Characterization of PKIγ, a Novel Isoform of the Protein Kinase Inhibitor of cAMP-dependent Protein Kinase

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Attempts to understand the physiological roles of the protein kinase inhibitor (PKI) proteins have been hampered by a lack of knowledge concerning the molecular heterogeneity of the PKI family. The PKIγ cDNA sequence determined here predicted an open reading frame of 75 amino acids, showing 35% identity to PKIα and 30% identity to PKIβ1. Residues important for the high affinity of PKIα and PKIβ1 as well as nuclear export of the catalytic (C) subunit of cAMP-dependent protein kinase were found to be conserved in PKIγ. Northern blot analysis showed that a 1.3-kilobase PKIγ message is widely expressed, with highest levels in heart, skeletal muscle, and testis. RNase protection analysis revealed that in most tissues examined PKIγ is expressed at levels equal to or higher than the other known PKI isoforms and that in several mouse-derived cell lines, PKIγ is the predominant PKI message. Partial purification of PKI activities from mouse heart by DEAE ion exchange chromatography revealed two major inhibitory peaks, and isoform-specific polyclonal antibodies raised against recombinant PKIα and PKIγ identified these inhibitory activities to be PKIα and PKIγ. A comparison of inhibitory potencies of PKIα and PKIγ expressed in Escherichia coli revealed that PKIγ was a potent competitive inhibitor of Cα phosphotransferase activity in vitro (K_i = 0.44 nM) but is 6-fold less potent than PKIα (K_i = 0.073 nM). Like PKIα, PKIγ was capable of blocking the nuclear accumulation of Flag-tagged C subunit in transiently transfected mammalian cells. Finally, the murine PKIγ gene was found to overlap the murine adenosine deaminase gene on mouse chromosome 2. These results demonstrate that PKIγ is a novel, functional PKI isoform that accounts for the previously observed discrepancy between PKI activity and PKI mRNA levels in several mammalian tissues.

The cAMP-dependent protein kinases (PKAs)1 comprise a subfamily of serine/threonine kinases that are activated by increases in intracellular concentrations of cAMP. Members of this family play a central role in the coordination of cellular responses to both hormones and neurotransmitters. Upon activation of receptors coupled to adenylate cyclase, intracellular concentrations of cAMP rise, and cAMP binds to each of two regulatory (R) subunits of the inactive tetrameric holoenzyme complex, releasing C subunit. Once released, C subunit phosphorylates both cytoplasmic and nuclear substrates that can alter the rate of cell division, cellular morphology, membrane ion permeability, metabolic enzyme activity, or levels of gene transcription (1–3).

Extensive biochemical characterization and molecular cloning studies have identified three C subunit (Cα, Cβ, and Cγ) and four R subunit isoforms (RIα, RIIβ, IIα, and RIIβ) (4, 5). Although the amino acid sequences of Cα and Cβ are highly similar (6), they differ significantly in their tissue distributions and interactions with R subunits. Cα is widely expressed in mammalian tissues, and Cβ is expressed in cells of the nervous, endocrine, and reproductive systems (6, 7). Importantly, PKA holoenzymes formed with RIα and Cβ have a 5-fold lower K_i value for cAMP than RIα and Cα containing complexes (8). Like the C subunits, the R subunit isoforms also show heterogeneity due to differences in their tissue distributions, subcellular localizations, and interactions with C subunit. The RII isoforms are localized to different parts of the cytosol via their interactions with cAMP-dependent protein kinase-anchoring proteins (9, 10), and RI isoforms are generally cytoplasmic. Furthermore, holoenzymes containing RIα are less sensitive to increases in cAMP than those containing RIβ (11, 12). Several of these isoform-specific differences have been verified in whole animal studies (13–15).

In addition to the R subunits that inhibit the activity of the C subunit in a cAMP-regulated manner, there is a second level of regulation of PKA activity by protein kinase inhibitor (PKI) proteins. The PKIs are specific and potent inhibitors of the C subunit; however, unlike R subunits, PKI inhibition of C subunit is not relieved by cAMP. Due to the low levels of PKI activity found previously in tissues relative to C subunit activity and the high binding affinity of the PKIs for C subunit, it has been proposed that the PKIs may regulate basal PKA activity (16, 17).

To date, biochemical characterization and molecular cloning of cDNAs encoding PKIs have demonstrated that at least two distinct PKI genes are expressed in mammals, PKIα and PKIβ (18–21). The PKIα isoform is highly expressed in heart, skeletal muscle, cerebral cortex, and cerebellum (18, 21), whereas the PKIβ isoform is most highly expressed in testis (21). Similar to R subunits, both PKI isoforms are pseudosubstrate, competitive inhibitors (19, 22, 23) and inhibit the C subunit through interactions within the substrate binding site of the C subunit (24). Specific amino acids conserved between PKIα and
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PKIγ (Phe10, Arg15, and the pseudosubstrate sequence (Arg18, Arg18-Ans20, Ala21)) (25, 26) have been demonstrated to be important for binding and inhibition of the C subunit. Although both isoforms have a high affinity for C subunit, the murine PKIiso and PKIγ isoforms differ significantly in their inhibitory potency, and individual residues important for this difference have been identified (16).

In addition to inhibiting C subunit phosphotransferase activity, the PKIs also serve to localize C subunit in the cell. It has been demonstrated that C-PKI complexes are more rapidly exported out of the nucleus than C subunit alone and that this process is both temperature- and ATP-dependent (27). Specifically, a nuclear export signal (NES) has been identified on PKIγ corresponding to a leucine-rich sequence conserved between PKIα and PKIγ (28).

Previous attempts to understand the cellular roles of the PKIs have been complicated by the heterogeneity of PKI activity in mammalian tissues. To help resolve this problem, we sought to identify the nature of PKI activity in tissues that expressed low levels of PKIα and PKIβ. In this report we describe the identification and characterization of a cDNA sequence that encodes a novel PKI isoform which is abundant, widely expressed, and a potent inhibitor of the C subunit of PKA. Because of its similarity to the known murine PKI isoforms, we have named it PKIγ. Like other members of the PKI family, PKIγ inhibits cAMP-dependent gene transcription and nuclear accumulation of the C subunit. The results of this study suggest that PKIγ is found at physiologically significant levels in many tissues and functions in a manner similar to previously characterized PKI isoforms.

