A phosphatidylinositol (PI) 4-kinase cDNA was cloned from a rat brain cDNA library. This cDNA encoded a protein of 2041 amino acids with a calculated molecular weight of 231,317. The deduced amino acid sequence shared the identity of 52.3 and 34.4% in the presumed catalytic domain with two yeast PI 4-kinases, STT4 and PIK1, respectively, and showed 31.7% identity to p110catalytic domain with two yeast PI 4-kinases, STT4 and PIK1, respectively, and showed 31.7% identity to p110

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otide numbers represent those of STT4). The sequences for the primers were designed according to the mammalian codon usage: CGGAATTC-GGTA(G/T)CA(G/T)GA(T/C)GA(T/C)GA(T/C)GA(T/C)GA(T/C)GA(T/C)
for 5' primer and GGTAATTC(G/A)GA(A/G)ATCC(G/A)GA(A/G)ATCC(G/A)
for 3' primer. The 5' ends of the 5' and 3' primers contained an EcoRI restriction sequence for the subsequent cleavage of cloned DNA fragments. PCR amplification was performed with AmpliTaq DNA polymerase according to the following schedule: 94°C for 30 s, 45°C for 1 min, 72°C for 2 min for 35 cycles, followed by further incubation for 7 min at 72°C. PCR products were electrophoresed on a polyacrylamide gel, and an amplified DNA (about 400 base pairs) was excised from the gel and subcloned into pUC118 after digestion with EcoRI. Sequencing analysis revealed that 4 of 20 clones (pMP1, 2, 12, and 18) were identical and showed homology with STT4 and PIK1.

cDNA Cloning—A rat brain cDNA library was constructed as described previously (14). Clones (3 × 106) derived from the cDNA library were screened by hybridization with 382-base pair DNA fragment of pMP1. Hybridization was carried out at 42°C in a buffer containing 50% formamide, 5 × SSC (saline sodium citrate), 1 × Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50 mM sodium phosphate (pH 7.2), and 250 μg/ml heat-denatured salmon sperm DNA for 16 h. The membranes were washed twice at room temperature in 2 × SSC, 0.1% SDS for 10 min, followed by two washes in 0.1 × SSC, 0.1% SDS at 42°C for 30 min and finally at 35°C for 2 h. After hybridization, 10% nitrocellulose sheets were cut into pieces. Positive clones from the nitrocellulose sheets containing large cDNA inserts were selected and subcloned into pUC118. The cDNA inserts of these clones showed an identical digestion pattern of the restriction enzymes except for some length difference in their extreme 5' portions, and one clone containing the largest cDNA insert (pRPIK7, 4.2 kb) was chosen for further sequence analysis on both strands by the dideoxy chain termination method (15) with a 373A DNA sequencer (Applied Biosystem) according to the supplier's instructions. By another round of screening with a 5' portion of the pRPIK7 as a probe, a further elongated clone, pRPIK20 (6.4 kb), was isolated. The 5' missing end was obtained by a rapid amplification of cDNA ends (RACE) PCR (5' Amplifinder RACE kit, Clontech). For the sequences obtained, both strands were sequenced by the dideoxy chain termination method.

RNA Extraction and Northern Blot Analysis—Total RNA was extracted from several tissues of adult rats as described previously (16). Each of the total RNA samples (30 μg) were denatured with formaldehyde and size-separated by agarose gel electrophoresis. The RNA was transferred and fixed to a nylon membrane (Nytran, Whatman) by ultraviolet light. The membrane was baked at 80°C for 2 h, prehybridized in a solution containing 70:100:15:25 (v/v/v) chloroform:1 M NaCl:0.1 M sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml pepstatin, 50 mg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated for 40 min at 4°C with gentle rocking and centrifuged at 10,000 × g for 20 min at 4°C. Equal quantities of soluble protein were immunoprecipitated for 1 h at 4°C with a monoclonal antibody (anti-FLAG-M2, Kodak) specific for the FLAG marker peptide. Protein A-Sepharose beads pre-clogged with 2 mg/ml bovine serum albumin were then added to the cell lysates for 1 h of incubation. Beads were washed twice with 1% Nonidet P-40 in the lysis buffer and then with the lysing buffer. Thereafter, PI kinase activity was performed by the methods described (21).

