would be interesting directions in the future study.

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