MATERIALS AND METHODS

Isolation and Sequencing of a cDNA Clone Encoding Murine PKIγ—A full-length cDNA sequence coding for murine PKIγ (I.M.A.G.E. Consortium Clone 419982;Genbank accession W91205) (29) was identified in a search of the expressed sequence tag data base for protein sequences homologous to murine PKIs using the basic local alignment search tool algorithm (30). This I.M.A.G.E. Consortium (LNL) cDNA clone was obtained from Research Genetics Inc. It was sequenced in both directions by manual sequencing using Sequenase (U.S. Biochemical Corp.). Sequences sequencing by DNASTAR software. The murine PKIγ sequence has been submitted to the GenBank database.

Northern Blot Analysis—Plasmids AR-1 and MtPKI.pcr were linearized with BamHI and BglII and used to transfect using the basic local alignment search tool algorithm (30). The resulting precipitate was removed by centrifugation for 15 min at 30 °C. The assay was initiated by addition of Kemptide substrate (30 μM), incubated for an additional 20 min, and then terminated.

Partial Purification of PKI Activities from Mouse Heart—PKI activities were partially purified by a modification of previously described procedures (19, 37, 38). Mouse hearts (2.5 g) (Pel-Freeze) were frozen in liquid nitrogen, pulverized, and added to 8 ml of homogenization buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin (Sigma), cells were scraped into separate tubes and sonicated twice for 10 s. C subunits were separated by nondenaturing gel electrophoresis and stained essentially as described (35). Extracts were heated to 95 °C for 5 min. Various concentrations of heat-denatured extract were added to recombinant Ca (1 nm) (32) in a phosphotransferase assay mix for 10 min at 30 °C. The assay was initiated by addition of Kemptide substrate (30 μM), incubated for an additional 20 min, and then terminated.

RNase Protection Analysis—RNase protection analysis was performed essentially as described previously (31). The polymerase chain reaction (PCR) was performed with oligonucleotides 5’ GGA GAT CTC CAC CAT GAC TGA TGT GGA AAC TAC G 3’ and 5’ GGA GAG TCT TTA GCT GTG GTT GAC TGC GCT GCC GCT TCT AGA CTT GGC TGC 3’ (Biomedical Core Facilities, University of Michigan) with pMAL-PKIγ (32) as a template to amplify a fragment consisting of the full ORF of murine PKIγ flanked by BglII sites. This amplified fragment was digested with BglII, isolated, and ligated into the BglII/BamHI sites of psP73 (Promega) to create psP73.mPKIγ. This construct was linearized with BglII and used to generate PKIγ antisense RNA probes. Mouse multiple tissue Northern blots (CLONTECH, Palo Alto, CA) were hybridized at 60 °C with antisense cRNA probes for 10–14 h. Following hybridization, blots were washed for 2 h at 70 °C in 0.5 × Sf containing 0.1% sodium pyrophosphate, dried, and autoradiographed as described previously (31).

Chloroplas BglII digestion of the reaction mixture was performed with (ω−)UTP-radioabeled antisense RNA probes. The radiolabeled probes were incubated with 10 μg of total RNA or varying amounts of sense RNA (0, 0.3, 1, 3, 10, or 30 μg) for 24 h at 50 °C. Yeast tRNA was added to sense RNA samples to bring the RNA total to 5 μg. The samples were then treated with DNase A (200 units/ml) and RNase T1 (200 units/ml) (Sigma). The protected fragments were isolated and electrophoresed through 6% polyacrylamide sequencing gels. PhosphorImager quantitation was performed in a PhosphorImager apparatus and analyzed with IMAGEQUANT software (Molecular Dynamics).

