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Molecular Cloning of Neuronally Expressed Mouse βPix Isoforms

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Pix, a Pak-interacting exchange factor, is known to be involved in the regulation of Cdc42/Rac GTPases and Pak kinase activity. In this study, we cloned the cDNAs encoding two βPix isoforms from mouse brain cDNA library. Both of the cloned genes, designated βPix-b and βPix-c (GenBank Accession Nos. AF247654 and AF247655, respectively), have a novel insert region consisting of 59 amino acid residues. In βPix-c, 75 amino acid residues are deleted in the proline-rich region at the carboxyl-terminus of βPix. *In situ* hybridization studies with insert region-specific probe in rat embryo show that insert region-containing isoforms are expressed mainly in the central nervous system. Moreover, temporal expression pattern of isoforms is correlated with the active neurogenesis period in the cerebral cortex and cerebellum. These results strongly suggest that βPix isoforms may play important roles in the cellular events required for brain development such as neuronal migration.

The Rho family GTPases, which include Rho, Rac, and Cdc42, control a wide range of biological events including changes in the actin cytoskeleton in response to extracellular stimuli, cell cycle progression, signaling to nucleus, and cellular transformation (1–3). The activity of the Rho family proteins cycles between active GTP-bound and inactive GDP-bound conformational states, regulated by three kinds of proteins: GTPase activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDIs) and guanine nucleotide exchange factors (GEFs). GEFs promote the exchange of GDP for GTP on Rho GTPases, whereas GAPs elevate intrinsic GTPase activity of Rho proteins and GDIs stabilize proteins in the GDP-bound form in the cytoplasm (4). Until now, more than 20 putative GEFs for Rho family GTPases have been identified and characterized by the presence of the Dbl homology (DH) domain, which could catalyze the guanine nucleotide exchange reaction. In biochemical studies using cultured cell lines, GEFs have been shown to be involved in the regulation of transformation, metastasis, cytoskeletal reorganization, and transcriptional activation (5). These proteins also play important roles in development; UNC-73A is required for cell migration and axon guidance in *Caenorhabtidis elegans* (6) and DRhoGEF2 mediates morphological changes during gastrulation of *Drosophila* embryo (7). Although Rho family GTPases appear to be expressed ubiquitously, several GEFs and GAPs show the restricted expression pattern in various types of tissues during different developmental stage (5). Therefore, it is suggested that these proteins play their own distinct roles in the control of various types of cellular events by regulating small GTPases.

Previously, we reported p85SPR (a SH3 domain-containing proline rich protein, which corresponds to βPix and p85Cool-1) as a widely expressed focal protein (8). In addition to tandem DH domain and Pleckstrin homology (PH) domain, βPix has a SH3 domain that directly mediates association with the fourth proline-rich region of p21-activated kinase (Pak) (9, 10). Paks were identified as serine/threonine kinases whose activity are regulated by Cdc42 and Rac (11–13), and have been implicated as important downstream effectors in cellular signaling governed by Cdc42/Rac GTPases (14–18). In several lines of evidence, Pix could also regulate Pak kinase activity by stimulating Cdc42/Rac or inducing conformational changes on Pak kinase (10, 19, 20). Here we report two novel splice variants, βPix-b and βPix-c, probably resulting from alternative splicing. To determine a possible role of βPix isoforms, we have investigated the expression pattern of the insert region-containing βPix isoforms in the developing rat brain. Our results suggest that βPix isoforms

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might be involved in neurogenesis, presumably via regulation of Cdc42/Rac signaling.

MATERIALS AND METHODS

cDNA cloning and sequencing. To identify βPix isoforms, a mouse brain λUniZAP cDNA library (Stratagene) was screened with the random primed DIG-labeled p85SPR cDNA probes (Boehringer-Mannheim). Several positive clones were further purified and plaque-purified phage clones were converted to pBluescript plasmid using helper phage ExAssist. Complete DNA sequence was determined by the dideoxy chain termination method using an Autoread DNA sequencing kit and an ALFexpress DNA sequencer (Amersham Pharmacia Biotech).

