Phosphatidylinositol 4-kinases (PtdIns 4-kinases) catalyze the phosphorylation of phosphatidylinositol (PtdIns) on the D-4 position of the inositol ring. The product of this reaction, phosphatidylinositol 4-phosphate (PtdIns4P) is a major precursor in the synthesis of phosphatidylinositolpolyphosphates (PIPs), including PtdIns3,4P2, PtdIns4,5P2, and PtdIns3,4,5P3, which participate in signal transduction, membrane trafficking, and cytoskeletal reorganization (1-5). Two classes of PtdIns 4-kinases, Types II and III, have been identified; the enzyme originally designated Type I was subsequently found to be a PtdIns 3-kinase (6). Mammalian Type II PtdIns 4-kinase (henceforth referred to as PI4KII) is a 55-kDa integral membrane protein believed to account for most of the PtdIns 4-kinase activity in cells (7). It can be distinguished from the Type III kinases (PI4KIIIs) by virtue of its lower $K_m$ values for ATP and PtdIns, its insensitivity to inhibition by wortmannin, and its sensitivity to adenosine and monoclonal antibody 4C5G (8). The two closely related Type III kinases, IIIα and IIIβ, belong to the PtdIns 3/4-kinase superfamily, which also includes the protein kinases TOR/ATM/DNA-PK (9, 10). Although PI4KII was identified more than 30 years ago (11) and purified to apparent homogeneity more than 10 years ago (12-16), it had, until now, been not possible to clone it. Here we report the amino acid sequence of rat PI4KII and show that it represents a large family of putative lipid kinases highly conserved from yeast to humans, but bearing little similarity to other known lipid or protein kinases. Recombinant rat PI4KII has the enzymatic characteristics expected of the Type II kinases, and, despite lacking an obvious transmembrane domain, behaves as an integral membrane protein. This tight membrane association is due to palmitoylation within a cysteine-rich segment in the center of the protein.

**EXPERIMENTAL PROCEDURES**

**Materials—**Q-Sepharose, SP-Sepharose, and DEAE-dextran were from Amersham Pharmacia Biotech; protein G-Sepharose was from Zymed Laboratories Inc.; t-o-phosphatidylinositol was from Avanti Polar-Lipids, Inc.; diC4-pedins, diC7-PtdIns(3)P, diC12-PtdIns4P, and diC24-PtdIns(5)P were from Echelon Research Laboratories Inc.; diethylaminoethylcellulose (DE52) was from Whatman; [γ-32P]ATP and [9,10-3H]palmitic acid were from PerkinElmer Life Sciences; cloning reagents (cat cDNA library, reagents for mutagenesis) were from Stratagen; monoclonal anti-Myc antibody 9E10 was obtained from Cell Signaling Technology; a polyclonal anti-PI4KII antibody 4C5G was from Upstate Biotechnology; dimethyl pimelimidate (DMP) was from Pierce. All other reagents, including ATP, adenosine, phenylarsine oxide (PAO), wortmannin, and protease inhibitors were from Sigma.

**Purification of PI4KII—**PI4KII was partially purified from membranes of bovine adrenal chromaffin granules (17), obtained by floating lysed granules over a 1.2 m sucrose cushion. Membranes were washed in a solution containing 20 mm Tris-Cl, pH 7.5, 0.1 mm EDTA, 1 mm dithiothreitol (buffer A), and a range of protease inhibitors: 0.2 mm phenylmethyl-sulfonyl fluoride and 10 μg/ml each of N2-p-tosyl-L-lysine chloromethyl ester, N2-p-tosyl-L-arginine methyl ester, N2-p-tosyl-L-arginine chloromethyl ketone, leupeptin, and pepstatin A (buffer A) plus 0.5 mm NaCl to remove loosely bound proteins, then solubilized in buffer A containing 0.5% sodium deoxycholate and 1% Triton X-100 and centrifuged. PtdIns 4-kinase was identified by assaying renatured gel slices (13).

The most active band was submitted for amino acid sequence analysis (Argo BioAnalytica, Inc.).

