Isolation and Molecular Cloning of Wortmannin-sensitive Bovine Type III Phosphatidylinositol 4-Kinases*

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Agonist-sensitive phosphoinositide pools are maintained by recently-identified wortmannin (WT)-sensitive phosphatidylinositol (PI) 4-kinase(s) (Nakanishi, S., Catt, K. J., and Balla, T. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 5317–5321). Two loosely membrane-associated WT-sensitive type III PI 4-kinases were isolated from bovine adrenal cortex as [3H]WT-labeled 110- and 210-kDa proteins. Based on peptide sequences from the smaller enzyme, a 3.9-kilobase pair (kb) cDNA with an open reading frame encoding a 90-kDa protein was isolated from a bovine brain cDNA library. Expression of this cDNA in COS-7 cells yielded a 110-kDa protein with WT-sensitive PI 4-kinase activity. Northern blot analysis of a human mRNA panel showed a single ∼3.8-kb transcript. Peptide sequences obtained from the 210-kDa enzyme corresponded to those of a recently described rat 230-kDa PI 4-kinase. A 6.5-kb cDNA containing an open reading frame of 6129 nucleotides that encoded a 7.5-kb transcript. The molecular cloning of these novel WT-sensitive type III PI 4-kinases will allow detailed analysis of their signaling and other regulatory functions in mammalian cells.

Phosphatidylinositol (PI)4-kinases were first recognized as the enzymes that provide PI 4-phosphate for the synthesis of PI(4,5)P2, the precursor of two important intracellular messengers, inositol 1,4,5-trisphosphate and diacylglycerol (1). Early studies indicated that multiple PI 4-kinase activities are present in cellular homogenates, based on their individual sensitivities to detergents (2, 3). It has been widely accepted that the major PI 4-kinase activity is present in the plasma membrane and regulates the synthesis of phosphoinositides destined for hormone-regulated hydrolysis (3). This tightly membrane-bound activity has been purified from detergent extracts of various membranes including the red blood cell membrane (4–7), which contains a ∼56-kDa enzyme termed type II PI 4-kinase. Another form of PI 4-kinase, the type III enzyme, was subsequently described in detergent extracts of bovine brain membranes and differed from the type II enzyme by its larger size (over 200 kDa based on sedimentation characteristics), and lower affinity for both ATP and PI (8).

Receptor-mediated regulation of PI 4-kinase activity has been indicated by the rapid increases of PI(4)P levels observed in agonist-stimulated cells (9–11), but few data are available to support direct regulation of this enzymatic activity by either G protein-coupled or growth factor receptors (12–14). Increased PI kinase activity is associated with activated receptor and non-receptor tyrosine kinases and viral oncoproteins (15–17), but this activity phosphorylates PI on the 3- rather than the 4-position of its inositol ring (18). This enzyme, termed type-I PI kinase or PI 3-kinase, contains a 110-kDa catalytic and an 85-kDa regulatory subunit. It produces 3-phosphorylated phosphoinositides by utilizing PI, as well as PI(4)P and PI(4,5)P2, as substrates (see Ref. 19 for a review). Several PI 3-kinases have been purified and cloned (20–24), and some of these, such as the yeast Vps34p, only phosphorylate PI and interact with a 150-kDa subunit. A 210-kDa form of PI 3-kinase has also been reported in Drosophila (24).

Recently, the fungal metabolite wortmannin (WT), a potent inhibitor of PI 3-kinases, has been used to define cellular functions that are regulated by PI 3-kinases. Although WT was not believed to inhibit PI 4-kinases (25–27), we observed that micromolar concentrations of the compound abolish the sustained formation of inositol 1,4,5-trisphosphate in agonist-stimulated cells by inhibiting a PI 4-kinase enzyme (28). Further characterization of this soluble (loosely membrane-bound) WT-sensitive PI 4-kinase activity demonstrated its similarity to the type III PI 4-kinase and showed that the bovine brain type III enzyme (as originally described) displays similar WT sensitivity (29). In contrast, the major cellular PI 4-kinase activity, the type II enzyme, was insensitive to WT and thus unlikely to participate in the synthesis of hormone-sensitive phosphoinositide pools (29). The sensitivity of the type III PI 4-kinase(s) to WT (although significantly less than that of PI 3-kinases) raised the possibility that they have structural similarities to PI 3-kinases.