RT PCR. Total RNA was prepared from adult male ICR mouse tissues using the guanidium thiocyanate phenol-based single-step method. First strand cDNAs were synthesized in a 50 μl reaction mixture containing 50 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM dNTPs, 10 mM dithiothreitol (DTT), 2 μg of oligo $(dT)_{12\text{-}18}$/ml, 4 μg of total RNA, 40 units of RNase inhibitor and 50 units of avian myeloblastosis virus reverse transcriptase (Promega). After 2 h incubation at 42°C, reaction was stopped by heating at 94°C for 5 min. The PCR was achieved by 30 cycles with 1 μl of first-round PCR products. The reaction proceeded for 30 cycles of 96°C for 20 sec, 60°C for 30 sec, and 72°C for 1 min. The two primers were used; primer 1242F (5′-AAGCAAAGAGATGCGAGC-3′), encompassing amino acids 382-388 of p85SPR and primer InsR (5′-TGTGTT-TGGGACACTGCATG-3′), which is specific for 177-bp insert region. The amplified products were separated on a 2% agarose gel stained with ethidium bromide.

In vitro transcription and translation. In vitro transcription and translation was performed by TNT Reticulocyte Lysate System (Promega). 1 μg of supercoiled plasmid was used according to the manufacturer's protocol. The 25 μl reaction mixture contained 12.5 μl of TNT rabbit reticulocyte lysate, TNT reaction buffer, T3 RNA polymerase, 20 units of RNasin and amino acid mixture without methionine, supplemented with [^{35}S]methionine as the radioactive precursor (1000 Ci/mmol, NEN). After 90 min incubation at 30°C, the reaction mixture was analyzed on an 8% SDS–polyacrylamide gel electrophoresis and visualized by fluorography.

Generation of anti-insert region antibody. The insert region was amplified with synthetic primers that contained *Eco*RI or *Xho*I sites (Forward: 5′-CGGAATTCTGGCAAGGCACTGACCTG-3′, Reverse: 5′-GGGCCTCGAGGGATGCAGAACCCATTCT-3′) and introduced into the pGEX4T-1 vector. The recombinant vector was transformed into *E. coli* DH5α and the fusion proteins were induced with IPTG. Rabbit polyclonal antibodies were raised against GST-insert region protein purified by glutathione affinity column.

In situ hybridization. In situ hybridization was performed essentially as previously described (21). In brief, Sprague–Dawley rat embryos and brains were frozen in pre-chilled isopentane. Sagittal or horizontal sections (12 μm thick) were cut and thaw-mounted onto gelatin-coated slides. The sections were fixed, treated with acetic anhydride, dehydrated, and finally air-dried. RNA probes for the insert region were generated by transcription with T7 or SP6 polymerases from a plasmid containing the 177 bp of the insert region into pGEM-T Easy vector (Promega). Transcription of antisense and sense probes was carried out using the Riboprobe (Promega) in the presence of [α-^{35}S]UTP (NEN). Sections were hybridized overnight with labeled probe, treated with RNase A, washed sequentially in SSC, briefly rinsed in a graded series of ethanol and dried. Slides were exposed to β-max film (Amersham) for 5 days and developed.

RESULTS AND DISCUSSION

Cloning of βPix cDNA Isoforms

Immunoblot analysis with the anti-βPix SH3 domain antibody indicated that there are at least four βPix isoforms in the brain (8, 9). Of these, p85SPR (designated βPix-a) is expressed in all tissues, whereas other isoforms show more restricted tissue distribution. To identify βPix isoforms, a mouse brain cDNA library was screened with a random labeled βPix-a cDNA probe. Two clones from several positives in this screen showed different restriction enzyme digestion patterns from βPix-a and were further analyzed by nucleotide sequencing. Both of the two different ORFs (designated βPix-b and βPix-c) are identical to βPix-a at the nucleotide level except for the 177-bp insert at the position of serine 555 (Fig. 1A). Because of the insertion, the serine 555 of βPix-a was changed to threonine and novel 59 amino acids were added. The insert region is composed of many serine and threonine residues which might provide possible phosphorylation sites. A search using the BLAST network for similarities between the translated insert sequence and known proteins revealed no overall identity or homology, and the 177 bp appear to be unique. However, 95% identity was shown in one human EST clone, which was cloned out from a human female infant brain (Accession No. R51701). In addition to the insertion, βPix-c had a 225-bp deletion from the region encoding the proline-rich region, a potential SH3 domain-binding site (Fig. 1B). An identical deletion pattern as that in βPix-c is found in certain human βPix EST clones derived from a human colon tumor (Accession No. AA877532).