Construction of PKI Mammalian Expression Vectors, Transient Transfection, and Determination of Inhibitory Activity—A mammalian expression plasmid encoding a carboxyterminal hemagglutinin (HA)-tagged murine PKIγ protein was constructed by PCR. A PCR fragment encoding PKIγ with a carboxyterminal 12CAs epitope (YPYDVPDYA) and a one amino acid glycine linker was generated using primers 5’ GGA GAT CTC CAC CAT GAC TGA TGT GGA AAC TAC G 3’ and 5’ GGA GAG TCT TTA GCT GTG GTT GAC TGC GCT GCC GCT TCT AGA CTT GGC TGC 3’ with psP73-PKIγ as a template. The resulting PCR fragment was digested with BglII, isolated, and ligated into BglII digested pCMV.Neo (34) to create pCMV.HA-PKIγ. Likewise, the pCMV.mPKIγ mammalian expression vector was constructed by PCR using the oligonucleotides 5’ GGG AGA TCT CCA CCA TGA TGG AAG TCG AGT CCC 3’ and 5’ GGG AGA TCT CCA CCA TGA TGG AAG TCG AGT CCC 3’ with psP73-PKIγ as a template. The resulting PCR fragment containing the coding region of PKIγ flanked by a BglII site and a BamHI site was ligated into pGEM-T (Promega) to create pGEM-T.mPKIγ, pGEM-T.mPKIβ, pGEM-T.pSP73.mPKIγ, pCMV.mPKIγ, or pCMV.mPKIβ, all of which were sequenced by Sequenase (U.S. Biochemical Corp.). Expression vectors were linearized with BglII and BglII to excise the PKIγ coding region (16). The pCMV.Neo, pCMV.mPKIγ, and pCMV.mPKIβ were sequenced to confirm the coding region sequence. The human PKIγ and murine PKIγ mammalian expression plasmids have been described previously (20, 35). The human PKIγ protein is 97% identical to the murine PKIγ amino acid sequence (35). HEK293 cells at 50% confluency in 10-cm plates were transfected using a calcium phosphate co precipitation method (36) with 25 μg per plate of either pCMV.HA-PKIγ, pCMV.mPKIγ, or pCMV.mPKIβ, at the same concentration. The transfectants were harvested 72 h after transfection, and the pCMV.hPKIγ, pCMV.mPKIγ, and pCMV.mPKIβ were sequenced to confirm the coding region sequence. The human PKIγ and murine PKIγ mammalian expression plasmids have been described previously (20, 35). The human PKIγ protein is 97% identical to the murine PKIγ amino acid sequence (35). HEK293 cells at 50% confluency in 10-cm plates were transfected using a calcium phosphate co precipitation method (36) with 25 μg per plate of either pCMV.hPKIγ, pCMV.mPKIγ, or pCMV.mPKIβ, at the same concentration. The transfectants were harvested 72 h after transfection, and the pCMV.hPKIγ, pCMV.mPKIγ, and pCMV.mPKIβ were sequenced to confirm the coding region sequence. The human PKIγ and murine PKIγ mammalian expression plasmids have been described previously (20, 35). The human PKIγ protein is 97% identical to the murine PKIγ amino acid sequence (35). HEK293 cells at 50% confluency in 10-cm plates were transfected using a calcium phosphate co precipitation method (36) with 25 μg per plate of either pCMV.hPKIγ, pCMV.mPKIγ, or pCMV.mPKIβ, at the same concentration. The transfectants were harvested 72 h after transfection, and the pCMV.hPKIγ, pCMV.mPKIγ, and pCMV.mPKIβ were sequenced to confirm the coding region sequence. The human PKIγ and murine PKIγ mammalian expression plasmids have been described previously (20, 35). The human PKIγ protein is 97% identical to the murine PKIγ amino acid sequence (35).
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ORFs of murine PKIα and PKIγ each with an amino-terminal hexahistidine tag. pMAL-PKIA and clone 419982 were used as templates in the PKIα and PKIγ PCR reactions, respectively. Fragments were digested with NcoI and BamHI, isolated, and ligated into pET9d (Novagen) that had been NcoI- and BamHI-digested. Escherichia coli (BL21 DE3/pET9d) transformed with pET9d.His6PKIγ and pET9d.His6PKIγ E. coli cultures (1 liter) were grown and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Pellets were resuspended in 20 ml of buffer A (20 mM Tris, pH 8.0, 300 mM NaCl, 0.1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. Each suspension was sonicated three times for 1 min, and then centrifuged to obtain a final concentration of 20 mg/ml. The bacterial lysate was centrifuged for 1 h at 100,000 × g, and the supernatant was loaded onto a 2-ml nickel affinity resin (Qiagen). The column was washed with 10 column volumes of buffer A containing 20 mM imidazole and eluted with a step gradient of imidazole in buffer A. The major peaks of His6PKIγ were isolated and purified using antibodies for rabbit production (Research Genetics Inc.).

Western Blotting—Antiserum raised against His-tagged PKIα and PKIγ were affinity purified on MBP-PKIα (32) and MBP-PKIγ (see below) nitrocellulose blots essentially as described (39, 40). Affinity purified anti-PKIα antibody recognized PKIα (14 kDa) but did not react with Western blots of cell extracts transfected with PKI expression constructs. Likewise, affinity purified anti-PKIγ recognized PKIγ (16 kDa), but no signal was detected in extracts from PKIα- or PKIβ-transfected cells. Fractions from the DEAE column were concentrated in microcentrifuges (Microcon-3, Amicon), denatured in SDS-PAGE buffer at 95 °C for 5 min, resolved on 15% SDS-PAGE gels, and transferred to 0.2-μm nitrocellulose membranes (BA-83, Schleicher and Schuell). Membranes were blocked for 4 h in PBS supplemented with 5% non-fat dried milk, 2% polyvinylpyrrolidone (PVP-40), and 0.1% Triton X-100 and subsequently incubated with either a 1:10 dilution of affinity purified anti-PKIα or anti-PKIγ in PBS supplemented with 0.5% bovine serum albumin and 0.1% Triton X-100 for 2 h. Filters were washed three times for 10 min with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), and then they were incubated with a 1:10,000 dilution of goat anti-rabbit alkaline phosphatase (Life Technologies, Inc.) in TBST supplemented with 5% non-fat dried milk as the secondary antibody for 2 h. Following the final set of three 10-min washes with TBST, the blots were developed with the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate system (Life Technologies, Inc.).

Construction of Prokaryotic Expression Vector For PKIγ and Determination of PKI Isoform ICα and Kγ—To create a MBP-PKIγ prokaryotic expression vector, the murine PKIγ coding region was amplified by PCR using oligonucleotides 5′-GGG GGA ATC TGG GAA GTC GAG TCC TCC 3′ and 5′-GGG GAA TTC TTA GGA TGA GGT GTT CGC ATC 3′ designed to create EcoRI recognition sites. The resulting PCR fragment was cut with EcoRI, isolated, and ligated into the EcoRI site of pMAL.cRI (New England Biolabs). This vector was sequenced and blast-digested using the primers 5′-GGG GGA TCC ACC ATG GAC TAC AAG GGC AAC GCC GCG GCC GCC AAG AA 3′ and 5′-AAG CGA GGC AGG GCC GCG GCC GGC AAC AA 3′ with pGEM-4.CcI, which is a mammalian expression plasmid. The resulting PCR fragment was digested with BamHI and BglII, isolated, and ligated into BglII-digested pCMVneo to create pCMV.Flag-Cα. To generate pCMV.Flag-Cα2 to digest the wild type Flag-tagged c expression vector pCMV.Flag-Cα3. To create the Ca2Y35S/F239S mutant expression vector, pET9d.Ca2Y35S/F239S (42) was digested with BglII and the 240-bp fragment containing the mutated sites was ligated into BglII-digested pCMV.Flag-Cα2 to generate pCMV.Flag-Ca2Y35S/F239S. Both expression plasmids were restriction mapped and sequenced.

