PKCu Is a Novel, Atypical Member of the Protein Kinase C Family*

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We have isolated the full-length cDNA of a novel human serine/threonine protein kinase gene. The deduced protein sequence shows strong homology to conserved domains of members of the protein kinase C (PKC) subfamily. Homologies reside in the duplex zinc-finger-like cysteine-rich motif and in the protein kinase domain. The lack of the Cz domain of the Ca²⁺-dependent PKCs and the presence of a unique NH₂-terminal sequence with a potential signal peptide and a transmembrane domain suggest that PKCu is a novel member of the subgroup of atypical PKCs. An open reading frame coding for 912 amino acids directs an in vitro translation product with an apparent Mr of 115,000. In vitro phorbol ester binding and autophosphorylation with lysates of cells overexpressing PKCu showed phosphor ester-independent kinase activity, autophosphorylation, and, in normal rat kidney (NRK) cells, predominant phosphorylation of a 30-kDa protein at serine residues. Southern analysis revealed that PKCu is a single copy gene located on human chromosome 21. There is constitutive low level expression of the human PKCu gene in normal tissues with a single transcript of 3.8 kilobases and elevated expression levels in selected tumor cell lines. These data suggest a role of PKCu in signal transduction pathways related to growth control.

Protein phosphorylation, a fundamental process for the regulation of cell growth and diverse cellular functions, is catalyzed by a multitude of protein kinases (1-4). A predominant role of protein kinases is in receptor-mediated signal transduction, where extracellular signals are amplified and propagated by a cascade of protein phosphorylation and/or dephosphorylation events that ultimately control the transcriptional activity of genes (5). Within the intracellular activation cascade, the Ca²⁺/phospholipid-dependent serine/threonine kinases known as protein kinase C (PKC)1 play an important role (6-9). They are typically activated by the second messenger diacylglycerol and participate in cellular responses to various agonists like hormones, neurotransmitters, and growth factors (6, 10). Molecular cloning of various PKC isoforms has established that PKC is a multigene family (11). To date, 10 members have been identified, which can be grouped in two major classes according to their dependence on Ca²⁺ ions. The first group, the conventional PKCs (cPKCs, β1, β2, and γ), require Ca²⁺ to be activated in the presence of phosphatidylserine (6), whereas the second group, the novel PKCs (nPKCs), are Ca²⁺-independent (12-14). The molecular basis for this functional heterogeneity is located in the conserved Cz region, which is absent in the nPKC subfamily (12-15). All PKC isozymes share a conserved catalytic kinase domain in the COOH-terminal region and an NH₂-terminal regulatory site (Cz) (6, 7). Common features of the Cz domain of cPKCs and nPKCs are a conserved pseudo-substrate site and two adjacent amino-terminal cysteine clusters that are responsible for phorbol ester binding (16). More recently, two novel members of the PKC family have been identified that do not fit into the above classification and might represent members of an independent subgroup. They have been termed atypical PKCs (αPKCz and λ), because they lack the Cz region and contain only one cysteine-rich motif in the Cz region (6, 17). αPKCs are dependent on phosphatidylserine but are not affected by diacylglycerol, phorbol ester, or Ca²⁺ (6). According to their different conditions of activation, each PKC subtype is likely to possess distinct functions in intracellular signaling pathways (7).

In search of new protein kinases participating in growth control and differentiation, we employed DNA probes, specific for the conserved catalytic kinase domain of serine/threonine protein kinases, to screen cDNAs derived from various human tissues and established cell lines. Here we describe the cloning of a novel serine-specific protein kinase gene, which contains a putative amino-terminal transmembrane region but also shows significant homologies to the catalytic and regulatory domains of the cytoplasmic kinases of the PKC multigene family (10-15, 17).

