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Neuronal Specific βPix-b Stimulates Actin-Dependent Processes Via the Interaction Between its PRD and WH1 Domain of N-WASP

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βPix, a Pak-interacting nucleotide exchange factor (Cool-1/p85SPR), is a Cdc42/Rac1-specific guanine nucleotide exchange factor (GEF) involved in various actin-related processes. Many previous studies have focused on ubiquitously expressed βPix-a, while the role of the neuronal-specific isoform βPix-b is still unknown, especially whether its role is distinct from or similar to βPix-a. Here we show that unlike βPix-a, overexpression of βPix-b stimulates actin-dependent comet formation in BHK21 cells. This effect is attributed to the interaction between its proline-rich domain (PRD) and the WH1 domain of N-WASP. In addition, we show that overexpression of βPix-b stimulates actin-dependent dendritic spine formation in rat hippocampal neurons in culture, a formation that is blocked by co-expression of the WH1 domain of N-WASP or the PRD of βPix-b. Knocking-down endogenous expression of βPix-b by shRNA reduced the number of dendritic spines, which were rescued only by PRD-containing βPix-b mutants. GEF activity of βPix-b is also required for these effects. The results show that neuronal-specific βPix-b stimulates actin-dependent processes in cells via the interaction between its PRD and the WH1 domain of N-WASP. Our results identify N-WASP as the first protein shown to interact with the PRD of βPix-b, raising the possibility that, as an N-WASP WH1-binding protein, βPix-b may regulate N-WASP's activity in cells.

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βPix, a Pak-interacting nucleotide exchange factor (Cool-1/p85SPR), was identified as a protein localized in focal adhesions (Oh et al., 1997), and was later demonstrated to act as a guanine nucleotide exchange factor (GEF) for Rac1/Cdc42 GTPases. It comprises a N-terminal Src homology 3 (SH3) domain followed by a Dbl homology (DH) domain, a Pleckstrin homology (PH) domain, a proline-rich domain (PRD), a GIT1-binding domain (GBD), and a leucine zipper (LZ) domain (Manser et al., 1998; Premont et al., 2000; Zhao et al., 2000; Kim et al., 2001).

βPix drives the formation of membrane ruffles and filopodia via Rac1 and Cdc42, respectively (Koh et al., 2001). In addition, βPix acts as an important regulator of the subcellular distribution of GIT1, leading to cell motility alteration and neurite extension (Za et al., 2006). It is also associated with signaling proteins, such as CAMKK and CaMKI, as well as PDZ-containing proteins, such as SHANK and Preso, promoting synapse formation and dendritic morphogenesis during neuronal development (Park et al., 2003; Lee et al., 2008; Saneyoshi et al., 2008).

Various βPix isoforms (βPix-a, -b, -c, -d, and -bL) have been reported (Oh et al., 1997; Kim et al., 2000; Rhee et al., 2004). Among them, βPix-a is ubiquitously expressed in most tissues. βPix-b and βPix-c contain a novel insert (INS) region and βPix-b is a specific and a major isoform expressed in the nervous system (Supplementary Fig. 1). βPix-d lacks an LZ domain but has an 11 amino acid addition at the carboxyl terminus. βPix-bL is generated by an alternative translation of βPix-b mRNA.

N-WASP is a critical regulator of Arp2/3-mediated actin polymerization and is involved in various actin related cellular processes such as actin tail formation and dendritic spine formation in neurons (Miki and Takenawa, 2003). Actin tails are implicated in a variety of movements within living cells such as intracellular motility of endocytic vesicles and other membrane-bound organelles, movements that are mediated by Cdc42/Rac1 GTPases. The formation of actin tails induced by *Shigella* and *Vaccina* virus involves the N-WASP-Arp2/3 complex, which is used to trigger actin nucleation and polymerization on the surface of virus membrane (Moreau

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et al., 2000; Suzuki et al., 2000). Overexpression of type I phosphatidylinositol-4-phosphate 5-kinase, which synthesizes PtdIns(4,5)P_2, also promotes N-WASP-Arp2/3-dependent actin polymerization from membrane-bound vesicles to form actin comets (Rozelle et al., 2000). Dendritic spines in neurons have an extensive actin cytoskeleton, and thus, proteins known to alter the arrangement of the actin cytoskeleton in dendritic spines, along with the mediators of those proteins, have been the primary targets of studies focusing on spine changes (Tada and Sheng, 2006; Hotulainen and Hoogenraad, 2010). As a downstream converging point in the actin-regulating pathway, the N-WASP-Arp2/3 complex plays a critical role in dendritic spine formation. For example, EphB receptors regulate dendritic spine formation via an N-WASP-dependent pathway (Irie and Yamaguchi, 2002). SPIN90/WISH also regulates dendritic spine and synapse formation via an N-WASP-Arp2/3 complex dependent mechanism (Lee et al., 2006). A recent series of deletion and knock-down studies in cultured neurons revealed a molecular mechanism by which N-WASP and the Arp2/3 complex regulate the development of dendritic spine and synapses (Wegner et al., 2008).

Although the roles of βPix-a in the regulation of actin dynamics have been extensively studied, the function of the neuronal-specific isoform βPix-b is still unknown, especially whether it has a function that is distinct from or similar to βPix-a. Here, we show that unlike βPix-a, overexpression of βPix-b stimulates actin-dependent processes such as actin tail formation in BHK21 cells and dendritic spine formation in neurons. Those results are attributed to the interaction of βPix-b with N-WASP as we show that the PRD of βPix-b interacts with the N-WASP WH1 domain. N-WASP is the first protein to be shown to interact with the PRD of βPix-b. Based on the results, we hypothesize that βPix-b, acting as a GEF, activates Cdc42, while through its PRD it binds N-WASP, which makes Cdc42 activate N-WASP to induce actin-dependent processes. Since N-WASP WH1-domain binding proteins are known to recruit and regulate the activity of N-WASP, our results also raise the possibility that βPix-b may be involved in the regulation of N-WASP's activity in cells.

Materials and Methods

Ethics statement

Animal experimental procedures were approved by the Institute of Animal Care and Use Committee of Seoul National University, Korea (Approval ID number: SNU-100930-5).