Transient Transfection of NIH 3T3 Cells and Luciferase Assays—NIH 3T3 cells were grown on 10-cm plates to 50% confluency and transfected using a standard calcium phosphate method (36) with 1 μg of pCMV.Flag-Cα or pCMV.Flag-Ca2Y35S/F239S, 1 μg of human chorionic gonadotropin-luciferase (HCG.Luciferase), 5 μg of pBS.gal, and the indicated amounts of pCMV.mPKIγ. The total plasmid DNA was brought to 25 μg with the parent vector pCMVNeo. Twenty-one hours after transfection, cells were washed twice with ice-cold PBS, scraped into homogenization buffer, sonicated, and assayed for luciferase and β-galactosidase activities as described (8).

Immunofluorescence—COS-1 cells or CV-1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum in 8-well tissue culture chambers on poly-l-lysine-coated glass slides (Lab-Tek) to 30% confluency and transfected using a standard calcium phosphate method (36). Cells were transfected with 2 μg of pCMV.Flag-Cα, 5 μg of pCMV.RIα, and neither PKI expression vector, 8 μg of pCMV.HA-PKIα, or 4 μg of pCMV.mPKIγ. Total plasmid concentration was determined by the method of Bradford (Bio-Rad Protein Assay). MBP-PKIα proteins were determined by the Bradford method (Bio-Rad Protein Assay). Antibodies raised against His6PKIα and His6PKIγ and the indicated amounts of pCMV.mPKIγ. The total plasmid DNA was brought to 25 μg with the parent vector pCMVNeo. Following a 12-h incubation with DNA precipitates, cells were washed with ice-cold PBS, fixed with 4% formaldehyde in PBS for 10 min at room temperature followed by a 1:1 mixture of methanol and acetone for 5 min. After washing three times with PBS, cells were incubated with an anti-Flag epitope antibody (M2) (Eastman Kodak) at a 1:2000 dilution in PBS supplemented with 1% bovine serum albumin, 1% horse serum, and 0.1% saponin (Sigma). After four washes with PBS supplemented with 0.1% saponin, a 1:3000 dilution of Cy3-F(ab′)2 fragment goat anti-mouse IgG (Jackson) was incubated with the cells for 1 h in the dark in PBS supplemented with 1% bovine serum albumin, 1% horse serum, and 0.1% saponin. Prior to examination by fluorescence microscopy (Olympus), cells were washed four times for 2 min in PBS plus 0.1% saponin and twice for 2 min in PBS. In control experiments, no fluorescence was detected in non-transfected cells.

RESULTS

Identification of cDNA Clone Encoding Murine PKIγ—A comparison of PKI activity of tissue extracts with PKIα and PKIβ tissue mRNA levels supported the idea that novel PKI isoforms might exist (18, 20, 21). For example, tissues such as kidney and liver show low levels of PKIα and PKIβ mRNA but significant amounts of PKI inhibitory activity (data not shown). To determine if evidence for other PKI isoforms existed, the amino acid sequence for murine PKIα (44) was used to search for homologous sequences in the NCBI GenBank expressed sequence tag data base using the basic local alignment search tool program. This search identified a murine full-length cDNA clone (I.M.A.G.E. consortium clone 419982) derived from a mouse embryo (embryonic day 13.5–14.5) cDNA library which encoded a protein having statistically significant homology (p < 0.001) to the murine PKIα amino acid sequence. The cDNA clone was fully sequenced (Fig. 1), shown to contain 1064 base pairs (bp), and characterized further.

Assuming that the first of two methionine codons encodes the initiator methionine, the murine PKIγ cDNA contains an ORF of 231 nucleotides and a 3′ untranslated region encoding a putative polyadenylation signal (AATAAA(1042–1047) and a poly(A) tail (Fig. 1). The PKIγ protein predicted by the ORF is 75 amino acids in length with a calculated molecular mass of 8.7 kDa and pi of 3.9. PKIγ shows relatively low amino acid homology to the other known murine PKI isoforms: PKIα (35% identity) and PKIβ (30% identity). However, most of the residues demonstrated to play a role in the high affinity of PKIα for the C subunit are conserved. Not only is the inhibitory
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PKIγ M E V E S S Y S D F I S C D O R T G R N A P V D I 26
PKIα M T D V E T T I L A D F I A S G R T G R N A H D I 26
PKIB M T D V E S V T S F S A S A R A G R N A L P D I 26
PKIC G G D S L E A V S R K L A D G M E L A G A E G 52
PKIC L V S A S G N S N E L A L K L A D I N T K E G 52
PKID Q S S L A T G S G S S U L P L L K L A V E D A K 52
PKIE Q A E S T P D K E A S Q P E S S D A N T S S 76
PKIF E D D G Q S T E S G F A E G A E A K S 76
PKIG T K N E E K D Q G P K T P L N E G K 71

Fig. 2. Amino acid sequence alignment of murine PKI isoforms. Predicted protein sequence of murine PKIγ is compared with the protein sequences of murine PKIα and murine PKIβ in the above alignment using DNASTAR software. The numbering of the three sequences begins with the predicted or known initiator methionine and is placed on the right of the diagram. Amino acid residues identical between any two of the three sequences are boxed. Residues known to be important in high binding affinity of PKIα for C subunit are indicated with a plus sign on the top line (Tyr2, Phe10, Arg15, and pseudosubstrate sequence). Hydrophobic residues shown to be important in the nuclear export of C subunit by PKIβ are marked by an asterisk on the bottom line.

RNase Protection Analysis of PKI Isoform Expression in Mouse Tissues and Mouse-derived Cell Lines—PKI activity has been extensively characterized in several tissues where PKIγ mRNA is detected in Northern blot analysis including skeletal muscle (21, 22), heart (38, 45), and testis (19, 37). However, it was surprising that PKIγ had not been identified previously. One explanation could be that PKIγ transcripts are widely expressed but of low abundance relative to PKIα and PKIβ transcripts. To quantitate PKIγ mRNA more accurately, RNase protection assays using isofom-specific, antisense RNA probes of similar length were performed. Antisense coding region probes of the PKI isoforms were chosen for these experiments so as to generate similar size protected fragments and to detect all possible coding region splice variants. For PKIα, a protected fragment of 228 bp was seen prominently in heart, brain, and skeletal muscle with weaker bands in lung, liver, and kidney (Fig. 3C). The presence of PKIγ mRNA in these organs is important since they have low levels of PKIα and PKIβ message (18, 20, 21).