EXPERIMENTAL PROCEDURES

Cloning and Characterization of cDNA Clones—A 145-bp PCR fragment (see "PCR Analysis") was used to screen a cDNA library derived from the human natural killer cell line YT, and cloned into the expression vector pCDM8. Colony hybridization was performed by transferring the bacteria to a nylon membrane (Schleicher & Schuell) and baking them on a prewet (5 × SSC, 5% SDS) Whatman paper in a microwave oven until filters were dry. Hybridization analysis with the 32P-labeled PCR fragment was performed according to standard procedures (18). A positive cDNA clone (pkyt1) was identified that contained a 150-bp 5’- and a 200-bp 3’-extension of the above PCR fragment followed by a 2.9-kb noncoding sequence. A 23-bp 32P-labeled oligonucleotide, derived from the 5’-region of this clone, was used to screen an oligo(dT)-primed human placenta cDNA library cloned into pZAP (Stratagene). Hybridization and stringent washing was performed as described (19). One positive clone, pBP4, was excised and characterized by restriction analysis. The complete nucleotide sequence of the pBP4 cDNA was determined by the dideoxy chain termination method (20) using the modified T7 DNA polymerase (Pharmacia LKB Biotechnology Inc.) with oligonucleotide primers designed according to the sequence obtained. Both strands were sequenced. The 5’-deletion mutant of the PKCu cDNA was constructed by digesting pBP4 with ApoI.
removing the 394-bp Apo1 fragment by agarose gel electrophoresis, and religating the larger pBl4 fragment after elution from the agarose gel. Sequence analyses and data base searches were done with the HUSAR program (21) at the German Cancer Research Center, Heidelberg, Germany.

**PCR Analysis**—128-fold and 192-fold degenerate oligonucleotides derived from the first 5' and the second 3' end of the PKCp cDNA were synthesized and used to transfect HeLa and GT2PA cells. PCR products were cloned into the mammalian expression vector pMAMneo (Invitrogen) or pBGMneo (27). 2 x 10^6 cells were transfected with 5 µg of this plasmid using Transfectam™ reagent (Serva) according to the manufacturer's instructions. Cells were kept in RPMI 1640 medium supplemented with 5% fetal calf serum and 900 µg/ml G418 to select for transformants. After 3 weeks, clone colonies were picked and expanded individually. pMAMneo-transfected HeLa and pBGMneo-transfected COS cells were induced by 2 µM dexamethasone (48 h) and 5 µM CadCl₂ (12 h), respectively. In parallel, expression of PKCp was analyzed by Northern blotting and RT-PCR.

**In Vitro Kinase Reaction and Phosphoamino Acid Analysis**—Cells were harvested and washed once with phosphate-buffered saline, and aliquots of 5 x 10^5 cells were resuspended in 50 µl of phosphorylation buffer (HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM glucose, 1 mM MnCl₂, 2 mM sodium orthovanadate). 50 µg/ml phosphatidyserine was added to each reaction mixture. 50 µg/ml FMA was added when indicated. Cells were lysed by shearing several times using a 1-ml Luer syringe and a G23 gauge. Phosphorylation of cellular proteins was carried out by adding 2 µl of 100 µM ATP, 1.5 µg of [γ-³²P]ATP (20 Ci/mmol) in a volume of 50 µl and incubated 15 min at 37 °C. Reactions were terminated by adding an equal volume of sample buffer, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane.

To analyze phosphoamino acids, pertinent regions of the SDS-PAGE were excised and eluted as described (28) and the [³²P]-labeled protein was hydrolyzed in 6 M HCl for 90 min at 110 °C. Supernatants were lyophilized, mixed with nonradioactive phosphoamino acid standards, and analyzed by two-dimensional electrophoresis on cellulose thin-layer chromatography plates (Merck, Silicagel) with 0.35 running buffer (pyridine-acetic acid:H₂O, 5:50:495).

**Phorbol Ester Binding Assay**—This was done essentially as described (29). Aliquots of 2 x 10^6 cells were homogenized in a buffer containing 20 mM Tris/Cl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml Trasylol, 10 µg/ml leupeptin, 30 µg/ml trypsin inhibitor and added to the reaction mixture containing, in a final volume of 200 µl, 20 mM Tris/Cl, pH 7.4, 10 mM MgCl₂, 50 mM NaF, 10 mM [³²P]PDBu, and other supplements as indicated. After overnight incubation at 0 °C, free [³²P]PDBu was separated from receptor-bound [³²P]PDBu by filtration through glass fiber filters. Filters were washed with buffer containing 20 mM Tris/Cl, pH 7.4, 10 mM MgSO₄, 1 mM CaCl₂ and measured in a digital autoradiograph (Berthold). Specific binding represents the difference between total and nonspecific binding estimated in the presence of 10 µM unlabeled PDBu.

