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Kinesin-1-dependent transport of the βPIX/GIT complex in neuronal cells

Eun-Young Shin[1], Chan-Soo Lee[2], Han-Byeol Kim[1], Jin-Hee Park[1], Kwangseok Oh[1], Gun-Wu Lee[1], Eun-Yul Cho[1], Hyong Kyu Kim[3] & Eung-Gook Kim[1,]*

Departments of [1]Biochemistry and [3]Microbiology, College of Medicine, and Medical Research Center, Chungbuk National University, Cheongju 28644, [2]Food Standard Division Scientific Office, Ministry of Food and Drug Safety (KFDA), Cheongju 28159, Korea

Proper targeting of the βPAK-interacting exchange factor (βPIX)/G protein-coupled receptor kinase-interacting target protein (GIT) complex into distinct cellular compartments is essential for its diverse functions including neurite extension and synaptogenesis. However, the mechanism for translocation of this complex is still unknown. In the present study, we reported that the conventional kinesin, called kinesin-1, can transport the βPIX/GIT complex. Additionally, βPIX bind to KIF5A, a neuronal isoform of kinesin-1 heavy chain, but not KIF1 and KIF3. Mapping analysis revealed that the tail of KIF5s and LZ domain of βPIX were the respective binding domains. Silencing KIF5A or the expression of a variety of mutant forms of KIF5A inhibited βPIX targeting the neurite tips in PC12 cells. Furthermore, truncated mutants of βPIX without LZ domain did not interact with KIF5A, and were unable to target the neurite tips in PC12 cells. These results defined kinesin-1 as a motor protein of βPIX, and may provide new insights into βPIX/GIT complex-dependent neuronal pathophysiology. [BMB Reports 2021; 54(7): 380-385]

INTRODUCTION

Beta PAK (p21-activated kinase)-interacting exchange factor (βPIX) is a guanine nucleotide exchange factor (GEF) for Rac1/Cdc42 GTPases (1) that regulates the dynamics of lamellipodial and filopodial actin structures in response to extracellular cues. Two distinct genes encode the mammalian PIX family members namely αPIX and βPIX (2, 3). αPIX and βPIX have similar domain structures, with the exception of an extra calponin homology (CH) domain at the N-terminus of αPIX (4). βPIX interacts stably and transiently with G protein-coupled receptor kinase-interacting protein (GIT) and Rac1/Cdc42 effector p21-activated kinase (PAK), respectively, thus generating a high-molecular weight complex designated here as the βPIX complex (5, 6).

The βPIX complex plays a key role in a wide range of biological processes, including neurite extension (7-9) and cell migration (9, 10), which requires the correct subcellular localization of the complex. The neuronal growth cone, found at the tip of axonal or dendritic projection, is a motile structure whose migration promotes neurite extension during the development of the nervous system and regeneration. At the growth cone, mostly near the actin-rich peripheral zone, the βPIX complex localizes and promotes growth cone motility (8, 11). The βPIX complex can also be found at synapses, in both presynaptic and postsynaptic areas, causing synaptogenesis including dendritic spine morphogenesis (12-15). The βPIX complex promotes neurite extension and synaptogenesis through dynamic cytoskeletal rearrangement. In contrast to these well-known functions of the βPIX complex, the mechanism for its subcellular translocation is still unknown.

Motor protein kinesin-1 (also known as conventional kinesin-1) mediates plus end-directed, microtubule-dependent transport. Several types of cargo for kinesin-1 has been identified, including protein complexes, organelles, and mRNA (16). Kinesin-1 is a tetramer composed of two identical kinesin heavy chains (KHCs) and two identical kinesin light chains (KLCs) (17). The mammalian genome contains three KHC genes namely KIF5A, KIF5B, and KIF5C. Among these KHC isoforms, KIF5A and KIF5C are neuron-specific, whereas KIF5B is expressed ubiquitously (18). Two major cargo-interaction sites have been recognized: (i) the C-terminal heptad-repeat region in the tail domain of KHC (19, 20) and (ii) the C-terminal tetratricopeptide repeat region in KLC (21). The former is responsible for direct binding to kinectin, RanBP2, SNARE proteins, GRIP1 and β-dystrobrevin (22).