Detection of βPix Isoforms on RNA and Protein Level

We performed RT-PCR using primers designed to specifically amplify the insert region-containing βPix isoforms. As shown in Fig. 2A, two PCR products obtained from reverse transcribed brain cDNA were the expected 695 bp and 470 bp, respectively. This suggests that two βPix isoforms do exist *in vivo* and that these clones were not generated by recombination during phage amplification. To confirm that the ORF sequence present in βPix-b and βPix-c clone codes for translatable protein, each cDNA was transcribed and translated in a rabbit reticulocyte *in vitro* translation system with incorporation of [^{35}S]methionine. The result showed 90-kDa and 84-kDa translation products of βPix-b and βPix-c, respectively, indicating the presence of functional coding sequences (Fig. 2B). We next investigated the tissue distribution of βPix-b and βPix-c using an affinity purified polyclonal anti-insert region antibody. Because of the low level of βPix-c expression, 1 mg of tissue extracts were immunoprecipitated with the anti-βPix SH3 domain antibody and

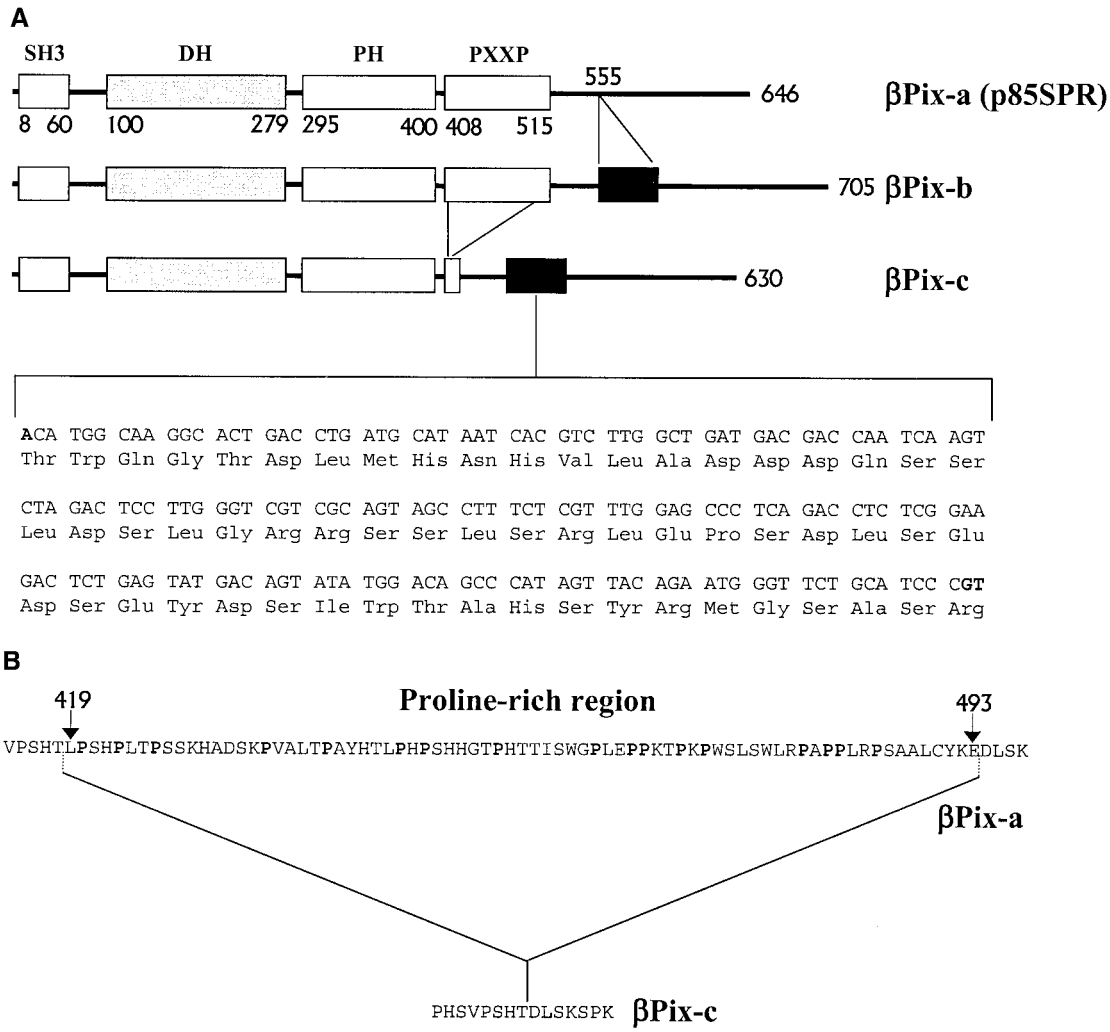

FIG. 1. Schematic representation of mouse βPix isoforms. (A) The coding region of βPix-b and βPix-c contains an additional 177-bp insert region denoted by black box. Deduced amino acid sequence of insert region is indicated. (B) 75 amino acids of proline-rich region of βPix-a are deleted in βPix-c.

the immunoprecipitates were subject to immunoblot analysis with the anti-insert region antibody. As shown in Fig. 2C, βPix-b of 90-kDa band was strongly detected only in the brain and testis, and βPix-c of 84-kDa band was mainly detected in the brain and testis but also recognized at lower levels in the lung and heart.