Plasmid construction, antibody, and reagent

DNA corresponding to the regions of mouse βPix-b was amplified by polymerase chain reaction and subcloned into indicated expression vectors. N-WASP was also amplified by PCR and subcloned into pEGFP, HA, mCherry and pET-23b vectors and its mutants used in this study corresponding to the residues of rat N-WASP. GFP-Actin, mRFP-Actin and pcDNA-HA-PIP5KIβ were kindly provided by Gilbert Di Paolo (Columbia University, New York, NY). The fidelity of all constructs was verified by sequencing. The following antibodies were used: Anti-βPix (Chemicon, Temecula, CA), anti-N-WASP (Cell Signaling Technology, Boston, MA), anti-GFP (Abcam, Cambridge, UK), and anti-HA (Covance, Princeton, NJ). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa 405 conjugated goat anti-mouse secondary antibody, Texas Red-X phalloidin, and Oregon Green 488 were from Molecular Probes (Eugene, OR) and all other reagents were from Sigma, St. Louis, MO.

Cell culture and transfection

BHK21 cells (Korean Cell Line Bank, Seoul, South Korea) and HEK293T cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Cells were co-transfected with HA-PIP5KIβ and various βPix-b or N-WASP mutants for 18–24 h using Lipofectamine 2000 reagent (Invitrogen). Primary rat hippocampal neurons were prepared as described (Chang and De Camilli, 2001). Briefly, hippocampi were dissected from embryonic day 18 Spraque–Dawley fetal rats, dissociated with papain, and triturated with a polished half-pore Pasteur pipette. Cells (2.5×10^5) in minimum Eagle's medium supplemented with 0.6% glucose, 1 mM pyruvate, 2 mM L-glutamine, 10% fetal bovine serum, and antibiotics were plated on poly-D-lysine coated glass coverslips in a 60 mm Petri dish. Four hours after plating, the medium was replaced with Neurobasal (Invitrogen) supplemented with 2% B-27 and 0.5 mM L-glutamine. Transfection of neurons was carried out using calcium phosphate method.

Microscopy and actin tail quantification

Live cells were observed with a sealed chamber containing oxygen-depleted (Oxyrase, Mansfield, OH) Tyrode solution (119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, pH 7.4 and 30 mM glucose) mounted on an Olympus IX-71 inverted microscope equipped with a 60× oil-immersion objective (1.35 NA) (Olympus, Tokyo, Japan) and a CoolSNAP-Hq CCD camera (Roper Scientific, Tucson, AZ) driven by MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA). Time-lapse sequences were constructed by image acquisition with 3 sec intervals. Dual-color simultaneous imaging was performed using Dual-View (Photometrics, Tucson, AZ) with a GFP-mRFP optimized dual filter set (Chroma, Rockingham, VT). Confocal images were acquired on an Olympus FV-1000 confocal microscope with 60×, 1.35 NA oil-immersion lens driven by FluoView 1000. Cells were excited with 488 nm (from an argon laser) and 559 nm light (from diode laser). For actin tail quantification, 16–24 h after transfection, BHK21 cells were fixed in 4% paraformaldehyde, 4% sucrose, PBS for 15 min, permeabilized for 5 min in 0.25% Triton X-100, PBS and blocked for 30 min in 10% BSA, PBS at 37°C. The cells were incubated with Texas Red-X phalloidin (Molecular Probes), 3% BSA, PBS for 45 min at 37°C. Quantification of the number of the cells containing actin tail(s) was assessed in at least three independent experiments in which at least 200 cells were counted. The percent difference was calculated as the difference between two values divided by the average of the two values, which is shown as a percentage. Statistical analyses were performed using SigmaStat (Systat Software, Point Richmond, CA).

GST-fusion protein purification and in vitro binding assay

For GST fusion protein purification, we were transformed into *Escherichia coli* BL-21, and the transformants were cultured in 2XYT medium supplemented with ampicillin. After overnight induction with 0.5 mM IPTG at 25°C, the cultures were sonicated in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF) and centrifuged at 15,000*g* for 15 min, and the supernatants were incubated with glutathione-agarose-4B beads (Amersham Biosciences, Uppsala, Sweden) at 4°C for 1 h. After washing three times with lysis buffer, the beads were subjected to SDS–PAGE and coomassie staining for quantification. To assess in vitro binding, the N-WASP-6xHis plasmid was transformed into *E. coli* BL-21, and the transformants were cultured in 2XYT medium supplemented with ampicillin. After overnight induction with 0.5 mM IPTG at 25°C, the cultures were sonicated in NTA-lysis buffer (1% Triton X-100, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM Imidazole, 1 mM PMSF) and centrifuged at 15,000*g* for 15 min, and the supernatants

were incubated with Ni-NTA Chelating Agarose CL-6B (Peptron, Inc., Daejeon, Korea) at 4°C for 1 h. After washing three times with NTA-washing buffer (0.1% Triton X-100, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM Imidazole, 1 mM PMSF), the beads were incubated at 4°C for 2 h with NTA-elution buffer (0.1% Triton X-100, 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 300 mM Imidazole). The eluted product was quantified by SDS–PAGE and subsequently used for in vitro binding experiment. The N-WASP-6xHis was incubated at 4°C for 2 h with purified GST or GST fusion proteins bound to glutathione beads in lysis buffer. The beads were then washed extensively and analyzed by SDS–PAGE and immunoblotting.

Co-immunoprecipitation and immunoblotting

To detect βPix-b binding to N-WASP in vivo, HEK293T cells were transfected using Liopofectamine 2000 (Invitrogen). The cells were washed twice with cold PBS and extracted at 4°C for 1 h in 1% Tx-100 buffer (10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin, and 1 mM aprotinin). They were then clarified by centrifugation at 15,000*g* for 15 min, and protein concentrations were determined with a Bradford protein assay reagent kit (Bio-Rad, Hercules, CA). Samples containing 1 mg of total protein were immunoprecipitated for 4 h with anti-GFP antibody, followed by an additional 2 h of incubation at 4°C with protein A-Sepharose beads (Amersham Biosciences). The beads were extensively washed with lysis buffer and subjected to SDS–PAGE and transferred to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% skim milk TBST for 1 h, washed, and probed with primary antibody for 2 h at room temperature. After extensive washing in TBST, the membrane was incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Proteins were visualized with ECL reagent (Amersham Biosciences).