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Expression of PKI Isoforms in Mouse Tissues—To compare directly the expression patterns of PKI isoforms in mouse tissues, poly(A)+ Northern blots were probed using antisense RNA probes specific for the known murine PKI isoforms (PKIα, PKIβ, and PKIγ). The PKIγ probe detected a single 1.3-kb transcript in all tissues examined. Unlike the PKIα and PKIβ transcripts, this message was expressed at high levels in all tissues tested. Highest levels were seen in heart, skeletal muscle, and testis; however, significant levels were also seen in spleen, lung, liver, and kidney (Fig. 3C). The presence of PKIγ mRNA in these organs is important since they have low levels of PKIα and PKIβ mRNA expression (Fig. 4A). PhosphorImager quantitation of sense RNA-protected frag-

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mRNA is expressed at comparable levels to PKIα and PKIβ in all mouse tissues tested. In several tissues such as heart, lung, liver, and kidney, PKIγ is the predominant PKI isoform transcript (Fig. 4, B–D).

To confirm the widespread nature of PKIγ transcripts, total RNA from several mouse-derived cell lines was isolated and assayed for PKI isoform transcript expression by RNase protection analysis. As for the mouse tissues, hybridization with the PKIγ probe generated a 233-bp fragment in all cell lines tested (data not shown). C12, C12, myoblasts, N1E-115 neuroblastoma and L929 cells showed the highest levels of expression, and TM4 Sertoli cells showed the least (Fig. 4E). Only the N1E-115 neuroblastoma cell line displayed a significant level of PKIγ transcript, whereas no bands were detected in any of the cell lines in the PKIβ RNase protection analysis (data not shown). Of interest, no evidence of PKI alternative splice variants was observed in these RNase protection experiments.

Separation and Identification of PKI Activities from Mouse Heart—Another possible explanation for why PKIγ was not detected during prior PKI purifications is that it possesses significantly different biochemical characteristics from the other two known isoforms. Analysis of PKIα, PKIβ1, and PKIγ amino acid sequences shows that the three proteins vary considerably in their predicted isoelectric points: PKIα has a pl of 4.4, PKIβ1 has a pl of 5.1, and PKIγ has a pl of 3.9. Previous purifications of PKIα and PKIβ1 have involved elution from a DEAE-cellulose column with a linear gradient of NaOAc (0–350 mM) at pH 5.0 (19, 38). Under these conditions, PKIβ1 eluted at lower ionic strengths than PKIα. Since PKIγ has a lower pl than PKIα, it would be expected to elute later in the gradient. No inhibitory peaks were eluted after PKIα, but it is possible that PKIγ was never eluted from the column. To test this hypothesis and to verify that the PKIγ protein is present in native mouse tissues, PKI activities from mouse heart were partially purified by a procedure similar to a previously described three-step purification scheme involving heat denaturation, acid treatment, and DEAE chromatography (19, 37, 38).

This purification was modified to extend the elution from 350 mM NaOAc to 1 mM NaOAc. Mouse heart was chosen for this experiment due to its high levels of both PKIα and PKIγ message as determined by Northern blot and RNase protection analysis. In vitro kinase inhibition assays were conducted on the mouse heart fractions obtained from the DEAE column.

Two peaks of inhibitory activity were detected, one that eluted at a theoretical salt concentration of 250–325 mM NaOAc (9.9–13.0 millisiemens/cm), and a second that eluted at a theoretical salt concentration of 450–575 mM NaOAc (16.0-20.0 millisiemens/cm) (Fig. 5A). To determine if fractions of these peaks were likely to contain PKIα or PKIγ, eukaryotic expression vectors for human PKIα and murine PKIγ were transiently transfected into HEK293 cells, and PKI activities were partially purified as for mouse heart. Assay of the frac-
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When samples from the heart inhibitory peak fractions were analyzed by Western blotting, the affinity purified anti-mPKIα antibody detected a single 14-kDa band in peak 1 fractions (Fig. 5B). This band was the same apparent molecular mass as transfected PKIα. Likewise, the affinity purified anti-PKIγ antibody recognized a single 16-kDa band in peak 2 fractions that co-migrated with transfected PKIγ (Fig. 5C). Importantly, the intensity of the 14-kDa band in peak 1 fractions and the intensity of the 16-kDa band in peak 2 fractions correlated directly with the C subunit inhibitory activity of the fractions showing that PKIα is the inhibitor responsible for the majority of inhibitory peak 1 and PKIγ is the inhibitor responsible for the majority of inhibitory peak 2. These results suggest that both PKIα and PKIγ are expressed at significant levels in mouse heart and that PKIγ can be distinguished from other PKI isoforms both immunologically and by its DEAE elution characteristics.

Kinetic Analysis of Murine PKIα and PKIγ—To study the inhibitory potency of murine PKIγ, full-length PKIγ was PCR-amplified, cloned into pMALTcRI, and expressed in E. coli as an MBP-PKIγ fusion protein. Previously, it has been shown that the presence of an amino-terminal MBP fusion does not affect the inhibitory efficacy of PKIα or PKIβ1 (16). To assess relative inhibitory efficacy, PKIγ was compared with murine PKIα (16) in in vitro kinase inhibition assays. C subunit phosphotransferase activity was measured in the presence of increasing concentrations of either PKIα or PKIγ. A representative experiment is shown in Fig. 6A, and average IC50 values obtained from similar experiments are listed in Table I. Measurement of IC50 values for PKIα and PKIγ at 30 μM Kemptide substrate revealed that PKIγ possesses a 14-fold greater IC50 value than PKIα (Table I). To measure more accurately the difference in inhibitory potencies between these two tight binding inhibitors, KI values were determined by Henderson analysis (41) (Fig. 6, B and C). The KI values for PKIα and PKIγ were determined to be 0.073 and 0.44 mM, respectively (Table I). The increase in slope with increasing Kemptide substrate concentrations in Fig. 6B suggests that as for PKIα and PKIβ1, PKIγ is a competitive inhibitor of C subunit (16, 19, 22, 23). Hence, PKIγ is a potent, competitive inhibitor of C subunit; however, it is 6-fold less potent than PKIα. PKIγ possesses all the amino acid residues previously shown to be important in the high affinity of PKIα for C subunit (Tyr5, Phe10, Arg15, Arg18, and Arg19) (16, 25, 26); however, it is possible that PKIγ contains negative binding determinants. Mutagenesis of Thr8 and Ser12 in PKIβ1 to alanines caused a 4-fold increase in the inhibitory potency of PKIβ1. It has been postulated that these residues may block formation of the amino-terminal inhibitory α-helix of PKIβ1 (16). Thus, it is possible that Ser8 and Ser12 of PKIγ are negative determinants of C subunit binding.