**Purification of PI4KII—**PI4KII was partially purified from bovine...
adrenal medulla following the procedure of Downing et al. (18). Briefly, tissue was extracted with buffer A containing 1 m NaCl, and the fraction of supernatant precipitated at 40% saturation of ammonium sulfate was dialyzed against buffer A containing 30 m NaCl. Dialyzed sample was loaded on a DEAE-cellulose column, and PI4KIII was eluted with a linear 30–500 m NaCl gradient.

Molecular Cloning and Expression of Rat Brain cDNA Encoding PI4KII—Using a 300-bp (EcoRI/HindIII) fragment from a mouse EST (GenBank™ accession number AI385489, obtained from ATCC™), a rat brain ZAP II cDNA library was screened using standard procedures (19), yielding 10 overlapping clones that covered the entire coding region of PI4KII. A 1.5-kilobase pair cDNA fragment encoding the full-length sequence was subcloned into pCMV5Myc vectors. The mycPI4KII cDNA construct was used as the template for generating mutants by site-directed mutagenesis using the QuickChange™ Site-Directed Mutagenesis Kit, and the presence of appropriate mutations was confirmed by DNA sequencing. The deletion mutant ΔCCPCC was created using the following primers: 5′-GGCTTCA-CAAGCTTCTAGGAGGATGGCC-3′ and 5′-GGCAATCTCGGCCAAGAACA-ACAGCTTCTGAAGCC-3′. Truncation mutant 92–487 was made with primers 5′-CCGGAATTCTACACGCCGTTCAGACCCACCGCGAG-3′ and 5′-CCCAGCTTCTACACGCCGTTCAGACCCACCGCGAG-3′. COS cells were transfected with cDNAs of wild type or mutant PI4KII in pCMV5Myc vectors using DEAE-dextran according to Schwartz and Rosenberg (20). Typical transfection efficiency was 20%.

Analysis of Recombinant PI4KII—COS cells transfected with Myc-PI4KII cDNA were washed in PBS; resuspended in solution containing 0.25 m sucrose, 20 m Tris-HCl, pH 7.5, 2 m EDTA, 1 m dithiothreitol, and protease inhibitors; and permeabilized by freezing and thawing. The suspension was centrifuged for 5 min at 1000 × g to obtain post-nuclear supernatant, which was subsequently centrifuged for 15 min at 200,000 × g to obtain cytosol (supernatant) and membrane (pellet) fractions. Pellets were extracted for 15 min on ice with a solution containing 20 m Tris, pH 7.4, 10% glycerol, 0.1 m NaCl, 1% Triton X-100, 1 m dithiothreitol, and protease inhibitors. Insoluble material was removed by centrifugation (15 min at 200,000 × g), and the supernatant, representing the membrane-bound pool, was analyzed by immunoblotting with anti-Myc antibody and assayed for PI4KII 4-kinase activity. The enzymatic activity was assayed using either total Triton X-100 extracts of cell membranes (Fig. 2 b and d–i) or immunoprecipitates obtained with anti-Myc antibody (Fig. 2 c). When membrane extracts were assayed, the activities of similar extracts from mock-transfected cells were subtracted from each data point. For immunoprecipitation of recombinant PI4KII, Triton X-100 extracts of membrane fractions described above were precleared by 30-min incubation with protein G-Sepharose and then incubated with anti-Myc antibodies chemically cross-linked to protein G-Sepharose with DMP.

PtdIns 4-Kinase Assay—PtdIns 4-kinase activity was measured by phosphorylation of PtdIns micelles using [γ-32P]ATP (10 mCi/mmol) as phosphate donor. Typically, kinase (in 20 m Tris, pH 7.5, 10% glycerol, 0.1 m NaCl, 1% Triton X-100, 1 m dithiothreitol, and protease inhibitors) was preincubated for 10 min with PtdIns prepared by sonication with 50 m Tris, pH 7.5, 1 m EGTA, 0.14% Triton X-100, and 0.5 mg/ml bovine serum albumin. Reactions were initiated by adding ATP and MgCl2 at final concentrations of 0.2 m and 15 m, respectively, and carried out at room temperature for 15 min. Phospholipids were extracted according to Bligh and Dyer (21) and separated by thin layer chromatography (TLC) in n-propyl alcohol/H2O/NH2OH (65:20:15) solvent system. For quantification, a range of [γ-32P]ATP concentrations were also spotted on TLC plates. Radioactive spots were detected by autoradiography, scraped, and radioactivity measured by scintillation counting.