In-gel digestion and peptide sample preparation

The SDS–polyacrylamide gels were silver-stained, and protein bands were excised. The resulting samples were washed three times with a 1:1 (v/v) solution of acetonitrile and deionized water for 10 min, dehydrated with 100% acetonitrile, washed with a 1:1 (v/v) solution of 100% acetonitrile and 100 mM ammonium bicarbonate, and dried using a SpeedVac. Then they were reduced with 10 mM tris(2-carboxyethyl)phosphine hydrochloride in 0.1 M ammonium bicarbonate at 56°C for 45 min and alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. The above washing step was repeated on the alkylated samples, which were dried, soaked in sequencing-grade trypsin solution (500 ng) on ice for 45 min, and immersed in 100 ml of 50 mM ammonium bicarbonate, pH 8.0, at 37°C for 14–18 h. The resulting peptides were extracted sequentially by agitation for 20 min with 45% acetonitrile in 20 mM ammonium bicarbonate, 45% acetonitrile in 0.5% trifluoroacetic acid, and 75% acetonitrile in 0.25% trifluoroacetic acid. The extracts containing tryptic peptides were pooled and evaporated under vacuum.

Micro-LC-MS/MS analysis and protein data base search

In gel digested proteins were loaded onto fused silica capillary columns (100 μm inner diameter, 360 μm outer diameter) containing 8 cm of 5 μm particle size Aqua C18 reverse-phase column material. The columns were placed in line with an Agilent HP 1100 quaternary LC pump, and a splitter system was used to achieve a flow rate of 250 nl/min. Buffer A (5% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 0.1% formic acid) were used to make a 90 min gradient. The gradient profile started with 5 min of 100% buffer A, followed by a 60 min gradient from 0% to 55% buffer B, a 25 min gradient from 55% to 100% buffer B, and a 5 min gradient of 100% buffer B. Eluted peptides were directly electrosprayed into anLTQlinear ion trap mass spect rometer (Thermo Finnigan, Palo Alto, CA) by applying 2.3 kV of DC voltage. Data-dependent scans consisting of one full MS scan (400–1,400 m/z) and five data-dependent MS/MS scans were used to generate MS/MS spectra of the eluted peptides. Normalized collision energy of 35% was used throughout data acquisition. MS/MS spectra were searched against an NCBI rat protein sequence data base using Bioworks version 3.1 and Sequest Cluster System (14 nodes). DTASelect was used to filter the search results, and the following Xcorr values were applied to the different charge states of peptides: 1.8 for singly charged peptides, 2.2 for doubly charged peptides, and 3.2 for triply charged peptides. Fragment ions in each MS/MS spectrum were manually assigned to confirm the data base search results.

RNA interference and rescue experiments

Small hairpin RNA (shRNA)s for βPix-b RNA interference were designed on the basis of mouse βPix-b cDNA sequence (Gene bank accession number NM_001113518), targeting to the region of nucleotides 719–737 (shRNA#1) and 2058–2076 (shRNA#2). These sequences are common to rat βPix-b cDNA sequence. Complementary oligonucleotides were synthesized separately with additions of a *Bam*HI site at the 5′ end and a *Eco*RI site at the 3′ end. The forward primer sequences were 5′-GATTCG-AGGAGCTGGAGAGACACATTTCAAGAGA-ATGTGTCTCTCCAGCTCCTCTTTTTT-3′ (shRNA #1) and 5′-GATTCGGCTGGTGAGGAAGGTTCTATTCAAGAGA-TAGAACCTTCCTCACCAGCCTTTTTT-3′ (shRNA #2); underlined letters represent the βPix shRNA sequence. The annealed cDNA fragment with βPix shRNA was cloned into the *Bam*HI–*Eco*RI sites of the pSIREN-DNR-DsRed-Express vector (Invivogen, San Diego, CA). The efficiency of shRNAs was tested in GFP-βPix-b expressed-HEK293T cells. For evading RNA interference, silent mutations within shRNA#1 targeting sequence (C724T, G726A, G729A, A730C, and A732C) in GFP-βPix-b were generated using QuickChange® Site-Directed Mutagenesis Kit (Stratagene, Austin, Texas). The fidelity of all constructs was verified by sequencing.

Results and Discussion

Overexpression of βPix-b stimulates PIP5KIβ-induced actin comet formation

Actin comets play a key role in the motility of endogenous cell organelles (Merrifield et al., 1999; Taunton et al., 2000). In fibroblasts, actin-based vesicle propelling can be visualized upon overexpression of phosphatidylinositol-4-phosphate 5-kinase (PIP5K), which generates PtdIns(4,5)P_2, or overexpression of constitutively active ADP-ribosylation factor 6 (Rozelle et al., 2000). Evidently, co-overexpression of a HA-tagged β isoform of type I PIP5K (PIP5KIβ) with GFP-actin in BHK21 cells results in the formation of PIP5KIβ-induced actin comets, which reflects the rapid rocketing motility of intracellular organelles (data not shown). When BHK21 cells are triply-transfected with GFP-βPix-b, mRFP-actin, and HA-PIP5KIβ, the GFP signal coincides spatio-temporally with the mRFP signal in the moving comet structures, indicating that they are indeed actin comets (Fig. 1A). We also show that overexpression of βPix-b increases the number of cells with actin tails by ~20% (Fig. 1C,D and Supplementary Table 1). Although BHK21 cells endogenously express the βPix-a isoform (Supplementary Fig. 1), the introduction of additional βPix-a with PIP5KIβ failed to stimulate the formation of actin tails (Fig. 1C,D), indicating that the role of βPix-b is distinct from that of βPix-a.