In view of the critical role of the βPIX complex in diverse biological processes, we aim to understand its targeting mechanism by identifying a motor protein capable of transporting this complex. The conventional kinesin-1 was identified as an interacting

*Corresponding author. Tel: +82-43-261-2848; Fax: +82-43-272-1603; E-mail: egkim@chungbuk.ac.kr

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partner of βPIX. By using biochemical and imaging analyses, we demonstrated that βPIX binds directly to KIF5 which is a heavy chain subunit of kinesin-1. βPIX interacted with all three isoforms of KIF5 through their tail domains. Thus, kinesin-1 may function as a universal transporter of the βPIX complex in diverse types of cells.

RESULTS

βPIX binds directly to KIF5A

To identify a molecular motor that transports the βPIX complex, we performed affinity purification using an anti-βPIX antibody and mass spectrometry of distinctively silver-stained bands. A band around 120 kD was identified as KIF5A, a neuronal isoform of kinesin heavy chain (KHC) (Fig. 1A, left), which was confirmed by immunoblotting (Fig. 1A, right). To confirm the association between βPIX and KIF5A, a series of co-immunoprecipitation assays were conducted. From the results, it was observed that KIF5A and KLC were co-precipitated with βPIX and GIT1 in the lysates from rat brains (Fig. 1B), cultured hippocampal neuron cells (Supplementary Fig. 1A) and PC12 cells (Supplementary Fig. 1B). However, no immunoprecipitation was noted between βPIX and KIF1A and KIF3A, indicating specificity of binding. PAK2 was detected upon precipitation

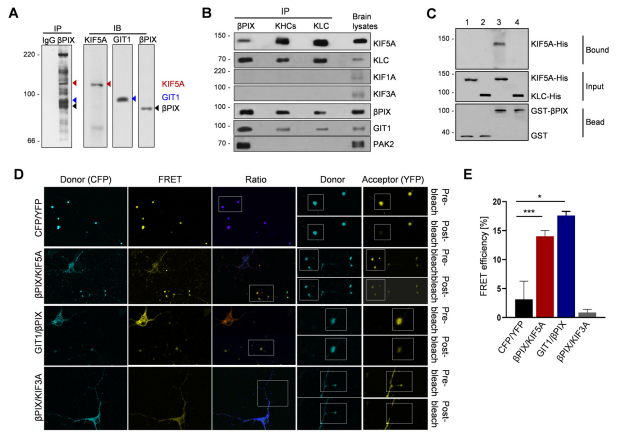

Fig. 1. Association of βPIX with KIF5A in the rat brain. (A) Mass spectrometry. Anti-βPIX immunoprecipitates were silver stained (left). Marked silver-stained bands (arrowheads) were subjected to mass spectrometry followed by immunoblotting (right, three columns). (B) Immunoblotting for the indicated proteins following immunoprecipitation with anti-βPIX, -KIF5A, or -KLC antibody. (C) GST pulldown assay. His-tagged proteins (input and bound), Glutathione-Sepharose immobilized GST-βPIX or GST protein (bead) were immunoblotted by anti-His, or GST antibody. (D) Representative FRET images from primary cultured rat hippocampal neurons co-transfected with plasmids encoding the indicated pairs of proteins. Acceptor (YFP)-bleached areas were indicated by white boxes. Negative control, CFP/YFP and βPIX-CFP/KIF3A-YFP; positive control, GIT1-CFP/βPIX-YFP. (E) Quantification of FRET efficiencies (mean ± SEM.). N = 20 cells/each condition. Student's *t*-test. *P = 0.0124, ***P < 0.0001.

when using anti-βPIX antibody. However, when using anti-KIF5A or anti-KLC antibodies, detection of PAK2 was not possible (Fig. 1B, Supplementary Fig. 1A, B). Further analysis revealed an interaction of αPIX complex with KIF5A. Fusion proteins of green fluorescent protein (GFP)-αPIX or βPIX co-precipitated with KIF5A, but no co-precipitation was noted with GFP alone (Supplementary Fig. 1C). Taken together, these results indicated that both αPIX and βPIX complexes specifically interacted with KIF5A.

Since βPIX makes a tight complex with GIT1, RNAi technology was used to determine whether it was βPIX or GIT1 which was responsible for interacting with KIF5A. When PC12 cells were treated with specific siRNAs for βPIX or GIT1, immunoblotting detected a marked downregulation of their expression (Supplementary Fig. 2). However, no GIT1 was detected in the KIF5A immunoprecipitates from βPIX-depleted cells (Supplementary Fig. 2A, middle), showing that there is no direct association between GIT1 and KIF5A. Conversely, silencing GIT1 did not impact the association of βPIX with KIF5A (Supplementary Fig. 2B, top), confirming the importance of βPIX in binding to KIF5A.