In Situ Localization of βPix Isoforms in Rat Embryo and Brain

To examine the spatial and temporal expression pattern, *in situ* hybridization with an insert region-specific probe was performed. In the rat embryo (embryonic day 20), the hybridization signal was detected only in the central nervous system, brain and spinal cord with a high level of developing cerebral cortex, olfactory bulb, and retina (Fig. 3A). In all cases sense probes were used to determine background signals (data not shown). Outside the brain and spinal cord, we could not detect insert specific signals, whereas the signal of pan-βPix using probe encompassing SH3 domain and DH domain was observed in the whole embryo (unpublished data). The central nervous system-specific expression of insert region-containing isoforms was maintained throughout the rat embryo development. To elucidate the developmental changes in the expression of the isoform, we examined the expression pattern of isoforms using various stages of horizontal sections of rat brain. As shown in Fig. 3B, the transcripts were predominantly expressed in the cerebral cortex, cerebellum, hippocampal formation, and olfactory bulb. However, the localized area and intensity of messages were not uniform but changed during brain development. Expression patterns in the cortex and cerebellum, especially, are correlated with the neuronal migration period. Although signals are detected

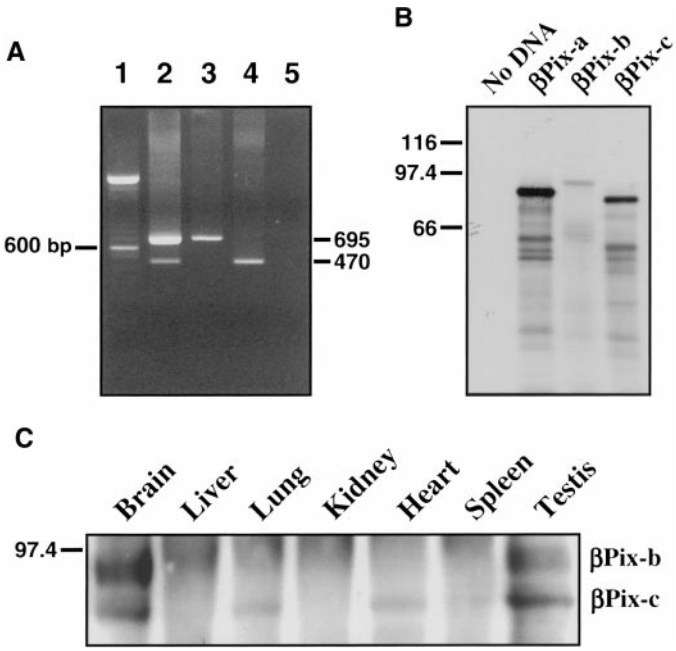

FIG. 2. Expression of βPix isoforms. (A) Detection of βPix-b and βPix-c transcripts by reverse transcript-PCR. Lane 1, DNA size marker (100-bp ladder); lane 2, PCR amplification from reverse transcribed mouse brain mRNA; lane 3 and 4, PCR positive control using βPix-b and βPix-c cDNA, respectively; lane 5, negative control PCR without DNA. (B) *In vitro* transcription and translation of each βPix isoform. (C) Immunodetection of βPix isoforms. Rat tissue extracts were immunoprecipitated with anti-βPix SH3 antibodies and blotted with anti-insert antibodies.

along migration of neurons from several layers, its expression becomes restricted to the outermost layer of the cortex and declines after neuronal migration. In the case of the cerebellum, signals change from the external granule layer (EGL) to the internal granule layer (IGL) and are maintained at a high level in the cerebellum of adult rat brain. This cerebellar distribution of insert region-containing βPix is shown to overlap with αPak (correspond to human Pak1) (22). Hybridization signals are also detected in hippocampal formation and olfactory bulb, other typical laminated structures in the adult brain. These results that insert region-containing βPix isoforms are highly maintained in cerebellum and hippocampus after establishment of neuronal connections suggest that βPix may also play roles in the maintenance of neuronal processes in these regions.