Palmitoylation—Cells were radiolabeled with [3H]palmitate (0.3 mCi/ml) for 2 h as described by Linder et al. (22), washed with cold PBS, then lysed with buffer containing 50 m Tris, pH 8.0, 150 m NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, and protease inhibitors for 15 min on ice. Lysates were centrifuged for 15 min at 200,000 × g, and Myc-tagged PI4KII was immunoprecipitated with anti-Myc antibodies as described above. Palmitoylation was detected by autoradiography.

Sequence Analysis—Sequence data base searches were performed using the nonredundant protein data base maintained at the National Center for Biotechnology Information (Bethesda, MD) were performed using the PSI-BLAST program (23) with various parameters run to convergence. The phylogenetic tree was constructed with the PHYLIP package (24) by the distance method (protdist pro-

Fig. 1. Purification and sequence of PI4KII. a, PtdIns 4-kinase activity of fractions eluted from an SP-Sepharose column with a 20–250 m NaCl gradient (upper panel) and Coomassie Blue-stained SDS gel of the same fractions (lower panel). b, assay of PtdIns 4-kinase activity of electrophoresed SP-Sepharose fractions. Pooled fractions from the SP-Sepharose column (cross-hatched in panel a) were electrophoresed on a 7.5% gel. The gel was cut into equal slices, which were then renatured and assayed (13), c, predicted amino acid sequence of rat ( ), PI4KII, and human and yeast homologs ( ). GenBank™ accession number AI385489; Sc, GenBank™ accession number AI385489 identified in the high-throughput genomic sequence data base at GenBank™.
FIG. 2. Characterization of PI4KII overexpressed in COS cells. 
a, distribution of Myc-tagged PtdIns 4-kinase between cytosol (S) and membrane (P) fractions in transfected cells as determined by immunoblotting with anti-Myc antibodies. b, PtdIns kinase activity in cytosol and Triton extract of membranes obtained from COS cells mock-transfected or transfected with rat PI4KII cDNA. c, phosphorylation of different substrates (PtdIns, PtdIns3P, PtdIns4P, and PtdIns5P) by anti-ATP concentration assayed at 80 nM ATP. d–i, the activity of Triton extracts of membranes from overexpressing cells were assayed with PtdIns as substrate. d, dependence of PtdIns 4-kinase activity on ATP concentration assayed at 80 μM PtdIns. e, dependence of activity on PtdIns (P) concentration assayed at 0.2 mM ATP. f, inhibition of PtdIns 4-kinase activity by adenosine. g, effect of PAO on expressed PI4KII and bovine adrenal PI4KIII. h, effect of wortmannin on PI4KII and PI4KIII. i, inhibition of activity by monoclonal antibody 4C5G. PtdIns activities of detergent extracts of membranes were in the range of 28–58 nmol/mg/min and 1.7–5.5 nmol/mg/ml from PI4KII-transfected and mock-transfected cells, respectively. PI4KII activity was on the level of 0.1–0.15 nmol/mg/min.

RESULTS AND DISCUSSION

To obtain amino acid sequence information, PI4KII was partially purified from bovine adrenal chromaffin granule membranes (14) (Fig. 1, a and b). As reported previously (12–16), a major 55-kDa electrophoretic band was prominent in SDS gels of active fractions from an SP-Sepharose ion exchange column. However, a more slowly migrating component, sometimes poorly resolved, was also evident. The gels were sliced, renumbered, and assayed for PtdIns 4-kinase activity. Only the upper, minor component of the 55-kDa doublet was active. The lower band was identified as cytochrome P450 by mass spectrometry.

The electrophoretic band containing PtdIns 4-kinase activity was excised for internal amino acid sequence analysis, and the sequences of five peptides were obtained. Although these sequences did not correspond to those of any known proteins, two peptide sequences were found within a 443-bp mouse EST clone. With this information, a 300-bp nucleotide probe was generated and used to screen a rat brain cDNA library. Analysis of the nucleotide sequences of overlapping clones allowed us to determine the complete amino acid sequence of rat PI4KII, corresponding to a protein of Mr 54,305 (Fig. 1c). All five of the original peptide sequences were found in this full-length sequence. Based on the rat sequence we identified predicted homologs in numerous genome data bases, some of which are presented in the phylogenetic tree in Fig. 4. The sequences of putative human and yeast (Saccharomyces cerevisiae) PI4KIIIs are aligned with the rat sequence in Fig. 1c.