**RESULTS**

Cloning and Sequencing of PKC-cDNA derived from the human cell line YT was initially employed to identify new protein kinase genes. Using two degenerate primers, deduced from the second conserved domain within the catalytic site of Ser/Thr protein kinases, a 145-bp fragment was amplified by PCR. Using an oligonucleotide derived from this sequence as a hybridization probe, a pCDM8-based cDNA library of YT cells was screened. One clone designated pkty1 contained the initially amplified PCR sequence on a 3.4-kb insert. From the 5'-region of pkty1, the oligonucleotide 5'-GCCGTCCTTTTCCATT-GACA-3' was used as a hybridization probe to screen a λZAP-based cDNA library derived from human placenta tissue to obtain a full-length cDNA clone. Gel hybridization and restriction analysis of the plasmid DNA revealed that it contained a 3.8-kb insert. This cDNA, designated pBl4, coded for a 236-bp 5'-untranslated region, an open reading frame of 912 amino acids followed by a TGA stop codon and a 787-bp untranslated 3'-region. Sequence comparison of clone pBl4 to the previously identified clone pkty1 revealed identities between nucleotide 2194 and nucleotide 2969, representing 158 amino acids of the protein kinase domain from the cDNA.

The coding region of the pkty1 cDNA sequence, identical with pBl4, ends with a GAAG where GAA codes for glutamine, which is identical with Glu-811 in the PKC-cDNA. Downstream of the second G, which is part of the Ala-812 codon in the PKCp gene (see Fig. 1), the sequence of the pkty1 gene continues with GTAA and a subsequent sequence that diverges completely from the pBl4 cDNA. Within the nucleotide se-
The PKCp cDNA Directs the Synthesis of a 115-kDa Protein—To confirm the predicted open reading frame, PKCp transcripts were synthesized in vitro and translated in a cell-free system in the presence of 32P-labeled methionine. The T7 sense transcript produced a polypeptide with an estimated M₀ of 115,000 as revealed from SDS-PAGE under reducing conditions (Fig. 3, lane 1). These data are in accordance with the calculated M₀ of the deduced protein sequence of 102,000. The apparent 13-kDa difference in molecular mass might reflect post-translational modifications of the molecule or unusual migration of the protein in SDS gels. To verify a translation initiation from the first ATG within the open reading frame, a deletion mutant of the cDNA was constructed by removing a 334-bp ApaI fragment spanning the 5'-untranslated region and the coding region up to amino acid 34. By translating the deleted cDNA in vitro, translation initiation should occur at position 80, now the first methionine in the predicted open reading frame, and result in a truncated polypeptide approximately 8 kDa lower in molecular mass as compared to full-length PKCp protein. This prediction could be experimentally verified (Fig. 3, lane 2). These data confirm translation initiation of PKCp cDNA at the first ATG (position 236) in the open reading frame. In addition to the 115-kDa protein, several lower molecular weight polypeptides were found. These products might have been obtained by translation of incomplete transcripts due to premature termination of in vitro transcription. Alternatively, initiation of transcription from internal methionine start sites (34) or posttranslational processing of a larger PKCp species may have occurred. No polypeptides of comparable size were synthesized from the antisense T3 transcript or in the absence of exogenous RNA (Fig. 3, lanes 3 and 4), indicating that the synthesis of the identified protein is directed by the PKCp RNA.

Analysis of PKCp Gene Expression—Northern blot analysis of human poly(A)+ RNA from various cell lines and human tissue using the 0.87-kb PstI PKCp cDNA fragment as a probe identifies a single transcript in two cell lines, Kym I and A549, as well as in several human tissues (Fig. 4, A and B). A transcript size of 3.8 kb indicates that the cloned cDNA is full-length (Fig. 4A). Moreover, constitutive expression of PKCp in all primary tissues examined indicates a broad distribution, although the mRNA level differed considerably between the various tissues. The highest steady state levels were detected in kidney, heart, and lung (Fig. 4A). Nevertheless, when compared to other genes, mRNA levels of PKCp in normal tissues appeared very low. Typically, a signal could only be detected after a longer (4-day) exposure of blots (Fig. 4, A and B), whereas GAPDH probes of similar specific radioactivity revealed signals after a 1-h exposure (data not shown). In contrast, Northern analysis of several established human tumor cell lines showed strong expression of PKCp in a lung carcinoma (A549) and a rhabdomyosarcoma (Kym I) cell line. For comparison with the respective normal tissue (Fig. 4A), a 4-day exposure of Northern blots from the two cell lines is also shown in Fig. 4B. Densitometric analysis of shorter exposures indicates 32fold and 40fold higher mRNA levels in the cell lines A549 and Kym I, respectively.