In the present study we report the purification of 110- and 210-kDa WT-sensitive, type III PI 4-kinase enzymes from the bovine adrenal cortex, and the molecular cloning of cDNAs encoding these novel enzymes.

**EXPERIMENTAL PROCEDURES**

Materials—DEAE-Sepharose, SP-Sepharose (bulk media), and heparin-Sepharose, butyl-Sepharose, MonoQ, and MonoS columns were from Pharmacia Biotech (Uppsala, Sweden). Phosphatidylinositol and ATP were from Fluka (Ronkonkoma, NY) and Sigma, respectively.
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[y-32P]ATP (6000 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were from Amersham, and [3H]wortmannin-17-ol (19.7 Ci/mmol) was from NEN Life Science Products. Reagents for SDS-PAGE were obtained from Bio-Rad, CA), and restriction enzymes were purchased from Life Technologies, Inc. or New England Biolabs (Beverly, MA). The T/α cloning kit from Promega (Madison, WI) and all other chemicals were of HPLC or analytical grade.

Isolation of PI 4-Kinase Activity—In the final purification, 60 bovine adrenal cortices were homogenized in three batches (20 each) in Buffer A (20 mM Tris/HCl, pH 7.3, 1 mM EDTA, 1 mM dithiothreitol, 100 μM AEBSF, and 10 μg/ml leupeptin) containing 1 mM NaCl as described previously (30), except that the volumes were scaled down to accommodate the smaller scale. All manipulations were performed at 4 °C unless otherwise indicated.

After centrifugation (100,000 g, 20 min), the supernatant was taken up to 40% ammonium sulfate (40% saturation) and the precipitated material collected by centrifugation (10,000 g, 20 min) and the pellets stored at −70 °C until further processing. Ammonium sulfate precipitates were dissolved in 100 ml of Buffer A and dialyzed overnight at 4 °C against 10 volumes of Buffer B containing 30 mM NaCl. The dialyzed protein solution was then diluted to 500 ml with Buffer A/30 mM NaCl and applied to a 5 × 70-cm DEAE-Sepharose column pre-equilibrated with the same buffer. After loading, the column was washed with 2 liters of Buffer A, 30 mM NaCl and eluted with a linear gradient of 0.05–0.5 mM NaCl in Buffer B at a flow rate of 10 ml/min. The resulting digest was separated at 0.25 ml/min with a gradient described by Fernandez et al. (31) on a narrow bore RP 8.2 × 150 cm Vydac 218TP525 column and guard column (Separations Group, Hesperia, CA) at 35 °C using a System Gold HPLC equipped with a model 570 autosampler, model 126 programmable solvent module, and model 168 diode array detector (Beckman, Fullerton, CA). The column effluent was monitored at 215 and 280 nm, and fractions collected at 30-s intervals were stored at −70 °C. Fractions (125 μl) containing tryptic peptides were applied in 30-μl aliquots to Biobrene (Applied Biosystems, Foster City, CA)-treated glass fiber filter and dried prior to amino acid sequencing on a model 477A pulsed-liquid protein sequencer equipped with a model 120A PTH analyzer (Applied Biosystems) using methods and cycles supplied by the manufacturer. Data were collected and processed by a model 610A data analysis system (Applied Biosystems) using software in the GCG-Swiss Protein Data base (University of Wisconsin Genetics Computer Group).

[3H]Wortmannin Binding Experiments—Aliquots of the fractions eluted from the various columns were incubated for 20 min at room temperature in a total volume of 100 μl of PBS containing 0.4 μCi of [3H]PI 3-kinase (New England Nuclear), 10 μM [3H]wortmannin-17-ol (19.7 Ci/mmol) was from NEN Life Science Products and ethanol preparation was used instead of trichloroacetic acid (since WT binding has been reported to be acid-labile), there was no difference in the protein labeling. After fixation and Coomassie staining, the gels were impregnated with EN3HANCE (NEN Life Science Products) solution, and after drying were exposed at −70 °C for 1–2 weeks with Hyperfilm (Amersham).

Molecular Cloning of PI 4-Kinases—A size-enriched cDNA library was constructed from the PCP 1 fraction from bovine adrenal cortex using the GeneRacer kit (Ambion). The cDNA was size-enriched on sucrose-fractionated (5–20% sucrose–fractionated (>2.5 kb) polyA)-selected RNA and the SuperScript™ plasmid system for cDNA cloning (Life Technologies, Inc.) according to the manufacturer’s instruction. This library contained 1.25 million primary clones and after amplification was stored in aliquots of glycerol stock at −70 °C.