We also tested which domain of βPix-b is responsible for the stimulatory effect on actin tail formation. βPix interacts with Pak, one of the key players in actin regulation, via the βPix SH3 domain. However, the SH3 domain-deleted mutant of βPix-b (ΔSH3-b) was still able to stimulate PIP5KIβ-induced actin tail formation (Fig. 1C,D). The catalytic activity of βPix-b as a GEF is

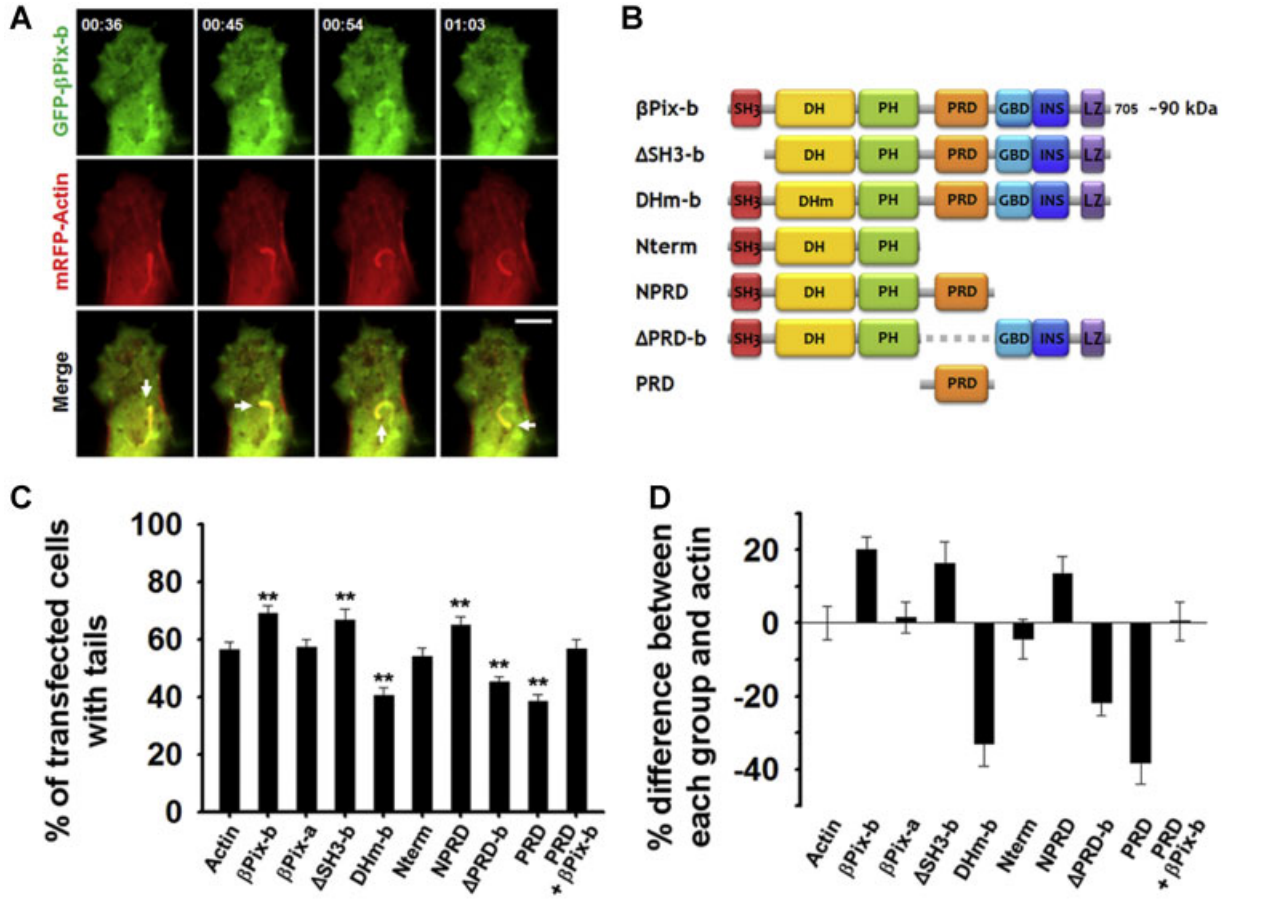
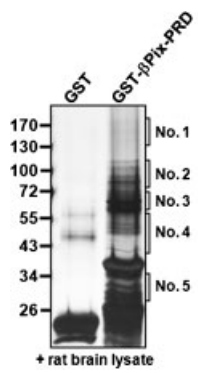


Fig. 1. βPix-b stimulates actin-dependent comet formation. A: A series of images were taken from a time-lapse movie of BHK21 cells triply transfected with GFP-βPix-b, mRFP-actin, and HA-PIP5KIβ. Arrows point to the comet head in each frame to follow the movement of comet-like structures. Scale bar, 5 μm. B: Schematic diagrams of full length βPix-b and various deletion mutants used in the experiments. C,D: Effect of βPix-b or various deletion mutants on actin tail formation. The percentage of transfected cells with actin tail(s) from three independent experiments is shown in (C). $^{**}P < 0.001$ compared with Actin (one-way ANOVA). D: The values in (C) were re-calculated to show the percent difference between each group and actin. Values are means ± standard deviations from at least three independent experiments in which >200 transfected cells were examined for each condition. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

required since the catalytic dead mutants (DHm-b, L238R/L239S) of βPix-b significantly inhibited the formation of actin tails (Fig. 1C,D). Expression of a C-terminal deletion mutant (Nterm) failed to increase actin tail formation while including PRD in Nterm (NPRD) did stimulate such formation, indicating that protein interaction via the PRD is important for the stimulatory effect of overexpressed βPix-b. Accordingly, co-overexpression of PRD with βPix-b negated the stimulatory effect of βPix-b (Fig. 1C,D). Overexpression of the PRD-deletion mutant (ΔPRD-b) or of the PRD itself of βPix-b also inhibited PIP5KIβ-induced actin tail formation.

The PRD of βPix-b interacts with the WH1 domain of N-WASP

Since the PRD of βPix-b appears to be an important domain for its stimulatory effect on actin tail formation, we searched for proteins that interact with βPix-b via the PRD using mass spectrometry. Among the proteins searched, N-WASP was pulled down by the PRD of βPix-b (Fig. 2). N-WASP or the hematopoietic WASP is reported to be essential for PIP5K-induced actin comet formation (Moreau et al., 2000). Here, we show that the WH1 domain of N-WASP can directly bind to the



	βPix-PRD binding partner cadidates
No. 1	Spectrin αII (fragment)
	Drebrin
No. 2	N-ethylmaleimide sensitive fusion protein
No. 3	N-WASP
No. 4	Tubulin α/β
	Protein phosphatase 2a
	Microtubule-associated protein 1A
No. 5	Syntaxin 1B
	Protein phosphatase 1γ

Fig. 2. Screening for putative βPix-PRD interacting partners. Rat brain lysates were incubated with GST and GST-βPix-PRD, and SDS–polyacrylamide gels were stained with silver staining. Specific bands were excised from the stained gel, analyzed by micro-LC-MS/MS, and identified by a protein database search. Candidate proteins are shown in the table.