In Situ Hybridization Histochemistry—Fresh frozen blocks of brains from adult (postnatal day 49) male and fetal (prenatal day 18) rats were sectioned at 30-μm thickness on a cryostat. The sections were mounted on silane-coated glass slides, fixed with paraformaldehyde, and pre-treated as described previously (14). The slides were prehybridized in a solution containing 50% formamide, 4 × SSC, 1 × Denhardt's, 1% sodium dodecyl sulfate (pH 7.2), 100 mM dithiothreitol, and 250 μg/ml heat-denatured salmon sperm DNA for 2 h at room temperature. Hybridization was performed at 42°C for 16 h in a moist chamber in the same solution (50 μl/slide), which contained an additional 10% dextran sulfate and 0.5–1.0 × 106 cpm of the same cDNA probe as used for Northern analysis labeled with [35S]dATP by nick translation or at the same amount of a control probe (pBR322, the plasmid vector). After hybridization, the slides were sequentially rinsed in 2 × SSC, 0.1% sarcosyl at room temperature for 30 min, three times in 0.1 × SSC, 0.1% sarcosyl at 42°C for 40 min each, and dehydrated in 70 and 100% ethanol. The sections were exposed to Hyperfilm-Max (Amersham Corp.) for 2–3 weeks.

Immunohistochemistry and Immunoblotting—The cells were fixed with 2% paraformaldehyde/0.1% or 0.01% Triton X-100 and were incubated with the anti-FLAG antibody. Sites of antigen-antibody reaction were visualized using the avidin-biotinylated peroxidase complex (ABC) system (Vector Laboratories) with diaminobezidine as a substrate. Some of the specimens, after immunoreaction, were postfixed with OsO4 and uranyl acetate and embedded in Epon. Ultrathin sections were examined under a transmission electron microscope (22). The lysates of the overexpressed cells were boiled for 4 min in Laemmli's sample buffer and subjected to SDS/7.5% polyacrylamide gel electrophoresis (24). The proteins were then electrothermally transferred to a nitrocellulose membrane (pore size, 0.45 μm). After blocking the nonspecific binding sites in the 5% skim milk (w/v) in phosphate-buffered saline, the membrane was incubated for 2 h at room temperature with the antibody against FLAG and then treated with peroxidase-conjugated anti-rabbit IgG antibody for 1 h.

RESULTS

The cDNA clone was isolated through PCR-mediated cDNA amplification by using primers for the amino acid sequences conserved between yeast STT4 and PIK1 and subsequent screening of a rat brain cDNA library. The primers used were degenerate oligonucleotides with mammalian codon usage against yeast amino acid sequences. The homologous DNA sequences were amplified from a rat brain cDNA library and subcloned into pUC118.

Sequence analysis of the PCR products revealed that four of 20 clones (pMP1, 2, 12, and 18) were identical and showed amino acid sequence identities of 52.3% and 34.4% with yeast STT4 and PIK1, respectively. A rat brain cDNA library containing 3 × 106 phage clones was screened by hybridization.
with the cDNA probe derived from the pMP1 cDNA under high stringency conditions. Four clones containing the large cDNA inserts were isolated from positive clones and subcloned into pUC118. Restriction map of these cDNA inserts revealed an identical pattern except for some size differences in the 5' end of the cDNA inserts. The nucleotide sequence of a representative clone containing the largest cDNA insert (pRPIK7, 4.2 kb) was determined. Another round of screening and a RACE were performed to obtain the 5' end of the coding sequence.

The nucleotide and deduced amino acid sequences of the composite cDNA are presented in Fig. 1. The putative initiation codon was preceded by an in-frame stop codon at nucleotides 2249, 2305, and 2323. The deduced amino acid sequence encoded a protein of 2041 amino acids with a calculated molecular weight of 231,317. By comparison with sequences in the protein and DNA data base, the nucleotide sequence of the composite cDNA from nucleotide 3562 throughout the rest of the 3' coding region starting with an ATG sequence was revealed to be 89.6% identical to and its deduced amino acid sequence was 98.2% identical to the sequence for the recently reported human PI 4-kinase of type II, termed PI4Kα (12). As expected from this identity, the present cDNA contained sequences encoding the ankyrin repeat domain, lipid kinase unique domain, pleckstrin homology domain, and presumed lipid kinase/protein kinase homology domain in the same turn as PI4Kα (12). The deduced amino acid sequence from the present composite cDNA also showed 52.3 and 34.4% identity in the presumed catalytic domain and 28.3% identity in the lipid kinase unique domain to STT4 and PIK1, respectively. In addition, this sequence showed 31.7% identity to p110α subunit of rat PI 3-kinase in the presumed catalytic domain and 28.3% identity in the lipid kinase unique domain (Fig. 2). As for the N-terminal half, which had no identity relation to the human PI4Kα, we found two proline-rich regions (amino acids 152–156 and 210–218) and a SH3 domain (25). Five leucine-rich portions were also found in the sequence, each of which, however, did not form a complete a-helix by Chou-Fasman analysis program (26).