Because PKCp-specific mRNA was readily detectable by Northern blot analysis in only two out of seven cell lines, RT-PCR was employed to increase sensitivity of detection. PKCp-specific primer for amplification of a 508-bp DNA fragment were used. As a positive control of equal cDNA amounts, a GAPDH-specific fragment was amplified in parallel (Fig. 4C). As expected from Northern blot analysis of Kym I cells, high

double underlined. The amino-terminal cysteine regions, designated CYS I and CYS II, are marked by a dotted line. Conserved amino acid residues in the protein kinase domain are marked by an asterisk. The putative splice site within the codon Ala-812 is marked by a triangle.
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**A**

- **Regulatory Domain**
- **KInase Domain**

![Diagram of PKC regulatory and kinase domains](image)

**B**

| Subtype | Cysteine Cluster 1 | Cysteine Cluster 2 | Cysteine Cluster 3 |
|---------|-------------------|-------------------|-------------------|
| cPKC   | PKCα              | PKCβ              | PKCγ              |
| nPKC   | PKCδ              | PKCε              | PKCζ              |
| aPKC   | PKCθ              | PKCι              | PKCκ              |

**C**

- **IL**
- **IIL**
- **III**
- **IV**
- **V**
- **VI**
- **VII**
- **VIII**
- **IX**

*Fig. 2. Homology of PKCα to members of the PKC family. A, schematic structure and localization of the cysteine clusters in PKCα and other PKC subtypes. B, alignment of the cysteine clusters from PKCα subtypes. Nonconservative substitutions are marked by an asterisk. C, comparison of protein kinase domain of PKCα to other PKC members and to other homologous protein kinases. Amino acids invariant in all protein kinases are marked by an asterisk.*
amounts of PKCp-specific transcripts were revealed by PCR (Fig. 4C, lane 2). In addition, high transcript levels were detected in the glioblastoma cell line 308 (lane 6). A low expression of PKCp was also revealed in the lymphoblastoid NK cell line YT (lane 3), in the erythroblastic cell line K562 (lane 4). A very faint band could be detected in the carcinoma cell line HeLa (lane 3) and in the T cell line Jurkat (lane 7). In all these cells, PKCp-specific mRNA was not discerned by Northern analysis. No transcripts were detected by PCR in the myelomonocytic cell line HL60 (lane 5). In addition, 5'-region specific primer for amplification of a 386-bp fragment coding for the putative leader and transmembrane sequence of PKCp were employed to compare transcript structure of Kym I cells with the cloned PKCp cDNA. A fragment of the expected size could be amplified from both cDNAs (Fig. 4D, lanes 1 and 2), which gives rise to identical cleavage products upon PvuII digestion (Fig. 4D, lanes 3 and 4).

The PKCp Gene Is Located on Chromosome 21 and Is Highly Conserved between Species—Southern analysis of human DNA with the 0.87-kb PsI fragment of pBpl4 revealed one specific band, indicating that PKCp is likely to be a single copy gene (Fig. 5A). Using the whole cDNA fragment as a hybridization probe, five to seven hybridization signals were obtained, depending on the restriction enzymes used for digestion of genomic DNA (data not shown). This suggests a genomic structure of PKCp consisting of five to seven exons.

A chromosomal assignment of the PKCp gene was achieved by Southern blot analysis of human-mouse somatic cell hybrids differing in the content of human chromosome 21. The hybrids N135 and N137, both containing human chromosome 21 (25), show the human PKCp-specific hybridization pattern, in addition to cross-hybridization with the homologous mouse gene, when probed with human PKCp-specific cDNA (Fig. 5A). In contrast, with the hybrid N148.26, which differs from N135 and N137 in the presence of chromosomes 6, 21, and 21, respectively, no human PKCp-specific signals were obtained (Fig. 5A). Accordingly, an assignment of PKCp to chromosome 21 can be made on the assumption that no translocations of small human genomic fragments to other chromosomes had occurred that would have gone unnoticed in initial caryotype analysis (25).