To confirm the interaction between KIF5A and βPIX at molecular level, fluorescence resonance energy transfer (FRET) analysis was conducted in hippocampal cells using the acceptor bleaching method. In FRET measurement, the donor and acceptor were CFP- and YFP-tagged, respectively. If a donor and acceptor protein were spatially arranged in close proximity, the intensity of the acceptor (YFP) fluorescence would increase over time following the photobleaching of the acceptor (YFP). Two pairs, CFP/YFP and βPIX/KIF3A, were used as negative controls as they do not exhibit biochemical interactions (Fig. 1B). No significant FRET was detected from the pair of CFP/YFP (3.13 ± 3.13%) or βPIX-CFP/KIF3A-YFP (0.87 ± 0.53%) (Fig. 1D, 1st and 4th row; quantified in E). GIT1 directly bound to the GIT binding domain (GBD) of βPIX via its Spa2 homology domain (SHD) and coiled-coiled region. Thus, the pair of GIT1-CFP/βPIX-YFP can function as a positive control. This pair showed a higher FRET efficiency (17.6 ± 0.72%) (Fig. 1D, 3rd row; quantified in E). The FRET efficiency for βPIX-CFP/KIF5A was 14.05 ± 1.09% as comparable to the positive pair of GIT1/βPIX (Fig. 1D, 2nd row; quantified in E). Collectively, these results indicated that βPIX indeed interacted with KIF5A in the rat brain and in cultured cells.

Analysis of the binding domains that mediated interaction between KIF5A and βPIX

A mapping analysis was conducted to determine which part of KIF5A/βPIX was responsible for binding. The motor, stalk or tail domains of KIF5A as illustrated in Fig. 2A were expressed in a His-tagged form and incubated with GST-βPIX or GST alone (control). GST-βPIX pulled down the tail region of KIF5A (Fig. 2B, top, lane 6) while GST control showed no specific binding (lane 3). We further determined whether the tail domains from KIF5B and KIF5C interacted with βPIX because

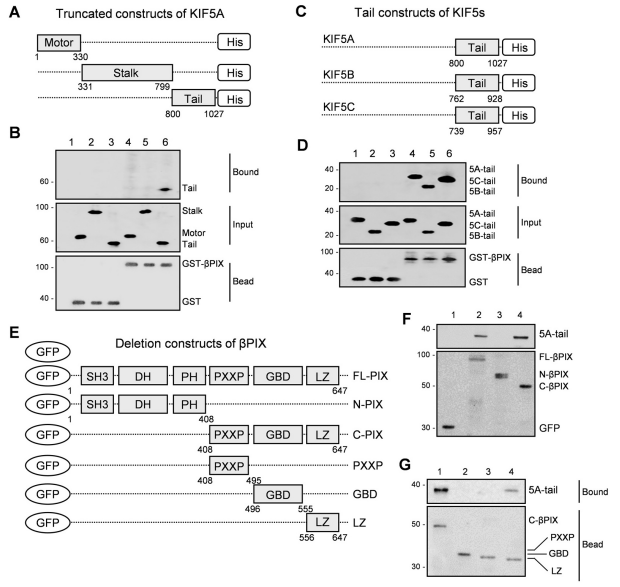

Fig. 2. Direct interaction between the LZ domain of βPIX and the tail domain of KIF5. (A) Schematic diagram of KIF5A constructs. (B, D) GST pulldown. Bound proteins were detected by immunoblotting with anti-His antibody (top). (C, E) Schematic diagram of the constructs for KIF5 tail (C) and βPIX (E). Each domain was expressed as His-tagged (C) or GFP-fused (E) protein. (F, G) Immunoprecipitation and immunoblotting. PC12 cells were transfected with plasmid encoding GFP-tagged βPIX construct (bottom) followed by immunoprecipitation with anti-GFP antibody. Bound KIF5A tail was detected by immunoblotting with anti-His antibody (top).