An oligonucleotide primer was designed from the peptide sequence, QLQSIWEQE, obtained from tryptic fragments of the 110-kDa PI 4-kinase, and an antisense primer based on the conserved KDRHNGN sequence that is common to all known PI 4-kinases. PCR amplification using this primer pair (5′-ctgcaRtctatgtagaacgg-3′ and 5′-ttttgaatcgtgtttggctt-3′) yielded a 300-bp product, which was ligated into the PGEM- Easy plasmid (Promega) and subjected to dideoxy sequencing. This DNA fragment, which encoded an amino acid sequence with high homology to PIK1, was random primer-labeled and used to screen the size-enriched bovine brain cDNA library. Homology search of the data base with the 300-bp sequence also revealed high homology to a rat EST (R46930) (Y-162) that was subsequently provided by Dr. Y. Yamada (NIDR, National Institutes of Health) and on sequencing showed extensive homology with PIK1. The 1.3-kb EcoRI/XhoI insert of this clone was ligated into the pcDNA3 plasmid from bovine adrenal cortex in subsequent experiments. Positive colonies were isolated and the plasmids cut with EcoRI/NsiI restriction enzymes to determine their insert-size. Several clones were isolated and sequenced, the longest of which (c354) contained a 3-kb insert. The missing 5′ end of the mRNA was obtained by 5′-RACE (version 2.0, Life Technologies, Inc.) following the manufacturer’s instructions. The full-length clone used for transfection studies was synthesized by life Technologies, Inc.) from cDNA prepared by reverse transcriptase (Superscript, Life Technologies, Inc.; Rethotherm, Epicentre Technologies) from mRNA isolated from cultured bovine adrenal glomerulosa cells. The primers used for this amplification were (5′-agttgccaagatggcagctcag-3′ and 5′-gagcaatctagattgcaacacag-3′) and the 3.3-kb product was ligated into the pCDNA3.1 (+) plasmid (Invitrogen, Carlsbad, CA) after digestion with XbaI/BamHI.

The isolation of the cDNA encoding the 210-kDa enzyme was initially attempted by colony hybridization of the brain cDNA library with a PCR-amplified fragment of the human PI4Kα, due to the homology indicated by the peptide sequences obtained from the 210-kDa bovine protein. The longest insert isolated was a 3.3-kb product that lacked a poly(A) tail due to the use of NotI enzyme during creation of the cDNA library and the presence of an internal NotI site after the stop codon in this sequence. The 3′-untranslated region was then obtained by 3′-RACE, and the 5′ end of the transcript was amplified with PCR using primers based on the rat sequence (32) that became available during the course of these studies. Completion of the 5′ sequence information was achieved by amplification from the cDNA library with the primers designed from the flanking NotI sites (NIDR, National Institutes of Health). Full-length expressable clones were created by long-range PCR amplification from the cDNA of bovine adrenal glomerulosa cells (primers: 5′-gagcttacctggc-ggcgccggcttgggagg-3′ and 5′-ggaggattccacaacagcagcctggcttgaattgc-3′) and ligation of the 6.3-kb product into the pCDNA3.1 (+) plasmid after digestion with BamHI and EcoRI.

Northern Blot Analysis—Northern blot analysis was performed uti-
zizing an mRNA panel of several human tissues (CLONTECH, Palo Alto, CA) and the random primer-labeled 1.8-kb fragment of the EcoRI digest of one of the clones (c365) (corresponding to nucleotides 1960–3750 of PI4KIIIα) or the full 2.9-kb insert of a partial clone of PI4KIIIα (c2D5) (3286–6200). After prehybridization at 42 °C in Hybrisol I (Oncor, Gaithersburg, MD), hybridization was performed overnight at 42 °C. The blot was washed several times with increased stringency with a final wash of 0.2 x SSC, 0.1% SDS at 55 °C for 10 min. The blots were analyzed both by autoradiography and by a PhosphorImager (Molecular Dynamics).