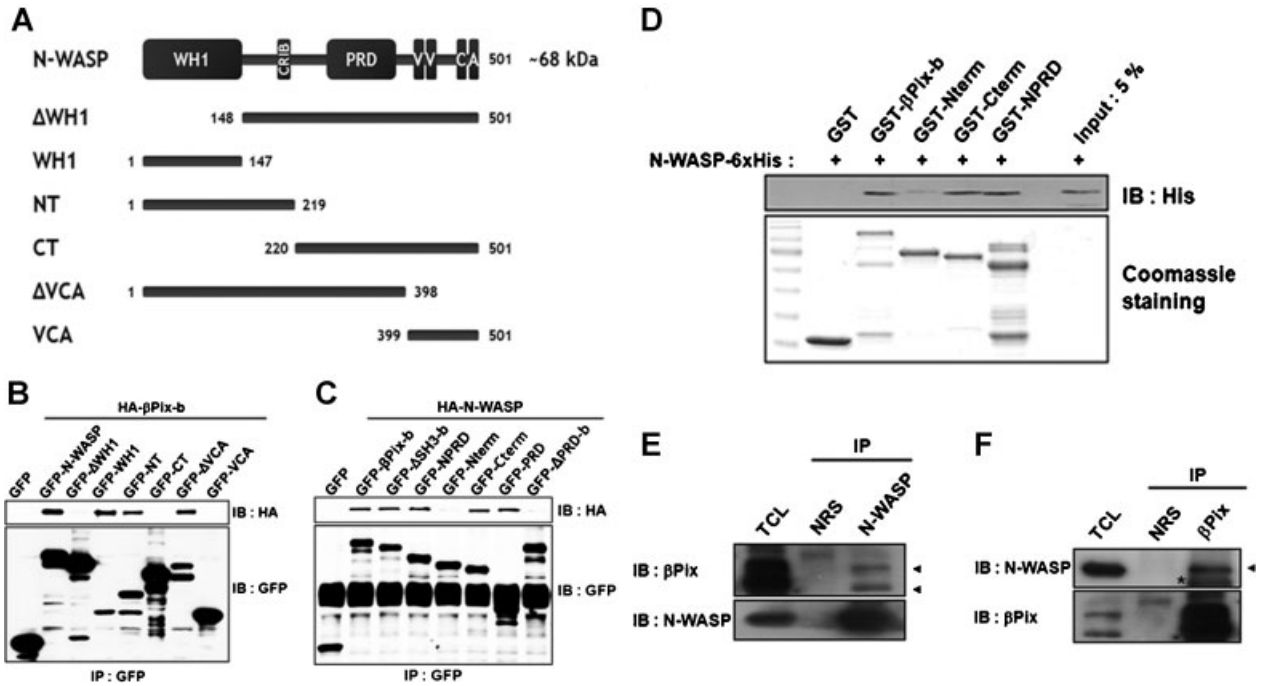


Fig. 3. βPix-b directly interacts with N-WASP via its PRD and WH1 domain of N-WASP. A: Schematic representation of full length N-WASP and various deletion mutants. WH1, WASP homology domain 1; CRIB, Cdc42/Rac1 interactive binding domain; PRD, proline rich domain; V, verprolin homology; C, cofilin homology; A, acidic region. B,C: βPix-b interacts with N-WASP in vivo. HEK293T cells were co-transfected with GFP-N-WASP, or its deletion mutants and HA-βPix-b (B), or with GFP-βPix-b or its deletion mutants and HA-N-WASP (C). Twenty-four hours after transfection, the cells were lysed and immunoprecipitated with anti-GFP antibody and immunoblotted with anti-HA antibody. IP, immunoprecipitaion; IB, immunoblot. D: In vitro binding assay was carried out with purified N-WASP-6xHis and GST-βPix-b, Nterm, Cterm, NPRD or GST alone, followed by immunoblotting with anti-His antibody. Input (5%): N-WASP-6xHis was used as a positive control. E,F: βPix-b endogenously interacts with N-WASP. Rat brain lysates were incubated with anti-βPix or anti-N-WASP antibody for immunoprecipitation, and immunoblotted with counter antibodies. Arrowheads in (E) indicate βPix-b (upper) and βPix-d (lower). Arrowhead and asterisk in (F) indicate N-WASP and antibody heavy chain, respectively. TCL. total cell lysate; NRS, normal rabbit serum.

PRD of βPix-b. When HEK293T cells were co-transfected with HA-βPix-b and GFP-N-WASP full-length or various deletion mutants, only those mutants containing the WH1 domain or full-length N-WASP interacted with βPix-b (Fig. 3B). When HEK293T cells were co-transfected with HA-N-WASP with GFP-βPix-b full length or deletion mutants, only PRD-containing mutants or full-length βPix-b interacted with N-WASP (Fig. 3C). The SH3 domain of βPix-b was not involved in N-WASP binding (Fig. 3C). In vitro protein binding assay results also showed that N-WASP binds to either βPix-b full-length or its PRD-containing mutants (Fig. 3D). In vivo immunoprecipitation assays confirmed that βPix-b binds to N-WASP and vice versa (Fig. 3E,F). Confocal imaging data provided further support for a physiological interaction of βPix-b with N-WASP in membrane ruffles (Supplemental Fig. 2).

Since the PRD of βPix-b is responsible for its stimulatory effect on actin comet formation, and N-WASP was found to be the binding partner of its PRD, we tested whether an interaction between βPix-b and N-WASP is required for the stimulatory effect of βPix-b on actin comet formation. First, consistent with a previous report (Moreau et al., 2000), expression of N-WASP with PIP5KIβ stimulates the formation of actin tails (Fig. 4). This is attributed to N-WASP WH1 domain-mediated binding to WIP since actin tail formation was blocked by N-WASP W54A mutant that cannot interact with WIP or by overexpression of WH1 domain (Fig. 4A,B and Supplementary Table 2). Next, when we overexpressed βPix-b together with N-WASP and PIP5KIβ, there was a small but significant increase in the formation of actin comets compared to that following expression of N-WASP with PIP5KIβ (Fig. 4A,B and Supplementary Table 2). This increase is not attributed to the N-WASP-WIP interaction because the W54A mutant of N-WASP, which binds to βPix-b but not to WIP, was still able to stimulate actin tail formation (Fig. 4 and Supplementary Fig. 3). Co-expression of the WH1 domain of N-WASP with βPix-b inhibits actin tail formation, indicating that the stimulatory effect of βPix-b on actin tail formation is mediated by its binding to the WH1 domain of N-WASP (Fig. 4A,B and Supplementary Table 2).