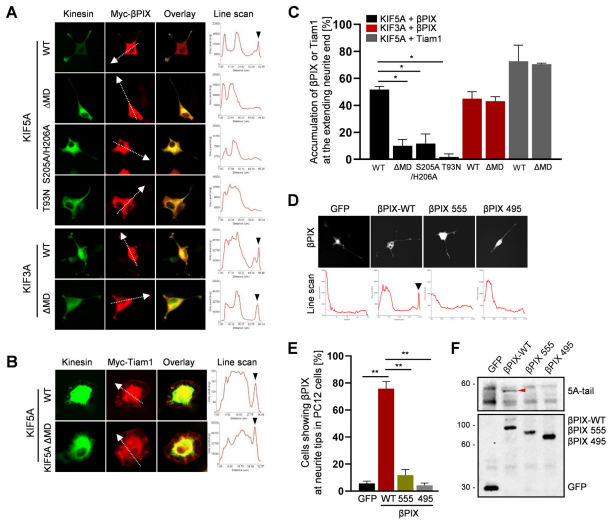

Fig. 3. KIF5A-dependent βPIX targeting to the neurite tip (A, B). Representative images for the localization of βPIX (A) and Tiam1 (B) in differentiated PC12 cells. Kinesin constructs were GFP-tagged, and βPIX and Tiam1 were Myc-tagged. Two days after co-transfected cells were stained with anti-GFP and anti-Myc antibodies. Arrowheads at the line scan indicate neurite tips. Note specific KIF5A-dependent localization of βPIX at the neurite tip. (C) Quantification of the localization of βPIX and Tiam1 in (A, B). Accumulation of βPIX, and Tiam-1 at the neurite tip was quantified by line scan analysis using MetaMorph software. Student's *t*-test. *P < 0.05, N = 30. (D) Representative images for the localization of GFP-tagged βPIX constructs in PC12 cells. (E) Quantification of the localization of βPIX constructs at the neurite tip in (D). Student's *t*-test. **P < 0.01, N = 20. (F) Association of βPIX constructs with KIF5A tail. PC12 cells were transfected with pEGFP-βPIX constructs for 2 days and were lysed and immunoprecipitated with anti-GFP antibody. Immunoprecipitates were incubated with purified His-tagged KIF5A tail followed by immunoblotting with anti-His antibody. Note that only βPIX-WT interacted with KIF5A tail (red arrowhead).

of their high homology. Each tail of the three KIF5 isoforms was expressed as a His-tagged protein (Fig. 2C) and incubated with GST-βPIX. Results showed that all three tail domains bound to βPIX (Fig. 2D, top, lanes 4-6). Conversely, to identify the binding domain in βPIX, various truncated forms of βPIX in GFP-fused proteins were produced (Fig. 2E) and transfected into PC12 cells followed by immunoprecipitation with an anti-GFP antibody. The tail of KIF5A was bound to both full-length βPIX (FL-βPIX) and C-terminal half of βPIX (C-βPIX) but not to the N-terminal half of βPIX (N-βPIX) (Fig. 2F). This result prompted to explore the subdomain of C-βPIX, thus, C-βPIX was fragmented into three parts, PXXP, GBD and LZ domains (Fig. 2E, bottom 3 rows). Incubations of these domains with His-tagged KIF5A revealed LZ domain as the binding partner (Fig. 2G). Taken together, these results indicated that the tail domain of KIF5 and LZ domain of βPIX mediated interaction between KIF5 and βPIX.

KIF5A-dependent targeting of βPIX to the neurite tip and neuronal synapses

To understand the functional significance of interaction between KIF5A and βPIX, we examined the effect of dominant negative mutants of KIF5A on targeting βPIX to the targeted sites. For this purpose, we produced KIF5A mutants without a motor domain (KIF5A ⊿MD), microtubule-binding capacity (S205A/H206A) or ATPase activity (T93N) that function in a dominant negative manner. Endogenous βPIX and KIF5A were abundantly expressed in the cytoplasm and co-localized at the neurite tips in differentiated PC12 cells (Supplementary Fig. 3A, top). Exogenous YFP-tagged βPIX and GFP-tagged KIF5A showed a similar behavior (Supplementary Fig. 3A, bottom). In cells expressing those KIF5A mutants, no significant accumulation of βPIX at the neurite ends was detected, as assessed by line scan (Fig. 3A, 2-4 rows; quantified in C). Their expression also caused some morphological changes in PC12 cells. For instance, retracted neurites with slender tapering ends were observed instead of finger-shaped growth cone-like structures. Unlike KIF5A mutants, expression of a motor less KIF3A did not affect the βPIX targeting the neurite tip. Localization of Tiam1, another Rac1 GEF, was also unaffected by a motor less KIF5A (Fig. 3B; quantified in C). These results

suggested that KIF5A may serve as a specific motor to translocate βPIX to the neurite tip.