Expression of the Enzyme in COS-7 Cells—COS-7 cells were grown to about 70% confluence in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum on 10 cm culture dishes. Cells were transfected with 5 ml of Opti-MEM medium containing 10 μg/ml LipofectAMINE (Life Technologies, Inc.) and 5 μg plasmid DNA (pcDNA3.1(+) containing the PCR-amplified clone of PI4KIIIα. After 8 h the medium was replaced with Dulbecco’s modified Eagle’s medium/10% fetal bovine serum and culture was continued for selected periods. PI 4-kinase activity was then measured in the soluble fractions after lysing the cells in 500 μl of ice-cold Buffer A containing 150 mM NaCl, followed by sonication and centrifugation at 14,000 x g for 30 min at 4 °C. For [3H]WT binding, the supernatants obtained from two such plates were combined, diluted, and applied to 1-ml DEAE-Sepharose columns, and after washing, eluted with 500 mM NaCl in Buffer A. This eluent was then concentrated on Amicon filters and subjected to PI 4-kinase activity measurement and [3H]WT binding followed by SDS-PAGE.

RESULTS

Soluble Extracts of Bovine Adrenal Cortex Contain Two Distinct WT-sensitive PI 4-Kinase—As reported previously (29), two PI 4-kinase activities with native molecular sizes of ~110 and ~200 kDa were identified and partially purified from the bovine adrenal cortex. The catalytic properties of the two components were indistinguishable, raising the possibility that the two peaks represent monomeric and dimeric forms of the same enzyme or that the larger is a heterodimer associated with another protein subunit. Based on the presumed similarity with PI 3-kinases, a purification procedure similar to that used for preparation of PI 3-kinases (33, 34) was employed by se-
Sequential chromatographies on DEAE-Sepharose, SP-Sepharose, heparin-Sepharose, butyl-Sepharose, MonoQ, and MonoS columns (Fig. 1). Two activities were clearly separated on MonoQ chromatography, and \(^{3}H\)WT-17-ol-binding (35) was used to correlate the PI 4-kinase activity of the effluent fractions with the \(^{3}H\)WT labeling of the proteins. As shown in Fig. 2, SDS-PAGE analysis of the WT-labeled proteins revealed that the two peaks of PI 4-kinase activity correlated with two labeled proteins of \(\sim 210\) and \(\sim 110\) kDa. The radioactivity bound to these proteins was only slightly reduced by preincubation of the fractions with 10 nM unlabeled WT (Fig. 3). Such treatment greatly reduced the labeling of PI 3-kinase(s) (data not shown), consistent with the lower affinity of these enzymes for WT. Similar treatment greatly reduced the labeling of PI 3-kinase (data not shown).

Due to their relatively low abundance, these proteins were not purified to homogeneity even after passage through multiple chromatography steps. However, WT labeling allowed their clear identification on SDS gels after Coomassie staining. The two protein bands of interest were then cut out from the gels and digested with trypsin. Only one unequivocal peptide sequence was obtained from the larger enzyme: EFDFFNK, which showed homology to a recently cloned human PI 4-kinase, PI4K\(^{\alpha}\) (36), and to the yeast PI 4-kinase, STT4 (37). Other peptide peaks from the digest were mixtures of peptides that yielded multiple sequences that were homologous to PI4K\(^{\alpha}\) (data not shown). Further purification of the peptides was not attempted because of the minute amounts available. Although the predicted molecular size of PI4K\(^{\alpha}\) is only \(97\) kDa, we concluded that it probably represents a smaller splice variant of the 210-kDa bovine enzyme, which would be the mammalian homolog of the yeast 200-kDa STT4.

Tryptic digestion of the smaller protein yielded seven unequivocal peptide sequences (LSEQALHTPTAFK, QLQSIWEQE, VENEDEPVR, LATLPTK, EF1K, EPVFIAAGDIR, and EPGYQA). None of these exhibited homology to any known protein sequence available in the database. However, a computer alignment of the three human PI 3-kinases (\(a, \beta, \gamma\)), and the 125-kDa yeast PI 4-kinase, PIK1, together with one of the sequences, QLQSIWEQE, revealed a possible alignment for the latter (see Fig. 8). This served as the basis for designing primers so that further sequence information could be obtained from this enzyme.