Based on the observation of the strong cross-hybridization with mouse DNA, 20 μg of genomic DNA from yeast was analyzed by Southern blotting with the same 0.87-kb PsI fragment used as a probe for human DNAs. As for mouse DNA, one specific signal was obtained in yeast, although under lower stringency hybridization conditions (Fig. 5B). These data show that PKCp or a closely related gene remained highly conserved during evolution.

Protein Kinase Activity of PKCp and Phorbol Ester Binding—In order to verify kinase activity of the cloned cDNA gene product and to identify potential cellular substrates, the coding region of PKCp was cloned in the expression vectors

![Fig. 3. SDS-PAGE analysis of ^35S-labeled protein after in vitro translation of the human PKCp mRNA. Lane 1, translation of the T7-synthesized PKCp sense mRNA; lane 2, translation of T7-synthesized PKCp deletion mutant initiating translation at methionine 80; lane 3, T3-synthesized antisense PKCp transcript; lane 4, no exogenous RNA. Molecular size markers (in kDa) are shown.](https://example.com/fig3)

![Fig. 4. Expression of the PKCp gene. A, Northern blot analysis of PKCp in selected human tissues. Positions of the size markers are indicated. B, Northern blot analysis of selected cell lines. The 28 and 18 S rRNA are indicated as size marker. 2 μg of poly(A)+ RNA were analyzed in each lane in A and B. Exposure time of the autoradiographs was 1 h. Both probes were of comparable specific radioactivity. C, PCR amplification of PKCp mRNA from different tissues. mRNA was isolated, transcribed in cDNA, and subjected to PCR analysis as described under "Experimental Procedures." Lane 1, YT; lane 2, Kym I; lane 3, HeLa; lane 4, K562; lane 5, HL60; lane 6, glioblastoma 308; lane 7, Jurkat; lane 8, cloned PKCp cDNA as a positive control. D, PCR amplification and restriction enzyme digest pattern of a 5'-specific cDNA fragment. Lanes 1 and 3, Kym I; lanes 2 and 4, pBpl4 cDNA as a positive control. Lanes 3 and 4, PCR fragments were cut with PvuII to verify identity of the amplified fragments.](https://example.com/fig4)
were digested with EcoRI (A, lane 1) or PstI (A, lane 2), fractionated, and transferred onto a nylon membrane. The membranes were probed with a 0.87-kb PstI fragment of the PKCμ human cDNA. A, chromosomal assignment of PKCμ. Genomic DNA of the human cell line YT and the mouse cell line L929 was compared to DNA derived from the hybrid cell line N135 (human chromosomes 6, 7, 14, and 21), N137 (human chromosomes 7, 14, and 21), and N148.26 (human chromosomes 7 and 14), respectively. The positions of the human- and mouse-specific fragments are indicated by closed and open arrowheads, respectively in the right margin. B, PKCμ is highly conserved in yeast DNA. Genomic yeast DNA was digested with EcoRI and analyzed as described above. Final washes were done at low stringency (55 °C, 0, 1 × SSC). The human and mouse blots represent overnight exposures; the yeast blot was exposed for 3 days.

pMAMneo for expression in NRK cells and in pBMGneo for expression in HeLa and COS cells. G418-resistant transfec-
tsants were assayed for PKCμ transcription by Northern Northern blot analysis of PKCμ. 20 μg of genomic DNA were digested with EcoRI (A, lane 1) or PstI (A, lane 2), fractionated, and transferred onto a nylon membrane. The membranes were probed with a 0.87-kb PstI fragment of the PKCμ human cDNA. A, chromosomal assignment of PKCμ. Genomic DNA of the human cell line YT and the mouse cell line L929 was compared to DNA derived from the hybrid cell line N135 (human chromosomes 6, 7, 14, and 21), N137 (human chromosomes 7, 14, and 21), and N148.26 (human chromosomes 7 and 14), respectively. The positions of the human- and mouse-specific fragments are indicated by closed and open arrowheads, respectively in the right margin. B, PKCμ is highly conserved in yeast DNA. Genomic yeast DNA was digested with EcoRI and analyzed as described above. Final washes were done at low stringency (55 °C, 0, 1 × SSC). The human and mouse blots represent overnight exposures; the yeast blot was exposed for 3 days.

