Evidence That a Phosphatidylinositol 3,4,5-Trisphosphate-binding Protein Can Function in Nucleus

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Kenichi Tanaka‡‡, Kaori Horiguchi‡, Toshinori Yoshida¶, Makio Takeda‡, Hideki Fujisawa¶, Kenichi Takeuchi‡, Masato Umeda, Sigeaki Kato**, Sayoko Ihara‡, Satoshi Nagata‡, and Yasuhisa Fukushima‡‡

From the ‡Laboratory of Biological Chemistry, Department of Applied Biological Chemistry, Graduate School of Agriculture and Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-0032, the ¶Toxicology Division, Institute of Environmental Toxicology, 4321 Uchimoriya-cho, Mitsuikaido-shi, Ibaraki 303-0043, the ‡‡Department of Inflammation Research, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo, 113-8613, and the **Institute of Moleculuar and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

PI3BP is a phosphatidylinositol 3,4,5-trisphosphate-binding protein (PI3BP) abundant in brain, containing a zinc finger motif and two pleckstrin homology (PH) domains. Staining of rat brain cells with anti-PI3BP antibody and determination of localization of PI3BP fused to the green fluorescent protein (GFP-PI3BP) revealed that PI3BP was targeted to the nucleus. Targeting was dependent on a putative nuclear localization signal in PI3BP. Generation of PI3 in the nucleus was detected in H2O2-treated 293T cells, nerve growth factor (NGF)-treated PC12 cells, and platelet-derived growth factor (PDGF)-treated NIH 3T3 cells. Translocation of phosphatidylinositol 3-kinase (PI3-kinase) to the nucleus and enhanced activity of PI3-kinase in the nucleus fraction were observed after H2O2 treatment of 293T cells, nerve growth factor (NGF)-treated PC12 cells, and platelet-derived growth factor.

Experimental Procedures

Cell Lines and Transfection—COS-7 cells and 293T cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% calf serum. Transfection was done by the calcium phosphate method as described by Shirai et al. (15) except that pH of the buffer was 7.0 instead of 7.15.

Primary Culture of Rat Brain—Pregnant mice were sacrificed by cervical dislocation on the 18th-day of gestation. After isolation of the embryos from the uterus, by cutting the outer layer of the pelvis, the fetal meninges were removed and the cerebral cortices were placed in DMEM containing 10% fetal bovine serum (Life Technologies, Inc.). Following the mechanical dissociation, cells were passed through a #100 mesh, and were suspended in DMEM supplemented with 10% fetal bovine serum for glial cell culture. For neuronal cell culture, the

Phosphatidylinositol 3-kinase (PI3-kinase) is an enzyme that is activated immediately after growth factor or differentiation factor stimulation of the cells (1) and that generates second messengers, phosphatidylinositol 3,4,5-trisphosphate (PI3P) and phosphatidylinositol 3,4-bisphosphate (PI3P2) (2–5). These 3'-phosphorylated phosphoinositides can activate serine, threonine kinases such as PKB/Akt, PKCs, and PDKs (6–9). They are also suggested to be involved in other events such as rearrangement of cytoskeleton and vesicle transport because these phenomena are sensitive to the PI3-kinase inhibitors and dominant negative mutants of PI3-kinase (10). Recently, it was reported that the 3'-phosphorylated phosphoinositides can activate guanine nucleotide exchanging factors of Ras and Rac, small G proteins involved in actin rearrangement and vesicle transport, respectively (11, 12). Therefore, G proteins as well as kinases are downstream of PI3-kinase.