The LZ domain of βPIX was responsible for binding to KIF5A (Fig. 2G). Thus, it could be difficult to properly target the neurite ends if βPIX is constructed without LZ domain. To test this suggestion, we employed two truncated mutants of βPIX without the LZ domain, namely βPIX 555 (aa 1-555) and βPIX 495 (aa 1-495) (Fig. 3D). As shown in Fig. 3D, the two mutants demonstrated a significant impairment in their migration to the neurite ends (i.e., detected in less than 11% of cells) (Fig. 3E). In contrast, more than 70% of cells expressing full-length βPIX (WT) marked a clear peak at the targeted location (Fig. 3E). Consistent with this result, co-immunoprecipitation revealed an interaction of KIF5A with only full-length βPIX amongst the βPIX constructs (Fig. 3F). Since βPIX can also target neuronal synapses and can subsequently lead to synaptogenesis, its targeting ability was therefore further examined herein. Endogenous βPIX and KIF5A co-localized in the cell body and small puncta along the dendritic neurites in cultured hippocampal neurons can be seen in Supplementary Fig. 3B, top. Exogenous βPIX and KIF5A also showed a similar behavior, suggesting their synaptic co-localization (Supplementary Fig. 3B, bottom). To examine the targeting of GFP-fused full-length βPIX (WT) and βPIX 555 to synapses, cells were stained for a postsynaptic marker PSD-95. The white merged dots along the dendrites represent co-localization of WT βPIX and PSD-95 (Supplementary Fig. 4A, middle row; quantified in B). In contrast, βPIX 555 and GFP control showed a significant decrease of approximately 50% in co-localization which could be attributed to their simple diffusion to the postsynaptic compartment. These results provided strong evidence for KIF5A as a specific transporter of βPIX.

DISCUSSION

Precise spatiotemporal targeting of the βPIX complex to the specific subcellular locations is essential for its cellular function. This study presented a unified mechanism underlying the transport of the βPIX complex to neurite tips and synapses in neuronal cells. We identified kinesin-1 as a binding partner of the βPIX complex; all KHCs bound the LZ domain of βPIX via their tail domain. Notably, both the tail and LZ domains shared a similar coiled-coil structure that facilitated the formation of protein dimers. Each binding domain thus appeared to play a key role in the formation of a large multi-complex. These conserved binding sites may also help kinesin-1 function as a transporter of the βPIX complex.

Molecular motors such as kinesin and dynein are essential in establishing and maintaining neuronal polarity by transporting axon- or dendrite-specific cargos in a tightly regulated manner (23). Upon suppression of kinesin-1 expression by antisense oligonucleotides, hippocampal cells displayed shorter neurites accompanied by defect in transport of GAP-43 and synapsin I to the neurite tips (24). Considering its catalytic function as a Rac1/Cdc42 GEF, KIF5-dependent targeting of the βPIX complex to growth cone-like structures at the neurite tips was consistent with its role in neurite outgrowth and axonal neuritogenesis. Abnormal dendrites with long, thin dendritic spines are typically observed in patients with mental retardation (MR) (25). Rho GTPases play an important role in actin-based cytoskeletal changes for morphogenesis of dendritic spines. Amongst thirteen genes whose mutations caused nonspecific X-linked MR, three genes encoding αPIX, PAK3 and oligophrenin1 were identified to directly regulate Rho GTPase signaling (26). βPIX in the dendritic spines was reported to interact with Shank, a scaffold protein in the PSD (27), and liprin-α through GIT1 (28). Although αPIX is also located in dendritic spines, its interacting partner is still unknown. Our current findings supported kinesin-1-dependent transport of both αPIX and βPIX. Considering their localization in the dendritic spines and similar role in actin dynamics, it seems intriguing why βPIX does not compensate for the functional loss of αPIX in mentally retarded patients. βPIX-deficient mice died during early embryonic development, but αPIX-deficient mice were alive and appeared normal after birth, suggesting its unknown function that cannot be compensated by βPIX. This warrants future study to elucidate their distinct functions, in particular in the dendritic spines.