Overexpression of βPix-b promotes dendritic spine formation through an interaction between its PRD and, possibly, the N-WASP WH1 domain

Since βPix-b is a specific isoform expressed in neurons, we overexpressed βPix-b in cultured hippocampal neurons and tested its effect on dendritic spine formation, a well-known actin-dependent process. Overexpressed βPix-b and N-WASP were co-localized at spines in cultured hippocampal neurons (Fig. 5A). When βPix-b alone was overexpressed in neurons, it increased dendritic spine formation by ~30% compared to that in controls. However, co-expression of βPix-b with the WH1 domain of N-WASP or the PRD of βPix-b failed to do so, indicating that the interaction between the βPix-b PRD and the N-WASP WH1 domain is responsible for the stimulatory effect of βPix-b on dendritic spine formation (Fig. 5B,C). Consistent with the report (Wegner et al., 2008), overexpression of N-WASP increased dendritic spine formation (Fig. 5). The

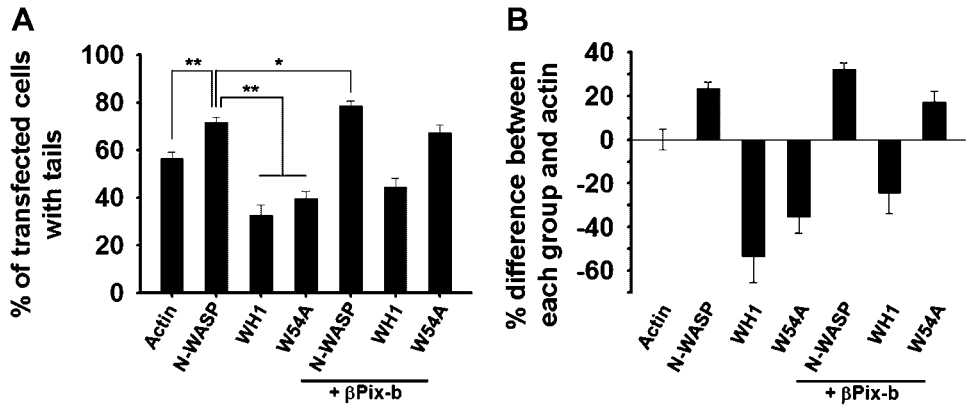


Fig. 4. Stimulatory effect of βPix-b on actin tail formation is mediated by its interaction with WH1 domain of N-WASP. A: The percentage of transfected cells with actin tail(s) from three independent experiments is shown. *$P < 0.01$; **$P < 0.001$ compared with Actin or N-WASP (one-way ANOVA). B: The values in (A) were re-calculated to show the percent difference between each group and actin. Values are means ± standard deviations from at least three independent experiments in which >200 transfected cells were examined for each condition.

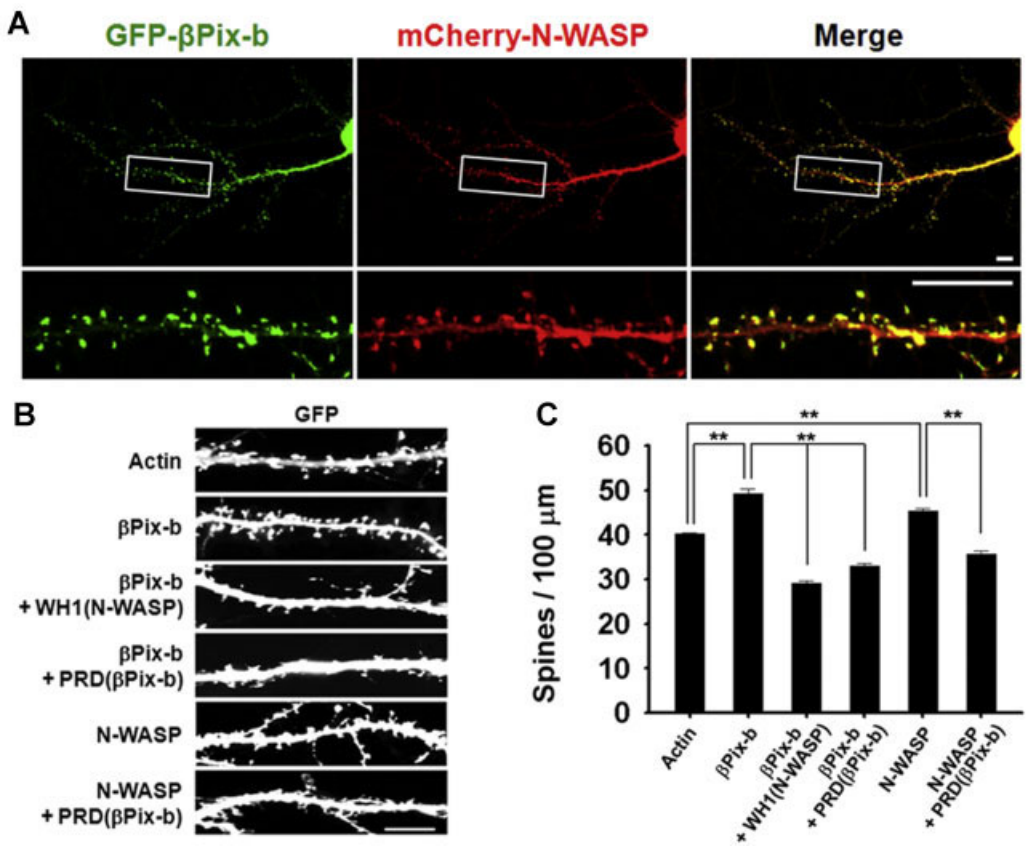


Fig. 5. βPix-b promotes dendritic spines formation through its interaction with N-WASP and the defects by βPix-b shRNA can be rescued by its PRD as well as GEF activity containing mutants. A: Cultured hippocampal neurons were co-transfected at DIV 13 with GFP-βPix-b and mCherry-N-WASP, and fixed at DIV 15. Scale bar, 10 μm. GFP-βPix-b and mCherry-N-WASP are colocalized at dendritic spines. B: Hippocampal neurons were transfected at DIV 13 with GFP-Actin, βPix-b, N-WASP, or co-transfected with GFP-βPix-b and mCherry-N-WASP, mCherry-WH1 (N-WASP), or mCherry-PRD (βPix-b), or GFP-N-WASP and PRD (βPix-b), and fixed at DIV 15. Scale bar, 10 μm. C: Quantification of the effect of βPix-b on the dendritic spines formation. Data were collected from 22 to 34 dendrites of 16–24 neurons for each group in the three independent experiments. **$P < 0.001$ (one-way ANOVA). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