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**Fig. 5.** Southern blot analysis of PKCμ. 20 μg of genomic DNA were digested with EcoRI (A, lane 1) or PstI (A, lane 2), fractionated, and transferred onto a nylon membrane. The membranes were probed with a 0.87-kb PstI fragment of the PKCμ human cDNA. A, chromosomal assignment of PKCμ. Genomic DNA of the human cell line YT and the mouse cell line L929 was compared to DNA derived from the hybrid cell line N135 (human chromosomes 6, 7, 14, and 21), N137 (human chromosomes 7, 14, and 21), and N148.26 (human chromosomes 7 and 14), respectively. The positions of the human- and mouse-specific fragments are indicated by closed and open arrowheads, respectively in the right margin. B, PKCμ is highly conserved in yeast DNA. Genomic yeast DNA was digested with EcoRI and analyzed as described above. Final washes were done at low stringency (55 °C, 0, 1 × SSC). The human and mouse blots represent overnight exposures; the yeast blot was exposed for 3 days.

**Fig. 6.** Kinase activity and Western blot analysis of PKCμ. A, in vitro phosphorylation of cellular proteins from PKCμ transfectants. Cellular proteins of PKCμ eDNA-transfected (lanes 1 and 3) and vector-transfected control (lanes 2 and 4) derived from COS cells (lanes 1 and 2) and HeLa cells (lanes 3 and 4) were phosphorylated, subjected to SDS-PAGE, transferred to a nitrocelulose membrane, and exposed overnight. B, Northern analysis of 10 μg of total RNA from the transfectants described above for PKCμ expression. Equal RNA load in each lane was verified by rehybridization with a GAPDH-specific probe (data not shown). C, immunoblot analysis of PKCμ. Total cell lysates from overexpressing COS transfectants (lane 1) were immunoprecipitated with a PKCμ-specific mouse serum, subjected to reducing SDS-PAGE, and compared to vector control transfectants (lane 2). In control blots, the mouse preimmune serum showed no reaction on immunoprecipitates from COS transfectants (data not shown).

lysates from HeLa or COS transfectants is shown in Fig. 8. On average (n = 4), an 1.2-fold increase was noted in these experiments for both cell types (HeLa, 1.13 ± 0.30; COS, 1.27 ± 0.23). Similar data were obtained with [3H]PDBu binding to intact cells (n = 2, data not shown). Together with the in vitro kinase assays, these data indicate that under the experimental conditions employed here PKCμ does not bind efficiently and is not activated by phorbol esters.
PKCp Is a Novel Atypical PKC

PKCs play important regulatory roles in a variety of cellular processes related to cell growth and differentiation. Typically, they are activated by external signals via receptor-mediated hydrolysis of membrane phospholipids, generating the second messenger inositol triphosphate and diacylglycerol (10). Inositol triphosphate, by mediating Ca\(^{2+}\) mobilization from internal stores, acts synergistically with diacylglycerol to activate the conventional, Ca\(^{2+}\)-dependent PKC subtypes (cPKC\(\alpha\), \(\beta\), \(\gamma\)) (10). The more recently identified novel PKCs PKC\(\delta\), \(\epsilon\), \(\eta\), and \(\theta\) (7, 12–15) lack Ca\(^{2+}\) binding sites, and their activation is Ca\(^{2+}\)-independent. PKC\(\zeta\) and \(\lambda\) differ from these two major PKC subgroups by lack of phorbol ester binding/activation capacity. They represent members of a newly defined subgroup of PKCs, the atypical PKCs (6).