To confirm that our derived sequence encodes an authentic Type II PtdIns 4-kinase, the Myc-tagged recombinant protein was expressed in COS cells, and its properties were characterized (Fig. 2). The expressed protein has an electrophoretic mobility consistent with its molecular weight of ~55,000, and, like endogenous PI4KII, it is found almost exclusively in the membrane fraction after high speed centrifugation (Fig. 2a), but can be solubilized by 1% Triton X-100. Homogenates of cells transfected with rat PI4KII typically have 10-fold higher specific activities than homogenates from untransfected or mock-transfected cells. Membrane fractions account for most of this excess activity (Fig. 2b) correlating well with the distribution of the overexpressed protein.

To determine the substrate specificity of PI4KII, the Myc-tagged kinase was immunoprecipitated with anti-Myc antibodies from overexpressing cells. As shown in Fig. 2c, the immunoprecipitated kinase did not phosphorylate PtdIns3P, PtdIns4P, or PtdIns5P to yield PtdInsP2; only PtdInsP was generated, presumably from trace contaminations of PtdIns in some of the PtdInsP preparations. Thus, PI4KII specifically phosphorylates only PtdIns.

The expressed kinase displays all of the enzymatic characteristics previously reported for the native PI4KII (3, 9, 30, 31):
it has relatively low $K_0$ values for ATP ($\sim 88 \mu M$) (Fig. 2d) and for PtdIns ($\sim 45 \mu M$) (Fig. 2e); it is inhibited by low concentrations of adenosine (Fig. 2f), but not by PAO (Fig. 2g) or wortmannin (Fig. 2h); and it is inhibited by monoclonal antibody 4C5G (Fig. 2i), which specifically recognizes the Type II kinase (8). In contrast, the Type III PtdIns 4-kinases have relatively high $K_0$ values for ATP (>400 $\mu M$) and PtdIns ($\sim 120 \mu M$), require millimolar adenosine concentrations for inhibition, and are sensitive to wortmannin (Fig. 2k) and PAO (Fig. 2q).

Although both native and recombinant forms of PI4KII behave as integral membrane proteins, sequence analysis does not reveal an obvious transmembrane domain. The only portion of the rat enzyme with a predominantly hydrophobic character is an alanine/valine-rich segment (residues 74–95) that is predicted to adopt an $\alpha$-helical conformation. However, a truncation mutant (92–487) lacking most of this hydrophobic segment still behaves as an integral membrane protein, i.e. it is soluble only in the presence of detergent (Fig. 3b). An alternative mode of tight membrane association is through covalently attached lipids. Rat PI4KII does not contain consensus myristoylation or prenylation motifs, but a cysteine-rich segment (CCPCC, residues 173–177) is a potential palmitoylation site. Indeed, wild type PI4KII and truncation mutant 92–487 are palmitoylated in cells, whereas a mutant lacking residues 173–177 (ΔCCPCC) is not (Fig. 3c). Both ΔCCPCC and the 92–487,ΔCCPCC double mutants behave as peripheral, rather than integral, membrane proteins; they can be solubilized by sodium carbonate, pH 10, though not by 1M NaCl (Fig. 3d). The ΔCCPCC mutant is catalytically inactive, but there is insufficient evidence to conclude from this that palmitoylation is essential for activity.

A search of protein sequence data bases using the PSI-BLAST program (23) identified PI4KII homologs in Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, S. cerevisiae, and other organisms (Fig. 4). The only prokaryotic sequences that belong to this family are from Mycobacterium, the organism in which inositol-containing lipids were first identified (32).

In addition to the putative orthologs of rat PI4KII, another family of more distantly related sequences was found using the PSI-BLAST program. This group is designated FJ-like putative kinases in Fig. 4 for its best characterized member, Drosophila Four-jointed, a membrane-bound or secreted protein with no known enzymatic activity, but apparently involved in development of ommatidia (33). Although it is much too early to assign lipid or protein kinase activity to Four-jointed protein or its mouse and human orthologs, the homology to known kinase catalytic domains merits further examination.

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