The present study has several limitations. Firstly, although αPIX and βPIX bind KIF5A (Supplementary Fig. 1C) and their subcellular targeting would be kinesin-1 dependent, the present study focused on βPIX. It remains to be determined whether αPIX can show a similar targeting behavior like βPIX. Secondly, it is uncertain how the PIX complex's kinesin-1-dependent mobility is controlled, for example, how to start or stop its movement. PAK2 was identified as a component of βPIX immunoprecipitates (Supplementary Fig. 1A), suggesting involvement of PAK2-mediated phosphorylation in the regulatory event. However, this kinase was not detected in KIF5A and KLC immunoprecipitates (Supplementary Fig. 1A), contradicting the former idea.

In conclusion, the present study defined kinesin-1 as a universal transporter of the βPIX complex based on the diverse binding and functional analysis. βPIX complex is known to regulate a number of key cellular activities in neuronal cells. Understanding its transport mode in association with kinesin-1 may provide novel insight into the pathophysiological function of the βPIX complex in neurons.

MATERIALS AND METHODS

Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (Invitrogen) and were kept at 37°C in a humidified 5% CO_2 incubator. Hippocampus from the brain of newborn (P1) Sprague-Dawley (SD) rat were dissected and incubated with papain in dissociation medium

(pH 7.4) (82 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.252 mM $CaCl_2$, 1 mM HEPES, 20 mM glucose, 0.5% phenol red) containing L-cystein for 20 min at 37°C and dissociated by pipetting in DMEM containing 10% FBS.

Mass spectrometry

Rat brain lysate was immunoprecipitated with anti-βPIX antibody immobilized onto CNBr-activated sepharose 4B. Immunoprecipitates were resuspended in SDS-PAGE sample buffer and were separated by SDS-PAGE. The gel was silver stained by using a kit according to the manufacturer's protocol (Bio-Rad, CA, USA). Specific bands excised from silver stained gel were subjected to mass spectrometry by a company (Genomine, Kyungbuk, Korea).

FRET microscopy

Cultured PC12 cells were co-transfected with a pair of plasmids for FRET analysis using LF2000, incubated for 36-48 h and fixed in phosphate-buffered saline (PBS) containing 3.7% paraformaldehyde for 10 min at RT. After washing with PBS, cells were mounted onto a glass slide. FRET measurement was performed on the Leica TCS SP2 confocal microscope according to the FRET acceptor photobleaching protocol (Leica, Wetzlar, Germany). The HCX PL APO 63× objective was used and excitation was provided by 20 mW multimode argon in laser lines. CFP was detected at 454 nm and YFP was detected and photobleached at 514 nm. Laser intensity was set to 20% for YFP detection but 100% for bleaching. The gain of the photomultiplier detectors was adjusted to obtain the optimal dynamic range. The CFP fluorescence was measured before and after YFP bleaching and FRET efficiency was calculated according to the equation; FRET efficiency [%] = $(CFP_{after} - CFP_{before}) \times 100/CFP_{after}$.

In vitro binding assay

pGEX4T-1-βPIX and pET-FL-KIF5 constructs were expressed in *E. coli* DH5α or BL21. Purified GST-FL-βPIX were immobilized onto glutathione-Sepharose 4 beads. GST-βPIX-immobilized beads were incubated with His-tagged FL-KIF5A, motor (aa 1-330), stalk (aa 331-799), or tail (aa 800-1027) in binding buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM DTT, 0.2% Triton X-100, 5 μM $MgSO_4$, and protease inhibitor) for 1 h, and then subjected to immunoblotting with antibodies to His and GST. In some experiments, GST-βPIX-immobilized beads were incubated with lysates from PC12 cells transfected with various βPIX constructs. Proteins bound to beads were subjected to SDS-PAGE and immunoblotted with the corresponding antibodies.

Immunoprecipitation and immunoblotting

Cells were lysed on ice with ice-cold 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). Lysates were immunoprecipitated with a primary antibody for 2 h followed by incubation with protein G Sepharose for 5 h. Immunoprecipitates were separated by SDS-PAGE, and transferred to PVDF membrane (Millipore, MA, USA) for 2 h. Membranes were blocked with 4% skim milk, incubated with a primary antibody for 1 h, and were blotted with secondary antibodies (PIERCE, IL, USA). Immunoblots were developed using enhanced chemiluminescence solution.

Statistical analysis

All experimental data were expressed as means ± SEM. Statistical significance was assessed using an unpaired *t*-test (*t*-test) using GraphPad Prizm software (version 10) for Windows. $P < 0.05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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