Based on the homologies in kinase and regulatory domains, the kinase described here, designated PKCp, is a novel member of this subgroup of atypical PKCs. Similar to the nPKCs and aPKCs, PKCp lacks the C\(_2\) region of Ca\(^{2+}\)-dependent PKCs (\(\alpha\), \(\beta\), \(\gamma\)) (Fig. 2A), suggesting that the kinase activity of PKCp is also Ca\(^{2+}\)-independent. Of note is the observation that two zinc finger-like domains are highly homologous (44–68\%) to those of other members of the PKC family with a complete identity in the positions of the histidine and cysteine residues. However, whole cell and total cell extract from stable transfectants overexpressing the PKCp cDNA showed, on average, only a 1.2-fold increase in phorbol ester binding (Fig. 8). In contrast, it has been shown previously that transient expression of phorbol ester responsive PKCs typically gives rise to a 3–10-fold increase in phorbol ester binding (29). Irrespective of a potential function of PKCp as a phorbol ester receptor, analyses of PKCp kinase activity clearly suggest phorbol ester independence. A constitutive autophosphorylation of PKCp was noted for all transfectants (Figs. 6 and 7); autophosphorylation was not increased in the presence of PMA.\(^{2}\) Moreover, in lysates of NRK transfectants, phosphorylation of the major substrate, a 30-kDa protein, was also not enhanced by PMA stimulation (Fig. 7C). We therefore conclude that PKCp is a phorbol ester-independent kinase, although within the phorbol ester binding domain, the overall degree of homology between PKCp and other PKCs is not different from that within the group of phorbol ester-responsive PKCs. However, it was noted that PKC\(\zeta\) and \(\mu\) differ at two positions in the first histidine/cysteine subdomain from all other PKC subtypes (Fig. 2B). It remains to be determined whether or not these non-conservative substitutions indeed negatively affect the phorbol ester binding capacity of the PKC isoenzymes \(\mu\) and \(\zeta\). Inefficient phorbol ester binding of PKCp might also be related to the unusual spacing of the tandem domains, which are 15–22 amino acids apart in phorbol ester binding PKCs, whereas in PKCp, the two domains are separated by 79 amino acids. Therefore, in addition to differences in the primary structure, it is conceivable that an appropriate spacing of both domains is required for efficient, high affinity phorbol ester binding. This reasoning is supported by the previous demonstrations that a single cysteine domain retains PMA binding, however, its binding affinity is 10–20-fold lower as compared to the native enzyme (16). Moreover, high affinity binding could not be reconstituted by coexpression of two complementary mutants, suggesting interactive cooperation of both domains in cis (16). Our data obtained here suggest that two cysteine domains not only have to be in cis, but also in close proximity to allow interaction and to create a high affinity phorbol ester binding site. Binding studies with the purified PKCp will allow a more precise definition of the phorbol ester binding capacity and its relevance for its regulation of kinase activity.

Aside from apparent lack of PMA responsiveness, additional arguments in favor of a novel function of PKCp are obtained from an analysis of the deduced NH\(_{2}\)-terminal structure of the gene product. A putative signal peptide and transmembrane domain suggest a potentially exclusive location of the mature PKCp protein at intracellular or cell surface membranes. This

\(^{2}\) F. J. Johannes and P. Oberhagemann, unpublished data.
would be in stark contrast to the phorbol ester-dependent PKCs, which, upon activation, only transiently translocate to the cell surface or nuclear membrane (7). Accordingly, the potentially permanent membrane location, lack of conventional regulatory domains of PKCs, and instead, two additional unique regions 5′ of the cysteine cluster and 5′ of the kinase domain, suggest a differential regulation of PKCμ function and a distinct biological role of this PKC subtype. As no typical targeting motifs for intracellular organelles have been detected, it is conceivable that PKCμ is directly associated with and activated by other cell membrane proteins and serves for example as a signal transducer for non-kinase receptors, such as cytokine receptors (35, 36). In this context, it is interesting that the highly conserved PKCμ gene appears to be located on human chromosome 21. Chromosome 21 has been shown earlier to carry several genes controlling cellular responsiveness to interferon α and γ (26, 37, 38). At present, it is unknown whether PKCμ has a functional relationship to these elusive interferon response controlling gene(s) or to any other cytokine receptor system.

A first indication of the biological role of PKCμ comes from expression studies. When compared to the respective normal primary tissues, which show differential but always rather low expression of PKCμ (Fig. 4A), a few tumor cell lines were identified that exert a significant overexpression of PKCμ. In particular, a 32-fold increase in steady state mRNA levels was noted in the lung carcinoma cell line A549 (Fig. 4B). Overexpression of PKCμ affects the phosphorylation pattern of endogenous proteins in transfected cells (Figs. 6 and 7). This may eventually lead to phenotypically apparent changes in cellular functions, morphology, or proliferative capacity. With the availability of stable transfectants and antibodies, it will be now possible to identify specific substrates and to analyze the functional role of PKCμ in cell growth and differentiation.

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Note Added in Proof—We have now obtained data indicating that enhanced kinase activity of PKCμ can be revealed upon phosphatidyserine/phorbol ester treatment in vitro when specific immunoprecipitates of PKCμ rather than whole cell extracts are analyzed. This finding suggests that phorbol ester responsiveness of PKCμ can be masked by other factors present in whole cell lysates.

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