We have identified PI3BP as a PI3P-binding protein, using a PI3P analogue column (13). It is abundant in brain, implying that it may be involved in the function of nerve systems. PI3BP binds to PI3P but not to PI3P2 or phosphatidylinositol 3,4-bisphosphate (PI3P2). It has a zinc finger motif homologous to that of Arf-GTPase activating protein (GAP) and two PH domains. Both PH domains are shown to be involved in binding to PI3P. Another PI3P-binding protein, centaurin α, is highly homologous to PI3BP (14). No GAP activity to Arf has been detected in either protein. Although the binding of centaurin α and PI3P to PI3P was specific, the role of the protein is unclear. To address this question, we determined the intracellular localization by immunological techniques, using monoclonal antibody to PI3BP as well as localization of green fluorescent protein (GFP) fusion proteins. Surprisingly, PI3BP was found to localize in the nucleus, where the generation of PI3P was detected after stimulation, suggesting a new pathway of signal transduction through PI3-kinase to PI3P in nucleus. PI3BP was exported out of the nucleus by expression of a constitutively active PI3-kinase. This suggests that PI3BP can shuttle between nucleus and cytoplasm depending on the activity of PI3-kinase.
cells were suspended in neurobasal medium containing 2% B27 supplement (both from Life Technologies, Inc.), 74 μg/ml l-glutamine and 25 μg/ml l-glutamate. They were plated in culture dishes coated with poly-L-lysine (100 μg/ml) and cultured in an atmosphere of 95% air and 5% CO2 at 36°C.

Plasmids Used in This Study—A cDNA fragment encoding the full-length PIP3BP was subcloned into pEGFP-c1, an expression vector for GFP fusion protein (CLONTECH), to produce pEGFP-PIP3BP, pEGFP-PIP3BP(NLS), pEGFP(NLS), and pEGFP-PIP3BP-PH. PIP3BP-NLS was constructed by deletion of amino acid 1–9 residues using the restriction site, Xhol, in the cDNA. Point mutants in the PH domains in PIP3BP (PIP3BP-PH) were introduced as described previously (13) by substituting Cys for Arg (residues 149 and 272) of PIP3BP by the Kunkel method (16). To obtain Myc-tagged PIP3BP, an Myc-tag sequence with an initiation codon, ATGGAGCAAGCGTGGATCAGAGAAAGATCT, was attached at the 5′ end of the cDNA of the PIP3BP. The resulting gene was expressed under the control of SRα promoter by an expression vector pMIKNeo (17). The expression vectors for constitutively active PI3-kinase (BD110) and a kinase negative mutant of PI3-kinase (BDKIN) were described previously (18). The BD110 protein has a structure similar to that of p110α reported by Hu et al. (19). The protein has an inter-SH2 domain of p85, which binds to the p110 amino terminus. BDKIN protein is a kinase negative counterpart of the BD110 protein with a point mutation in the kinase domain.

In Vivo Labeling of the Cells and Analysis of Lipids—In Vivo labeling of the cells and analysis of lipids were performed as described previously (20). A cDNA fragment encoding the full-length PIP3BP or mutant PIP3BP was subcloned into pBluescript SK(+) and the transcripts of T7 or T3 RNA polymerase labeled with digoxigenin were used as antisense or sense probes.

Production of the Monoclonal Antibody and Histocytocchemistry—A monoclonal antibody, mAb 13–14, was produced. GST fusion protein of PIP3BP (GST-PIP3BP) was expressed in Escherichia coli and purified with a glutathione-Sepharose column. Eight-week-old male mice were injected subcutaneously with the purified protein mixed with complete Freund’s adjuvant. Booster injections were given subcutaneously with the antigen mixed with incomplete Freund’s adjuvant two times with an interval of two weeks. After the final booster injection, which was given intravenously, spleen cells of the mouse were taken and fused with the SP2/O cells by a polyethylene glycol method (21). Ten days after fusion, culture supernatant of the hybridomas were examined for the reactivity to purified GST-PIP3BP protein by enzyme-linked immunosorbent assay. After several cycles of cloning, a hybridoma clone producing mAb 13–14 was established. The epitope for mAb 13–14 was determined to be the region between amino acid position 42–109, which is within the zinc finger motif. Immunostaining was carried out as described previously (22).