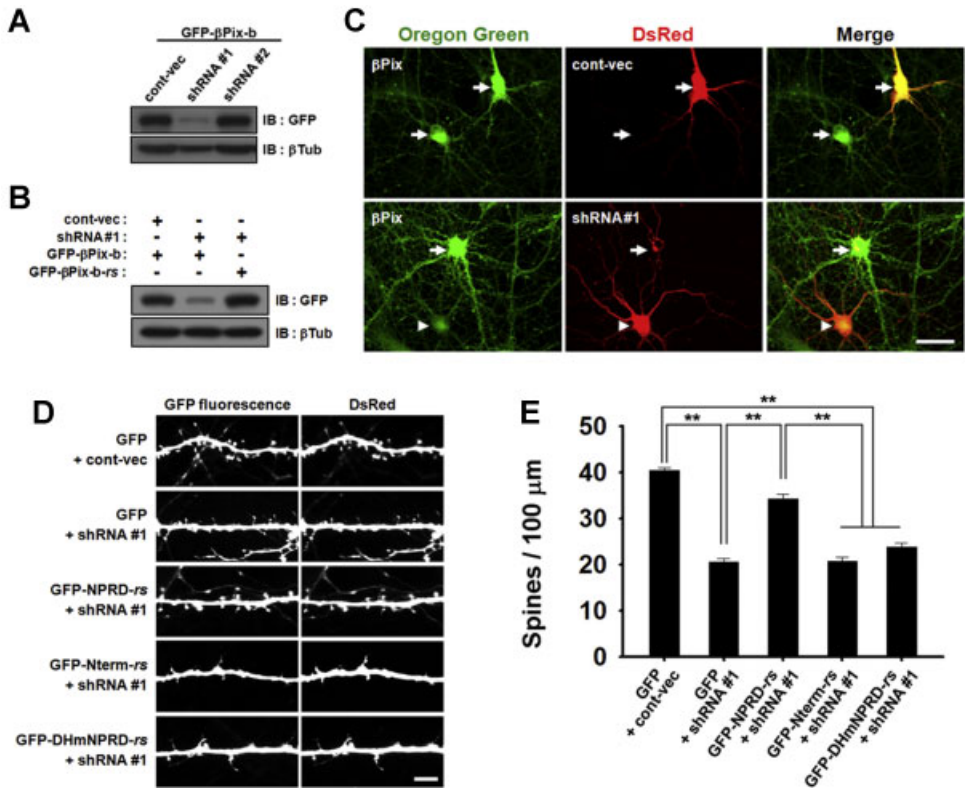


Fig. 6. Knocking-down endogenous expression of βPix-b by shRNA reduced the number of dendritic spines, which can be rescued only by PRD containing βPix-b mutants. A: HEK293T cells were co-transfected with GFP-βPix-b and shRNA#1, #2, or control vector (cont-vec), respectively. The shRNA#1, but not shRNA#2, successfully reduced the endogenous expression levels of GFP-βPix-b. B: HEK293T cells were co-transfected with various combination of constructs indicated (+). *rs* indicates silent mutant which is resistant to shRNA#1. While the expression of GFP-βPix-b was reduced by shRNA#1, that of GFP-βPix-b-*rs* was not affected by shRNA#1. C: Endogenous expression of βPix-b in cultured hippocampal neurons was reduced by shRNA#1. Hippocampal neurons were transfected with cont-vec or shRNA#1 at DIV 12, fixed at DIV 15, and immunostained with anti-βPix-b antibody, followed by Oregon Green 488-labeled secondary antibody. Oregon Green channel shows endogenous levels of βPix-b (stained with βPix-b antibody). DsRed channel shows the neurons transfected with control vector (cont-vec) or shRNA#1. Arrowhead indicated the neurons transfected with shRNA#1 that showed the reduced levels of βPix-b expression compared to non-transfected neurons (arrows). Scale bar, 40 μm. D: Hippocampal neurons were cotransfected at DIV 12 with GFP-NPRD-*rs*, Nterm-*rs*, DHmNPRD-*rs*, or GFP and shRNA#1 or cont-vec, and fixed at DIV 15. GFP channel shows GFP vector or various GFP labeled βPix-b constructs. *rs* indicates silent mutants which are resistant to shRNA#1. DsRed channel shows the neurons transfected with control vector (cont-vec) or shRNA#1. Scale bar, 5 μm. E: Quantification of dendritic spine number upon various co-expression indicated. Co-expression of NPRD-βPix-b-*rs* with shRNA#1 successfully negated the effect of shRNA#1 on dendritic spine formation. *rs* indicates silent mutants which are resistant to shRNA#1. Data were collected from 16 to 22 dendrites of 12–16 neurons for each group in the three independent experiments. $^{**}P < 0.001$ (one-way ANOVA). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

stimulatory effect of N-WASP on dendritic spine formation was inhibited by the co-expression of the PRD of βPix-b with N-WASP (Fig. 5B,C).