Molecular Cloning of the Two PI 4-Kinases—A primer was designed based on the comparison of the nucleotide sequences encoding QLQSIWEQE, and the corresponding nucleotide sequences of PIK1 and the three PI 3-kinases based on the putative amino acid alignment described above. This primer was used in combination with another primer designed on a conserved sequence (KDRHNGN) that is present in all of the then-known PI 4-kinases (PIK1, STT4, and PI4K\(^{\alpha}\)) but differs slightly in all PI 3-kinases (GDRHN\(X\)N). Amplification of a size-enriched bovine brain cDNA library with these primers yielded a 300-bp product with a nucleotide sequence showing extensive homology with PIK1. Screening of the size-enriched bovine brain cDNA library with this product yielded several clones, one of which contained a 3.0-kb insert (c354). Sequencing of this clone confirmed its homology with PIK1, especially within its catalytic domain, and revealed an open reading frame that encoded all seven peptide sequences obtained from the purified protein. Northern blot analysis showed that a
single transcript of ~3.8 kb was present in several tissues (Fig. 6A), and 5'-RACE was employed to capture the missing 5' region of the mRNA. The full-length transcript was found to be 3859 bp with an open reading frame of 2403 nucleotides that encodes a protein of 90 kDa (Fig. 4). Interestingly, one of the isolated clones contained an additional stretch of 48 nucleotides encoding a 16-amino acid serine-rich sequence that is intercalated within one of the peptide sequences obtained from the purified protein, and must represent a splice variant of the enzyme. This sequence was not present in clones that were obtained by PCR for mammalian expression studies and probably represents a minor variant of the enzyme (Fig. 4).

The peptide sequence (EFDFPNK) that was obtained from the larger enzyme indicated its similarity to the 200-kDa yeast PI 4-kinase, STT4 (37) and to a smaller human PI 4-kinase, PI4Ka (36). This and other less certain peptide sequences that were obtained from peptide mixtures of the enzyme digests of the 210-kDa bovine PI 4-kinase suggested that this enzyme is a mammalian homolog of the yeast enzyme, STT4, and a larger form of PI4Ka. Screening of the bovine cDNA library with a cloned fragment (1130–2908) of PI4Ka yielded several clones, one of which contained a 3.3-kb insert. Sequencing of these clones showed their homology with PI4Ka, but revealed a difference in the 5' end. While this work was in progress, the cloning of a rat 230-kDa PI 4-kinase that showed strong sequence homology with the sequenced bovine clones was reported (32). The 5' end of the bovine sequence was obtained by PCR, using primers based on the 5' sequence of the rat enzyme and the longest bovine cDNA clone, and 5'-RACE was used to determine the bovine sequence that corresponded to the 5' primer (rat) and 5'-flanking sequences. In addition, the 3'-untranslated region of the mRNA was obtained with 3'-RACE, since all the clones were truncated after the stop codon due to a Nots site in the sequence and the use of Nots during construction of the cDNA library. The full-length sequence of 6520 nucleotides that was reconstructed in this way encoded a 230-kDa protein that showed 92% homology at the protein level with the rat PI 4-kinase enzyme (32) (Fig. 5). Northern blot analysis showed the presence of a prominent ~7.5-kb transcript and minor amounts of smaller transcripts in some tissues, notably the placenta (Fig. 6B).

Expression of the 110-kDa PI 4-Kinase in COS-7 Cells—Because of the similarity of the larger bovine PI 4-kinase to the recently characterized 230-kDa rat enzyme, (32), our expression studies were focused on the smaller enzyme. A full-length clone of the 110-kDa enzyme was obtained using RT-PCR of mRNA isolated from bovine adrenal glomerulosa cells with long range PCR and the mammalian expression plasmid pcDNA3.1(+) (see “Experimental Procedures” for details). Plasmid DNA was expressed in COS-7 cells, and kinase activity was measured in the soluble fractions after homogenization and sonication of the transfected cells. As shown in Fig. 7, the transfected cells contained increased PI 4-kinase activity, which after chromatography on DEAE Sepharose minicolumns was completely inhibited by 1 μM WT. Control COS-7 cells that were treated only with LipofectAMINE also contained some endogenous WT-sensitive PI 4-kinase. Several [3H]WT-labeled bands were present in mock-transfected COS-7 cells and showed no change upon transfect-
FIG. 5. Nucleotide and predicted amino acid sequence of the 210-kDa type III bovine PI 4-kinase. Only the unequivocal peptide sequence obtained from the purified 210-kDa protein is shown underlined.
panels. PI 4-kinase. were subjected to analysis by a PhosphorImager after 24 h of exposure. Procedures.” After washing (0.2 SSC, 0.1% SDS, 55 °C), the membranes were hybridized with random-prime-labeled cDNA probes for the respective enzymes as described under “Experimental procedures.”