In Northern blot analysis of adult rat tissues using a probe composed of the nucleotide sequence from nucleotides 1277 to 3354 of the present composite cDNA, whose counterpart is not contained in the human PI4Kα, a single hybridization band of 7.8 kb in size consistent with nearly a full length of the present cDNA was detected intensely in the brain, kidney, and lung and less intensely in the small intestine, uterus, and adrenal gland, whereas it was detected weakly to faintly in the heart, skeletal muscle, thymus, spleen, and testis. No distinct hybridization band was detected in the liver (Fig. 3). For comparison, the same nylon membrane was hybridized with another probe composed of nucleotide sequence from 4206 to 6466, which corresponds to the sequence from nucleotides 654 to 2905 of the human PI4Kα. The hybridization pattern was the same as the former one, and no significant hybridization band was clearly detected at sizes about 3.5 kb, in which the smaller band had been described to occur for the human PI4Kα in the previous study by Wang and Cantley (12).

The lipid kinase activity of this novel molecule was measured open box and the lines represent the coding region and untranslated sequences, respectively. The PCR product (pMP1) used for screening a full-length cDNA, and the probes used for Northern blot analysis and in situ hybridization histochemistry are also indicated. b, nucleotide sequence of the composite cDNA and the deduced primary structure of rat PI 4-kinase. In-frame stop codons in the 5'-untranslated region are underlined. Proline-rich regions are doubly underlined. The leucine, isoleucine, and valine residues (shaded) making up leucine-rich portions are also shown.
Cloning, Expression, and Localization of 230-kDa PI 4-Kinase

Fig. 2. a, linear representation of rat PI4K, PI4Kα, two yeast PI 4-kinases, STT4 and PIK1, and rat PI 3-kinase p110α subunit. The lipid kinase unique domain, pleckstrin homology domain, catalytic domain, proline-rich region, and SH3 domain are shown. b, identity of the catalytic domain among the PI kinases described above. Conserved residues are shaded, and sequences used for PCR amplification are doubly underlined. c, comparison of the SH3 domain of rat PI4K with other SH3 domains. SH3 domains of phospholipase Cα, PI 3-kinase p85 subunit, c-src, and spectrin. Consensus residues of the SH3 domain, as defined by Musacchio et al. (25), and identical ones are shaded.

Fig. 3. Northern blot analysis of the rat PI4K mRNA in various rat tissues. Total RNAs (30 μg lane) were electrophoresed and transferred to a nylon membrane. The blot was hybridized with 32P-labeled probe. Lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, lung; lane 6, skeletal muscle; lane 7, small intestine; lane 8, spleen; lane 9, thymus; lane 10, testis; lane 11, uterus; lane 12, adrenal gland. Size markers (arrowheads) represent 28 and 18 S rRNAs.

PI(3)P according to Walsh et al. (22). It was revealed that the products catalyzed by the present molecule were almost exclusively PI(4)P (Fig. 4c). The kinase activity was not changed in the presence or the absence of calcium ion (data not shown).

After separation of the lysate into soluble and particulate fractions, the enzymatic activity for the present molecule was recovered predominantly from the particulate fraction (Fig. 4d). The predominant recovery in the particulate fraction was true in case of transfection with the epitope-tagged cDNA (data not shown). In the immunoblotting by the antibody against the FLAG tag, a single immunoreaction band was dominant in the particulate fraction (Fig. 4e). The activity for this molecule was markedly stimulated in the presence of Triton X-100 with increasing concentration from 0.1 to 0.2% but inhibited slightly as the concentration of Triton X-100 exceeded 0.2% (Fig. 5a).

On the other hand, equal amounts of COS-7 cell lysates containing the FLAG-tagged cDNA for this molecule were immunoprecipitated with the anti-FLAG antibody and assayed under various concentrations of adenosine and a constant concentration of 0.3% Triton X-100. The activity for this molecule was relatively insensitive to increasing concentration of adenosine (Fig. 5b).

When COS cells were overexpressed with the epitope-tagged cDNA and immunostained for the FLAG tag, cells immunoreactive for FLAG accounted for approximately 2–5% of the total cell population, and they appeared randomly dispersed in each culture dish. The immunoreactive products were densely aggregated in forms of caps in juxtaposition to the nuclei (Fig. 6a).

No significant immunoreactivity was discerned in any other cell regions such as the cell margins or nuclei. No immunoreactivity was detected in any cells when the transfection was made with the cDNA without the tag (data not shown). In immunoelectron microscopy, the immunoreactive products were localized in the cytoplasmic surface of the membranes of Golgi vesicle and vacuoles and their adjacent cytoplasm. No immunoreactive products were seen on the plasma membrane (Fig. 6c). As a reference, the FLAG epitope tag was fused to the 3' half of cDNA for the present molecule corresponding to the human PI4Kα-cDNA, and COS cells were transfected with the tagged cDNA and immunostained. The resultant light microscopic image was the same as that observed with the full-length cDNA, and the immunoreactive product was largely confined to perinuclear region (Fig. 6b).