Fractionation of the Cells—The cells were collected by centrifugation and resuspended in a buffer containing 20 mM Tris-Cl (pH 7.5), 10 mM CaCl2. After homogenization in a Dounce homogenizer, they were centrifuged at 1,000 × g for 5 min. After removal of the supernatant, two cycles of the same procedure were done to remove any non-nuclear membranes from the nucleus. The resulting pellet was used as a nuclear fraction. The supernatant was further ultracentrifuged at 100,000 × g for 30 min. The supernatant and the pellet were used as cytosolic and membrane fractions, respectively.

In Vivo Labeling of the Cells and Analysis of Lipids—Cells were labeled with [32P]orthophosphate (1 mCi/ml) for 4 h in a phosphate-free MEM supplemented with 25 mM Hepes-NaOH and treated with various stimuli. After fractionation of the cells, the lipids were extracted as described previously (23) and analyzed by TLC as described previously (24). High performance liquid chromatography analysis using SAX5 column (Whatman) was done to confirm the result of TLC (25).

RESULTS AND DISCUSSION

Localization of PIP3BP in Brain—In situ hybridization and immunostaining was carried out to determine the expression of PIP3BP in rat brain with mAb 13–14, anti-PIP3BP monoclonal antibody. In situ hybridization of sagittal cryostat sections (8 μm) of adult rat cerebral cortex was done using antisense probe (a) and sense probe (b). The nucleus was stained with methyl green. Sagittal cryostat sections (8 μm) of adult rat cerebral cortex were stained with hematoxylin and eosin (c) or immunostained with mAb 13–14 and stained with hematoxylin (d). Immunoreactivity was visualized by alkaline phosphatase and new fuchsin. Detection of PIP3BP in neuronal cells in culture. PIP3BP was detected from cell lysates (15 μg/lane) of neuronal cells (lane 1) and glial cells (lane 2) by immunoblotting using mAb 13–14. The position of PIP3BP is indicated by an arrowhead. Anti-neuron-specific enolase (αNSE) and anti-glial fiber acidic protein (αGFAP) were used as controls for the separation of the cells (bottom part). C, localization of endogenous PIP3BP in neuroblastoma, Neuro2A cells. Phase contrast image (a) and immunostaining of endogenous PIP3BP (b) in Neuro2A cells with mAb 13–14 are shown. D, nuclear localization of GFP-PIP3BP. COS-7 cells were transfected with the expression vectors for GFP-PIP3BP(a), GFP-PIP3BP(NLS) (b), GFP(NLS) (c), and GFP(d). Cells were observed under fluorescence microscopy 24 h after transfection.

FIG. 1. PIP3BP is in the nucleus of neuronal cells. A, in situ hybridization of PIP3BP gene and immunostaining of rat brain with mAb 13–14, anti-PIP3BP monoclonal antibody. In situ hybridization of sagittal cryostat sections (8 μm) of adult rat cerebral cortex was done using antisense probe (a) and sense probe (b). The nucleus was stained with methyl green. Sagittal cryostat sections (8 μm) of adult rat cerebral cortex were stained with hematoxylin and eosin (c) or immunostained with mAb 13–14 and stained with hematoxylin (d). Immunoreactivity was visualized by alkaline phosphatase and new fuchsin. B, detection of PIP3BP in neuronal cells in culture. PIP3BP was detected from cell lysates (15 μg/lane) of neuronal cells (lane 1) and glial cells (lane 2) by immunoblotting using mAb 13–14. The position of PIP3BP is indicated by an arrowhead. Anti-neuron-specific enolase (αNSE) and anti-glial fiber acidic protein (αGFAP) were used as controls for the separation of the cells (bottom part). C, localization of endogenous PIP3BP in neuroblastoma, Neuro2A cells. Phase contrast image (a) and immunostaining of endogenous PIP3BP (b) in Neuro2A cells with mAb 13–14 are shown. D, nuclear localization of GFP-PIP3BP. COS-7 cells were transfected with the expression vectors for GFP-PIP3BP(a), GFP-PIP3BP(NLS) (b), GFP(NLS) (c), and GFP(d). Cells were observed under fluorescence microscopy 24 h after transfection.