Mammalian Expression and C Subunit Inhibitory Activity of PKIγ—Expression experiments were performed to demonstrate that the PKIγ cDNA sequence was capable of producing a relatively heat- and acid-stable inhibitor of PKA. In addition, these expression experiments sought to characterize the ability of PKIγ to inhibit cAMP-dependent gene transcription and C subunit nuclear accumulation. pCMV.mPKIγ, a PKIγ mammalian expression vector, was constructed. Initially, HEK293 cells were transiently transfected with pCMV.mPKIγ, pCMV.hPKIα, pCMV.hPKIα, pCMV.mPKIβ1, or pCMV.neo. Cell extracts were treated with validated and increasing amounts were added to an in vitro kinase inhibition assay to determine if they could inhibit recombinant C subunit. HEK293 cells transfected with either of the three PKI isoform expression vectors contained at least 10-fold higher levels of C subunit inhibitory activity than those.
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The 50% inhibitory concentrations (IC50 values) are derived from titration of recombinant C subunit with PKI isoforms in the presence of 30 μM Kemptide substrate (see "Materials and Methods"). A representative experiment is shown in Fig. 6A. The Ki values were obtained from Henderson analyses (Fig. 6, B and C). IC50 and Ki values are expressed as the average ± S.D. from at least three experiments.

| PKI isofrom | IC50 (nM) | Ki (nM) |
|-------------|----------|--------|
| PKIα        | 0.085 ± 0.017 | 0.073 ± 0.017 |
| PKIγ        | 1.2 ± 0.2 | 0.44 ± 0.06 |

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**Inhibition of recombinant Ca by murine PKIα and PKIγ**

A, IC50 determinations. Protein kinase activity was measured with 30 μM Kemptide substrate in the absence or presence of increasing concentrations of PKIα (○) and PKIγ (●). Activity was expressed as the percentage of Ca-specific activity in the absence of inhibitor. The curves were fitted using the average values of triplicate assay points from representative experiments, and the error bars represent the standard deviation from the mean. The experiments were performed four times for each inhibitor. Average IC50 values are reported in Table I. B, determination by Henderson analysis. Protein kinase activity was measured in the absence or presence of PKIα or PKIγ at the following Kemptide substrate concentrations: 5 μM (○), 20 μM (●), 40 μM (▲), and 60 μM (▼). The data for a representative experiment of PKIγ are shown. I, is the total inhibitor concentration, and V0 and Vi are the reaction velocities in the presence and absence of inhibitory proteins, respectively. C, replots of the slopes from Henderson analyses versus Kemptide substrate concentration for PKIα (○) and PKIγ (●). The experiments were performed three times for each inhibitor. Average Ki values are reported in Table I.

transfected with the parental vector pCMV.neo. The extracts from cells transfected with pCMV.mPKIα showed greater inhibitory activity than pCMV.mPKIβ-transfected cells but less inhibitory activity than the pCMV.hPKIα-transfected cells (data not shown). The presence of the PKIs in cell extracts was verified with PKI-specific antibodies (data not shown).

**In Vivo Inhibition of cAMP-dependent Gene Transcription by PKIγ**—To verify the ability of PKIγ to inhibit C subunit in vivo, NIH 3T3 cells were transiently transfected with a constant amount of the Ca expression vector, either pCMV.Flag-Cα3 or pCMV.Flag-CαY235S/F239S, and increasing amounts of pCMV.mPKIγ. Each plate also received a constant amount of a cAMP-responsive reporter plasmid (HCG.luciferase) (46) and pRSV.βgal to control for transfection efficiency. In this experiment, luciferase activity was used as an in vivo measure of free cellular C subunit. Transient transfection of either C subunit alone produced a 35-fold increase in luciferase activity (data not shown). One to two μg of pCMV.mPKIγ was required to completely inhibit luciferase activity in cells transfected with pCMV.Flag-Cα3. NIH 3T3 cells expressing the Flag-tagged CoY235S/F239S mutant, a Ca mutant with reduced affinity for PKIα and PKIβ1 (42), showed significantly less inhibition of luciferase activity at all pCMV.mPKIγ concentrations (Fig. 7). It has been shown previously that three PKIα residues outside the pseudosubstrate sequence, Tyr7, Phe10, and Arg15, contribute significantly to the high affinity of PKIα for C subunit. Specifically, Phe10 of PKIα interacts with a hydrophobic pocket on the surface of the C subunit consisting of residues Tyr235 and Phe239 (24, 42). The reduced ability of PKIγ to inhibit the CoY235S/F239S mutant is consistent with the conservation of Phe10 between PKIα and PKIγ (Fig. 2). Interestingly, even though CoY235S/F239S is 2000-fold less sensitive to inhibition by PKI in vitro (18), it is still significantly inhibited in vivo. This is likely due to the fact that in vivo concentrations of C subunit reach micromolar levels, whereas nanomolar concentrations of C subunit are used in in vitro experiments.