RNA (CLONTECH) were hybridized with random-prime-labeled cDNA for the 110-kDa (PI4KIII b, upper panels) and the 210-kDa (PI4KIII a, lower panels) bovine adrenal PI 4-kinase. Membranes containing 2 μg of poly(A)+-selected human RNA (CLONTECH) were hybridized with random-prime-labeled cDNA probes for the respective enzymes as described under “Experimental Procedures.” After washing (0.2 SSC, 0.1% SDS, 55 °C), the membranes were subjected to analysis by a PhosphorImager after 24 h of exposure.

The most prominent of these was a large protein of ~250 kDa that is probably a member of the family of PI kinase-related enzymes (38). The endogenous 210-kDa kinase of COS-7 cells showed only very faint labeling, and the endogenous 110-kDa labeled band is probably a mixture of PI 3- and 4-kinases.

Phosphorylation of phosphatidylinositol by PI 4-kinases has long been considered as the initial reaction for the synthesis of membrane phosphoinositides that serve as precursors for the agonist-stimulated formation of inositol 1,4,5-trisphosphate and diacylglycerol. While the most abundant cellular PI 4-kinase, the tightly membrane-bound type II enzyme, was believed to be the most likely candidate to perform this function, our recent studies revealed that the sustained formation of hormone-sensitive inositol pools requires the participation of a WT-sensitive PI 4-kinase enzyme (28). Our analysis of the sensitivity of the various cellular PI 4-kinases to WT also showed that only the type III and not the type II enzymes show such sensitivity (29). Purification of the WT-sensitive, loosely membrane-associated type III PI 4-kinase from the bovine adrenal cortex revealed it to be a mixture of two major activities with molecular sizes of ~200 and 110 kDa (29). [3H]WT labeling and subsequent SDS-PAGE analysis confirmed the existence of two separate proteins of 210 and 110 kDa, both of which showed catalytic properties characteristic of type III PI 4-kinases and similar WT sensitivities (29).

Based on the expected similarity between these enzymes and PI 3-kinases, we employed a purification scheme that was successful for the isolation of PI 3-kinases (35, 34). Although these separation methods were also applicable to the PI 4-kinases, the stronger interaction of these enzymes with the ion exchangers did not allow as efficient separation from the majority of proteins as in the case of PI 3-kinases. Nevertheless, a sufficient amount of protein was obtained to permit petide sequences to be obtained from direct digests of the SDS gel slices containing the two proteins of interest.

Isolation and analysis of cDNA clones encoding these two enzymes confirmed their identity with the purified proteins and revealed that they were mammalian homologs of the yeast STT4 and PIK1 enzymes. Although the overall homology compared with the respective yeast enzymes is low (24% and 16% on the protein level for the bovine 110-kDa enzyme versus PIK1 and the 210-kDa enzyme versus STT4, respectively), these enzymes show a large degree of conservation within their lipid kinase/protein kinase and lipid kinase unique domains. However, sequence comparison within the kinase domain clearly defined two groups of the type III PI 4-kinases (Fig. 8 and Ref. 36). The marked homology of the 210-kDa enzyme with the much shorter (97 kDa) human PI4Ko enzyme described by Wong and Cantley (36) is consistent with the possibility that the latter might represent a splice variant of the human homolog of the bovine enzyme. The reason for the difference between the catalytic properties of the 210-kDa enzyme (type III) and those reported for the expressed PI4Ko (type II) is not clear at present. However, while these studies were in progress, a novel cDNA that encodes a 230-kDa rat PI 4-kinase was isolated by homology cloning (32) and the purification and partial cloning of a 200-kDa bovine brain PI 4-kinase was reported (39). Although their WT-sensitivities were not examined, both of these larger enzymes were identified as type III PI 4-kinases that are very similar or identical to the 210-kDa enzyme reported in the present study. Based on these findings, we propose the term PI4KIII a to denote the identity of this larger WT-sensitive enzyme as a type III form with homology to human PI4Ko, and PI4KIII b for the 110-kDa bovine PI 4-kinase.