To further assess the function of endogenously expressed βPix-b, we knocked-down βPix-b expression in neurons. Suppression of βPix-b expression by shRNA was confirmed by immunoblotting in HEK293T cells transfected with GFP-βPix-b, and further confirmed by immunostaining in neurons (Fig. 6A–C). When endogenous βPix-b was knocked down, the density of dendritic spines was largely reduced (Fig. 6D,E). By introducing silent mutations of βPix-b in a shRNA targeting sequence (designated "rs" which stands for "resistant to shRNA"), we tested which domain(s) of βPix-b could rescue the shRNA-mediated defect in spine formation. Figure 6D,E shows that NPRD-*rs* (Nterm + PRD-*rs*), but not Nterm-*rs*, successfully rescued the defect in spine formation resulting from βPix-b knock-down, indicating that the effect of βPix-b on dendritic spine formation requires its PRD. Expression of NPRD-*rs* containing DH mutant (DHm-NPRD-*rs*) failed to rescue the shRNA-mediated defect, suggesting that the GEF activity is also required (Fig. 6D,E).

Although the above results do not directly indicate that the effect of βPix-b on dendritic spine formation is attributed to the interaction between βPix-b and N-WASP, our results from biochemical investigation (Fig. 3), as well as our overexpression and knock-down studies (Figs. 5 and 6) together show that the stimulatory effect of βPix-b on dendritic spine formation may be mediated by βPix-b binding to N-WASP via a PRD–WH1 domain interaction.

A recent finding showed that N-WASP regulates the formation of dendritic spines and synapses via its interaction with the Arp2/3 complex (Wegner et al., 2008). Here, we show that N-WASP increases dendritic spine formation (Fig. 5B,C) and, in our hypothetical model (Fig. 7), we suggest that N-WASP regulates dendritic spine formation via its interaction with Arp2/3. Therefore, both our results and those of Wegener show that N-WASP and its interaction with the Arp2/3 complex is a key regulator of dendritic spine formation. Since the N-WASP–Arp2/3 complex interaction is a converging point for many actin-regulating factors, its activity profoundly affects neuronal morphology, including spines. Indeed, EphB receptors and SPIN90/WISH proteins regulate dendritic spine formation

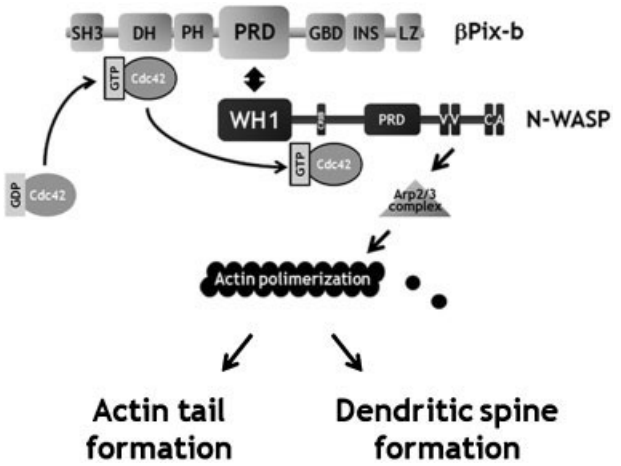


Fig. 7. Hypothetical model of βPix-b on N-WASP dependent actin regulation. In this study, we showed that βPix-b interacts with N-WASP and stimulates PIP5KIβ-induced actin tail formation in BHK21 cells, and dendritic spine formation in neurons. They do so via the interaction between PRD of βPix-b and WH1 domain of N-WASP. Our results suggest that as a GEF, βPix-b activates Cdc42 and through its PRD, it binds and brings N-WASP close to Cdc42 which in turn facilitates the binding of activated Cdc42 with N-WASP, thus stimulates Arp2/3 dependent actin assembly to induce actin-dependent processes such as actin tail formation and dendritic spine formation.

via a common downstream factor, the N-WASP-Arp2/3 complex (Irie and Yamaguchi, 2002; Lee et al., 2006). What we report here is a novel signaling pathway through which neuronal-specific βPix-b stimulates actin-dependent processes in cells via the interaction between its PRD and the WH1 domain of N-WASP; moreover, βPix-b may regulate N-WASP's activity in neuronal cells.

βPix isoforms have a common N-terminal region while their C-terminal regions show structural differences. In contrast to βPix-b, βPix-a and βPix-c lack an INS region and a PRD, respectively (Kim et al., 2000). These differences may be related to the alteration of the effects of βPix isoforms on actin-dependent tail and dendritic spine formation reported here. Our results show that the PRD is essential for the stimulatory effect of βPix-b on actin-dependent processes; however, the reason why overexpression of βPix-a, which contains a PRD, failed to stimulate actin tail formation is not clear. Interestingly, we found that, while βPix-b is highly concentrated in the dendritic spines, βPix-a presents evenly in the cytosol of hippocampal neurons (Supplemental Fig. 4). Since we have shown that the GEF activity is also required for βPix-b's effect, the lack of an INS region in βPix-a may affect its GEF activity or its localization. Alternatively, since the INS region contains many serine and threonine sites, the stimulatory activity of βPix may be regulated by phosphorylation in its INS region, although that speculation requires further investigation.

Several proteins such as Pak, SPIN90, and Cbl-b, have been identified as binding partners of the SH3 domain of βPix (Manser et al., 1998; Lim et al., 2003; Schmidt et al., 2006), but, to date, none has been reported to interact with the PRD of βPix. Our results identify N-WASP as the first protein shown to interact with the PRD of βPix-b. We further show this interaction to be important for βPix-b-induced actin-dependent processes.

Previous reports have shown that the WH1 domain of N-WASP binds to the PRD of WIP, and that a N-WASP-WIP complex is important for the regulation of N-WASP-dependent actin polymerization, including that related to filopodia, ruffle, and actin tail formation (Martinez-Quiles et al., 2001; Anton et al., 2003). WIP keeps N-WASP in an inactive state and inhibits Cdc42-mediated N-WASP-induced activation of the Arp2/3 complex in vitro. Moreover, TOCA-1 was found to be essential for the release of N-WASP from the inhibitory effect of WIP (Ho et al., 2004). We show that βPix-b is a novel binding partner of the WH1 domain of N-WASP. We further show that co-expression of βPix-b with an N-WASP W54A mutant (unable to interact with WIP) was still able to stimulate PIP5KIβ-induced actin tail formation (Fig. 4). Thus, βPix-b may recruit N-WASP to the site of actin assembly along a pathway that is independent of WIP in order to induce actin-dependent processes (Fig. 7). Whether βPix-b plays any role in N-WASP-mediated actin regulation is of great interest and needs further investigation.

Acknowledgments

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