**PKIγ Blocks Nuclear Accumulation of C Subunit**—Both PKIα and PKIβ are capable of actively exporting C subunit from the nucleus (27). In the case of PKIα, this export of C-PKI complexes has been shown to require a nuclear export signal (NES) consisting of a hydrophobic sequence that is conserved between PKIα and PKIβ1 (28). Amino acid alignment of PKIγ with PKIα and PKIβ1 reveals the possible presence of a NES in PKIγ (Fig. 2). The most significant difference observed was a glycine at amino acid residue 39 where both PKIα and PKIβ1 contain leucines. To test the functionality of the NES of PKIγ, a transient transfection assay was developed. An amino-terminal Flag-tagged Ca expression vector was constructed to determine the localization of C subunit in transfected cells. To verify that the Flag-tagged C subunit was catalytically active and that the Flag epitope was accessible both when the C subunit was free and when it was bound to a PKI, HEK293 cells were transiently transfected with the Flag-tagged C subunit alone or with a combination of Flag-tagged C subunit and PKIγ. Western blot analysis of these HEK293 cell extracts with the M2 anti-Flag antibody detected a single band with an apparent molecular mass of 41 kDa, the size expected for full-length C subunit (data not shown). No band was seen in extracts of HEK293 cells transfected with the parental vector alone (data not shown). When extracts of HEK293 cells transiently transfected with or without the Flag-tagged C subunit vector alone were subjected to immunoprecipitation with the M2 anti-Flag antibody, only the immunoprecipitates isolated from extracts...
made from cells transfected with pCMV.Flag-Ca3 showed significant C subunit activity (data not shown). Importantly, boiled M2 antibody immunoprecipitates from extracts of HEK293 cells co-transfected with Flag-tagged C subunit and PKIα showed significant C subunit inhibitory activity (data not shown). Moreover, immunoprecipitation of this inhibitory activity was dependent on the presence of Mg²⁺ and ATP in the immunoprecipitation buffer, both critical to the tight binding of C subunit to PKI (47). Hence, a catalytically active Flag-tagged C subunit is produced in cells transiently transfected with the pCMV.Flag-Ca3 expression vector, and it is recognized by the M2 anti-Flag monoclonal antibody when free in solution and when bound to PKIα.

To study the effect of elevations in cAMP on the cellular localization of C subunit in the presence and absence of PKIγ, CV-1 cells were transiently co-transfected with expression vectors for RIIα, amino-terminal Flag-tagged Ca, and either no PKI, PKIα, or PKIγ. Enough RIIα expression vector was used in each transfection to completely inhibit transfected C subunit phosphotransferase activity (data not shown). Likewise, in transfections including PKIα or PKIγ, a sufficient amount of PKI expression vector was included so as to fully inhibit transfected cAMP-dependent kinase activity (data not shown). Following transfections, CV-1 cells were stimulated for 40 min with forskolin and isobutylmethylxanthine and then analyzed by indirect immunofluorescence with the M2 anti-Flag monoclonal antibody. Consistent with previous results, the Flag-tagged C subunit was localized to the cytoplasm in untreated cells expressing Flag-tagged C subunit and RIIα (Fig. 8A) (48). When identically transfected cells were treated with forskolin, C subunit was found to accumulate in the nucleus (Fig. 8B) (48). Co-transfection of PKIα or PKIγ expression vectors with the Flag-tagged C subunit and RIIα had no discernible effect on the cytoplasmic localization of C subunit in non-stimulated cells (Fig. 8, C and E). However, both PKIα and PKIγ prevented the nuclear accumulation of C subunit in stimulated cells (Fig. 8, D and F) (49). Similar results were obtained in identically treated COS-1 cells (data not shown). In both cells lines, no significant anti-Flag immunofluorescence was observed in cells transfected with the parental vector alone (data not shown).

DISCUSSION

This study describes the identification, expression, and characterization of a new member of the PKI family of inhibitor proteins. The PKI family now includes at least three members: PKIα, PKIβ, and PKIγ. The amino acid sequence of PKIγ is 35% identical to PKIα and 30% identical to PKIβ suggesting that these three PKI isoforms are encoded by distinct genes. All members of the family possess an amino-terminal inhibitory region that includes a pseudosubstrate sequence and a central region containing a leucine and hydrophobic amino acid-rich NES. Most of the amino acid differences between the PKI isoforms occur in the carboxyl-terminal one-third of the molecule. All three PKI isoforms are approximately the same amino acid length suggesting that this size is important for physiological function.

The high degree of amino acid similarity between PKIγ and the other known PKI isoforms in the amino-terminal inhibitory region suggests that the PKI isoforms may also have common biochemical characteristics. For instance, we anticipate that PKIγ will require Mg²⁺ and ATP for high affinity interactions with C subunit (47), that it will inhibit both Ca and Cβ to similar extents (8), and that it will be specific for PKA (50, 51). To understand the role of the PKIs in the PKA signal transduction system, it will be important to understand the differences between the PKI isoforms. Even though PKIα and PKIβ share many of the same amino acids in their inhibitory region, they differ significantly in their inhibitory potencies. As determined by Henderson analysis, murine PKIβ possesses a 32-fold higher Kᵢ for Ca than murine PKIα (16). This difference has been demonstrated to be due in part to the absence of a tyrosine at position 7 of PKIβ (16). Because Tyr⁷ of PKIα is conserved in PKIγ, we anticipated that PKIγ would bind and inhibit Ca with a subnanomolar Kᵢ. Data from this study demonstrate that indeed PKIγ is a tight binding inhibitor of C subunit with a Kᵢ of 0.44 nM. Still, it is 6-fold less potent than PKIα. This decreased potency is probably due to potential negative determinants such as Ser⁶⁹ and Ser¹⁰². It is unlikely that a 6-fold difference in inhibitory potency could make a
significant difference in a stimulated cell where free C subunit levels are micromolar; however, at resting cellular levels of cAMP, where there is little active kinase in the cell, this difference in inhibitory potency between PKI isoforms could be significant (16, 17).

Unlike the other PKI isoforms, which do not contain any cysteines, PKIγ possesses a single cysteine residue at amino acid position 13. Assuming that PKIγ has an overall structure similar to PKIα (24), Cys13 of PKIγ would be located at the boundary of the amino-terminal α-helix and β-turn regions, two regions implicated in the high binding affinity of PKIα for C subunit. Previously, the β-turn region of PKIα has been hypothesized to be important in the proper positioning of Arg15 with Glu203 of the C subunit (24, 32). Since the β-turn region of PKIα is believed to be flexible and not in a fixed conformation until binding to C subunit (24), it was possible that modification of this cysteine residue could affect the ability of PKIγ to bind and inhibit C subunit. However, attempts at selective modification of Cys13 with selective sulfhydryl-modifying reagents such as N-ethylmaleimide, iodoacetic acid, and iodoacetamide failed to show specific decreases in PKIγ inhibitory activity (data not shown).