At present, *in vitro* and *in vivo* data suggest that two members of the Dbl family, Tiam-1 and Sif (*still-life, Drosophila* homolog of Tiam-1), are reported to be involved in neuronal migration and neurite outgrowth (23, 24). Surprisingly, βPix isoforms and Tiam-1 are highly expressed in specific neuronal populations like the cerebral cortex, cerebellum and hippocampus in the developing brain, although the *in situ* hybridization pattern of βPix isoforms is not exactly identical to that in Tiam-1. The similarities in the expression pattern between βPix isoforms and Tiam-1 suggest that two different GEFs may essentially have overlapping functions in the same molecular environments and affect the same cellular events. Therefore, it is possible that the insert region-containing βPix isoforms and Tiam-1 may regulate neuronal migration or neurite extension by locally regulating small GTPases, Rac and/or Cdc42. This possibility is strongly supported by the previous studies that Rac1 is involved in cellular migration and neurite extension (25, 26). Alternatively, these proteins may be localized at distinct subcellular sites or respond to different upstream signals to differentially activate Rac1 and regulate distinct processes of neuronal differentiation. In addition to the role of βPix in Cdc42/Rac signaling, βPix can associate

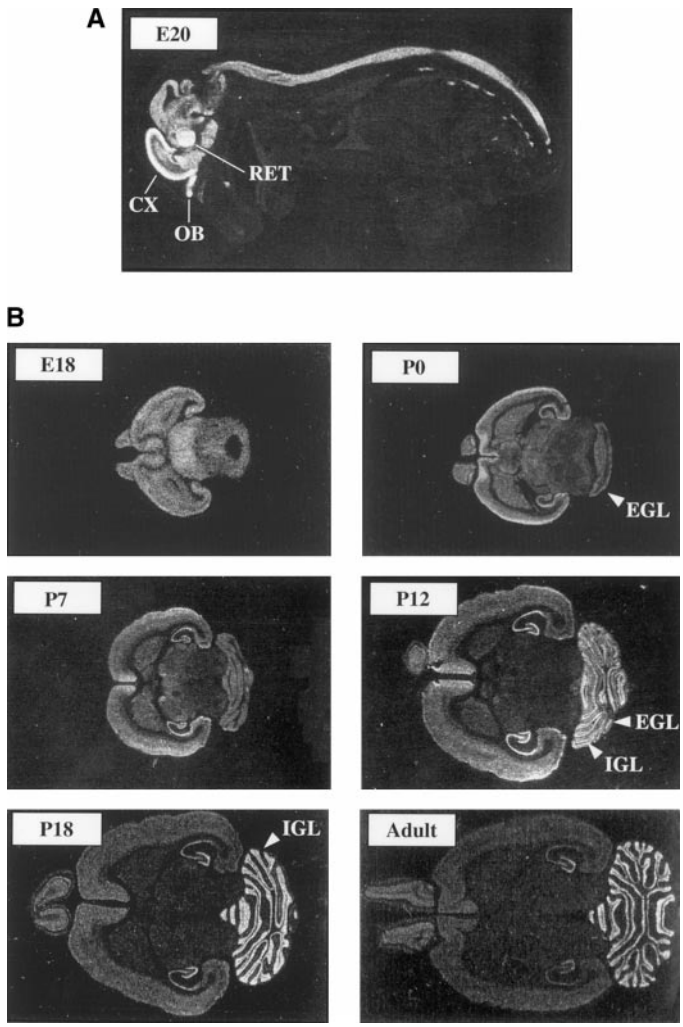

FIG. 3. Localization of βPix isoforms by *in situ* hybridization. (A) Hybridization with the 177-bp insert region-specific antisense riboprobe in the midsagittal section of rat embryo (E20). CX, cerebral cortex; OB, olfactory bulb; RET, retina. (B) Horizontal sections of rat brain during development (E18, P0, P7, P12, P18, and adult). EGL, external granule layer; IGL, internal granule layer.

with and regulate Pak kinase, a downstream effector of Cdc42/Rac1. Recently, Hing *et al.* (27) have demonstrated that Pak acts as a critical regulator of axon guidance under the control of Dock, the *Drosophila* homolog of Nck. The observation that βPix isoforms and brain-enriched Pak isoforms co-localize at specific areas in the brain suggests that insert region-containing βPix isoforms might associate with distinct Pak isoforms and perform their own specific roles in different areas. Three βPix isoforms reported here differ in primary structure at carboxyl-terminal region. These differences may affect the abilities of βPix isoforms to interact with other signaling molecules. We are currently investigating the functional roles of the insert region and proline-rich region by comparing the biochemical properties among βPix isoforms. Future experiments will address the functional differences of βPix isoforms.

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