Primary neuronal and glial cultures were prepared separately from embryonic day 18 rat brains. Cell fractionation was
correctly done because neuron-specific enolase (NSE) was specifically found in neuronal fraction, and the glial fiber acidic protein (GFAP) was found in the glial fraction (Fig. 1B, bottom part). Expression of PIP3BP was examined by immunoblotting using mAb 13–14. As shown in Fig. 1B, PIP3BP was detected exclusively in neuronal cells. No detectable amounts of PIP3BP were observed in glial cells. These results suggest that PIP3BP is localized in the nucleus of the neuronal cells in rat brain. Immunostaining using mAb 13–14 showed that native PIP3BP was also detected in nucleus of neuroblastoma, Neuro2A cells (Fig. 1C).

**PIP3BP Is Targeted to the Nucleus**—To confirm the nuclear localization of PIP3BP, COS-7 cells were transfected with a construct coding for PIP3BP fused to the green fluorescent protein (GFP-PIP3BP), and the localization of the protein in the intact cells was analyzed. The GFP-PIP3BP fusion protein was almost exclusively detected in the nucleus, supporting the immunostaining data (Fig. 1D, a). Similar results were obtained using PC12 cells and neuroblastoma Neuro 2A cells (26, 27) (data not shown). Nuclear localization signal-like motif, KERRK, was found in the amino terminus part of PIP3BP. We tested whether or not this sequence directs the protein to the nucleus. GFP fused to amino-terminal 14 amino acids of PIP3BP, MAKERRKAVLELLQ, localized exclusively in the nucleus (Fig. 1D, c). A deletion mutant lacking amino acid 1–9 (GFP-PIP3BP (−NLS)) was diffusely distributed all over the cells (Fig. 1D, b), suggesting that the targeting mechanism of the protein to the nucleus was absent. GFP alone was detected in all parts of the cells (Fig. 1D, d). These results suggest that the amino acid 1–14 of PIP3BP targets the proteins to the nucleus. Fractionation of COS-7 cells transfected with an expression vector for Myc-PIP3BP by homogenizing and centrifugation revealed that PIP3BP free from GFP was also located in the nucleus (see below).

**PIP3BP Is Generated in the Nucleus**—The above results suggest that PIP3BP may play a role in the nucleus. We therefore determined whether or not PIP3BP was generated in the nucleus. Various cells were stimulated by agonists and fractionated, and the lipids were analyzed by TLC. PI 3-kinase is strongly activated to give a strong signal of PIP3 in 293T cells treated with 10 mM H2O2. We first used this system. As shown in Fig. 2A, generation of PIP3 in the nucleus was detected in those cells as well as in the membrane. The fractionation was confirmed by Western blotting of Src and Myc, which are membrane and nuclear proteins, respectively (Fig. 2C). When the nuclear fraction of the H2O2-treated 293T cells was prepared and incubated with [32P]ATP-MgCl2, generation of PIP3 was clearly detected; it was not seen in that of the untreated cells (Fig. 2B). The presence of PIP3 in the samples was confirmed by high-performance liquid chromatography analysis of the lipids, using a SAX5 column (data not shown).

In the H2O2-treated 293T cells, tyrosine phosphorylation of the proteins was extremely elevated, suggesting the activation of many signaling pathways (28). Fractionation of the cells revealed that the level of p85 in the nuclear fraction was markedly elevated after H2O2 treatment and considerable tyrosine phosphorylation on nuclear p85 was detected (Fig. 2C), suggesting that the activation of PI 3-kinase activity in the nucleus may be because of relocalization of the enzyme. PIP3 was also detected in the nucleus in 293T cells transiently transfected, constitutively active PI 3-kinase, NGF-treated PC12 cells, and PDGF-treated NIH 3T3 cells (Fig. 2A). Recently, several groups have used PIP3-binding proteins, such as ARNO and GRP1, fused to GFP, as a means to visualize changes in

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exportation of the nucleus to understand the role of the protein.

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