The original goal of this study was to identify novel PKI isoforms from tissues showing significant levels of PKI activity but low levels of PKIα and PKIβ mRNA. Due to the relatively tissue-specific localization of PKIα and PKIβ mRNAs, previous models of PKI function had assumed that some tissues and cell types did not require PKI for cAMP-mediated signal transduction. Assuming equivalent translation rates, results from Northern blot analysis and RNase protection analysis indicate that PKIγ is widely expressed and may be the predominant PKI isoform in several tissues, including kidney and liver. In addition, determination of PKI isoform message levels in cultured cells showed that PKIγ was expressed in all cell lines studied, and it was the major PKI transcript in all cell lines tested. These cell lines could afford the opportunity to examine the role of PKIγ in the regulation of cAMP signaling.

PKI activity was first reported in 1965 as a heat-stable, trypsin-labile component of rabbit skeletal muscle extracts (52). The original observation that the inhibitory activity was stable to heat and acid treatment was used to devise a purification scheme (53). Rabbit skeletal muscle extracts were heated and loaded onto a DEAE column. PKI activity was eluted from this column using 0.25 M sodium acetate. Subsequently, this procedure was used to isolate PKI activity from a wide variety of tissues from many species. Using the results of these purifications and tissue purifications of C subunit, the level of PKI relative to C subunit in different tissues was calculated (54). For example, it was previously estimated that PKI activity in rat heart was sufficient to inhibit approximately 20% of the total heart C subunit. Similar results were obtained in other tissues with most tissues estimated to have significantly less total inhibitor than total C subunit. Importantly, this purification scheme limited the estimate of inhibitor activity from various tissues to those proteins that had the same chromatographic properties on DEAE-cellulose as the original material isolated from skeletal muscle.

To verify the existence of PKIγ protein in mouse tissues, PKI activities from mouse heart were partially purified and identified in this study. Two inhibitory peaks were resolved following heat denaturation, acid treatment, and DEAE chromatography of the heart extracts. PKIα and PKIγ were identified as the proteins responsible for inhibitory peak 1 and peak 2, respectively. It is likely that the previous purification procedure used to estimate PKI activity in tissues did not detect PKIγ due to its higher affinity for DEAE-cellulose than the other PKI isoforms.

Interestingly, in the purification of PKI activity from rat testis, the yield of inhibitory activity after DEAE-cellulose chromatography is significantly lower than after other steps in the purification. This loss of PKI activity could reflect a selective loss of the PKIγ isoform (37). Since the PKIγ mRNA is abundant and widely expressed, previous studies significantly underestimated the PKI activities in several tissues. Recent results further challenge the belief that all cells have less total PKI than total C subunit. In situ hybridization analysis of mouse brain suggests that there is considerable heterogeneity of both PKIα and PKIβ mRNA among different mouse brain regions (31). If this heterogeneity is also true of the PKI protein, then several regions of brain may have sufficient PKI to inhibit all of the C subunit present (i.e. cerebellar Purkinje cells and CA2 hippocampal neurons).

Results from this paper demonstrate that mouse heart contains significant protein levels of at least two PKI isoforms, PKIα and PKIγ. These results do not rule out the possibility of other uncharacterized PKIs, which may not bind DEAE-cellulose, or may not elute over the 5–1000 mU NaOAc gradient used in this study. Furthermore, multiple peaks of PKI activity have been detected by DEAE-cellulose chromatography of testicular extracts (19). Even though the heat stability of the known PKI isoforms has greatly aided in their purification, there is no a priori reason why all PKI isoforms should be heat stable.

During the course of these experiments the full-length PKIγ cDNA nucleotide sequence was used to search the NCBI GenBank data base. Significant sequence homology (p < 0.001) was detected between the 3' end of the mouse PKIγ gene and the 3' end of the mouse adenosine deaminase (Ada) gene (55). Direct comparison of the two cDNA sequences demonstrated that the two genes overlapped in a tail-to-tail orientation with their coding sequences on opposite strands (Fig. 1), an uncommon occurrence in mammalian genomes. Adenosine deaminase is an important enzyme in purine metabolism, and adenosine deaminase deficiency is a major cause of autosomal recessive severe combined immune deficiency disease (56). Although tightly linked, there is no obvious functional relationship between PKIγ and adenosine deaminase. To our knowledge no patients with deletions in the 3' end of the Ada gene have been identified. The mouse Ada gene has been localized to mouse chromosome 2 approximately 94 centimorgans from the centromere (57). The mouse PKIγ (Pkracn2) has been localized to mouse chromosome 10 (58), and the mouse PKIα gene has not been mapped. Interestingly, the PKIγ mRNA transcript was first observed during the characterization of the mouse Ada gene as an abundant, widely expressed 1.3-kb transcript transcribed from the antisense DNA strand that was co-amplified with the Ada gene during gene amplification (55). The low levels of amino acid identity and the distinct chromosomal localizations of the known PKI isoforms suggest that the members of the PKI family are distantly related.

PKIγ mRNA is widely expressed and abundant in mammalian tissues but has not been characterized previously due to its low sequence homology with the other known PKI isoforms and its high affinity for DEAE-cellulose. The identification of a widely expressed, abundant PKI isoform suggests that PKI activity may be more critical to general cell function than previously believed. PKIγ is differentially expressed in adult tissues and shows distinct interactions with C subunit, suggesting it may serve distinct roles. The availability of purification methods and antibodies capable of differentiating between PKIγ and other PKI isoforms should help clarify their specific roles. Future studies should determine what, if any, specific functions the non-conserved carboxyl-terminal region of these...
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proteins play. Verification of these specific roles will require the development of model systems selectively deficient in the expression of the three PKI genes.

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