The cDNA sequence encoding the smaller WT-sensitive enzyme, PI4KIII b, contained all seven peptide sequences in an open reading frame for a 90-kDa protein. Although this enzyme is a homolog of the yeast PIK1, its sequence similarity is confined largely to the C-terminal third of the molecule that contains the catalytic domain (Fig. 8A). Unlike the yeast enzyme, in which the lipid kinase unique domain is located near the N-terminal end, in the bovine enzyme this domain is more distantly positioned from the N terminus (Fig. 8B). Interestingly, in all three PI 3-kinases (α, β, and γ), as well as in the larger PI 4-kinases, this domain is even further away from the N-terminal part of the molecule (36). PI4KII b also contains a proline-rich sequence in its N-terminal region (Fig. 8). This sequence may promote the interaction of this enzyme with SH3 domains (40), but may also serve as an N-terminal processing signal as suggested in the case of cytochrome P450 enzymes (41). This kinase could also interact with membranes through the putative myristoylation site at its N-terminal end. In contrast, interaction of the larger enzyme, PI4KII a, with membranes may also be aided by its putative plekstrin homology domain located between its lipid kinase unique and lipid kinase/protein kinase catalytic domains as described for PI4Ko and STT4 (36). The shorter bovine PI 4-kinase, PI4KII b, showed closest homology to P3Kα of the PI 3-kinase family members. We found no sequence homology with PI 3-kinases that would suggest the interaction of this protein with the p85 regulatory protein of PI 3-kinase, and no indication was found...
during purification for the existence of heterodimers of the PI 4-kinases. The relatively large difference between the calculated molecular size of the protein and its apparent size on SDS-PAGE raises the possibility that the enzyme undergoes posttranslational modification. Similarly, the yeast enzyme PIK1 shows a larger apparent size on SDS gels (125 kDa) than its calculated molecular size (119 kDa).

Both the 110- and 210-kDa enzymes were found to be sensitive to WT, although at higher concentrations than those needed to inhibit PI 3-kinases (29). A recent study identified Lys-802 of PI3Kα as the site to which WT binds covalently within the putative ATP-binding domain (42), probably with...
the help of additional interactions with other residues, including Glu-821, Ser-919, and His-936 of PI3Kα (42). Although the residue corresponding to Lys-802 is highly conserved among all PI kinases, including the two enzymes described in this study, WT sensitivities show great variations even within PI 3-kinases. This suggests that the stabilization of WT binding by other residues is an important determinant of the inhibitory potency of this compound. Interestingly, none of the additional residues named above are conserved between PI 3- and PI 4-kinases. It is important to note that the WT sensitivity of these enzymes is dependent on the experimental conditions, in particular on ATP concentration, incubation time, and pH. Since both of the enzymes have a high $K_m$ for ATP, it is quite likely that at the prevailing ATP concentrations in intact cells, their WT sensitivity is even lower than under in vitro conditions. These factors must be considered when interpreting data on the WT sensitivity of cellular responses.

The physiological roles and modes of regulation of the multiple PI 4-kinases are not clear at present. However, our data on the WT sensitivity of the maintenance of agonist-sensitive PI(4,5)P_2 pools indicate that one or both of the currently described enzymes participate in this process. In the yeast, deletion of PIK1 but not STT4 is lethal. The latter mutant has an osmolarity-dependent phenotype (37, 43) and increased staurosorine sensitivity, indicating its possible connection with PKC-dependent pathways. PIK1 has also been cloned from Saccharomyces cerevisiae with the aid of antibodies raised against the nuclear pore complex and is presumably also present in the nucleus (44), but its function (if any) there is not known. The localization of the epitope-tagged 230-kDa rat PI 4-kinase was largely Golgi-associated in overexpressing COS cells (32), but this could reflect the artificially high production of the protein in such cells. The role of the very abundant 4-kinase was largely Golgi-associated in overexpressing COS cells (32), but this could reflect the artificially high production against the nuclear pore complex and is presumably also present in the nucleus (44), but its function (if any) there is not known.

In summary, the present results describe the identification, isolation, and molecular cloning of two WT-sensitive PI 4-kinase enzymes from the bovine brain and adrenal cortex. These enzymes are mammalian homologs of two yeast PI 4-kinases, PIK1 and STT4, that appear to have clearly distinct functions in yeast.

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Addendum—Shortly after the completion of this study, Meyers and Cantley (51) and Nakagawa et al. (52) reported the cloning of a WT-sensitive human and rat PI 4-kinase, respectively, (40) that are homologs of the bovine PI4KIIIβ described in this study.

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