By in situ hybridization histochemistry of brain on prenatal day 18, the expression of this novel molecule was detected intensely throughout the mantle zone of fore-, mid-, and hind brain. In the cerebrum, the expression was intense in the...
cortical plate, whereas it was weak in the vetricular zone, and no expression was seen in the intermediate zone (Fig. 7a). On postnatal day 49, the expression was evident more or less throughout the gray matter of the entire brain, among which the hippocampal pyramidal cells, the dentate granule cells, and the cerebellar granule cells expressed the mRNA intensely and the olfactory mitral and granule cells and the cerebral cortex expressed it moderately. Although the expression was weak in the diencephalon and brain stem, no significant expression was detected in the cerebellar medulla (Fig. 7b). When the expression signals in sections from fetal and adult brains were compared by simultaneous exposure of both sections to one and the same Hyperfilm-β max, the expression appeared much higher in the fetal brain than the adult brain, especially in the brain stem. When sections of brains at fetal and adult stages were hybridized with the control probe, a cDNA fragment of about 800 base pairs (PstI–HindIII) from the pBR322 plasmid vector without any insert cDNA, no significant hybridization signals were detected anywhere in the sections (data not shown).

DISCUSSION

We have cloned a mammalian PI 4-kinase species that localizes in hippocampal pyramidal and dentate granule cells and most neuronal cells throughout the gray matter of the brain. Judging from the enhanced activity of PI 4-kinase by Triton, the relative insensitivity to inhibition on the enzyme activity by adenosine, the calculated molecular weight in accord with that so far reported for the purified counterpart enzyme, and the membrane association of the enzyme activity, it is strongly suggested that this enzyme molecule is the first cloned mammalian PI 4-kinase of type III.

When compared with PI4Kα, a human type I PI 4-kinase reported by Wang and Cantley (12), this rat PI 4-kinase molecule has several distinct features: a much higher molecular weight (230 versus 97 kDa) and relative insensitivity to adenosine. On the other hand, several similarities are noted: an activation of kinase activity by detergents, especially an identity (89.6 and 98.2%, respectively), of the 3' half coding region of cDNA and its deduced amino acid sequence for the present rat PI 4-kinase to PI4Kα, and consequently a similar tissue distribution of mRNA between the two molecules except for testis. The similarity is noted in the Northern blotting with either one of the two cDNA probes for the present PI 4-kinase: its 5' coding region, whose counterpart is not contained in the...
PI4Kα, and its 3' coding one, which corresponds to PI4Kα. Although only a single band of 7.8 kb size is evident in any tissues even with the 3' probe in the present study, this may represent a much lower expression of the smaller transcripts due to the species difference. The smaller transcript was weekly detected even in their Northern blotting of human materials.

The association with particulate fraction of this PI 4-kinase activity as observed in the COS expression system does not seem to mean that this enzyme is an intrinsic membrane protein, because no amino acid sequences sharing the significantly extended hydrophobicity is found in its primary structure by the hydropathy analysis (27).

Although there has been no careful analysis of type III versus type II PI 4-kinase activities in subcellular fractions, the activity of PI kinase has been found in intracellular membrane, such as Golgi apparatus and lysosomes in addition to the plasma membrane (28–32). It has also been reported that brain coated vesicles have both type II and III PI 4-kinase activity, whereas red cell plasma membrane has only type II PI 4-kinase activity (6, 7). In this regard, the present study provides the first clear evidence for the localization of PI 4-kinase having the enzymatic characteristics of type III in the Golgi apparatus membranes, although we have to admit a requirement for this conclusion; the exogenously expressed protein should be localized to its normal intracellular compartment in the transfected cells. If this localization is further confirmed in some normal in situ cells immunohistochemically using a specific antibody against this novel molecule itself, it implies that PI 4-kinase play direct roles in the vesicular traffic, the main Golgi function, as suggested by Liscovitch et al. (33), rather than in the

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some yet unknown cytosolic molecules involved in the signal transduction. It may be possible that the prolinerich domain makes a self-association with the SH-3 domain found in this PI 4-kinase in an intramolecular manner.

As noted in the introduction, the calculated molecular weight of human PI4Kα, which was recently reported to show the activities of type II PI 4-kinase by Wang and Cantley (12), was much larger than that of most species of type II PI 4-kinase that had previously been purified from various tissues. In this regard, we should notice again the remarkable identity in the deduced amino acid sequence of human PI4Kα, which was recently reported to show the activity of type II PI 4-kinase from a variety of tissues and species (37) that a monoclonal antibody inhibiting the enzyme activity of type II PI 4-kinase from a variety of tissues and species failed to inhibit that of type III PI 4-kinase in favor of the latter possibility. On this latter assumption, the search for other PI 4-kinase species is under way.

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