Mouse Protein Kinase C-δ, the Major Isoform Expressed in Mouse Hemopoietic Cells: Sequence of the cDNA, Expression Patterns, and Characterization of the Protein†

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ABSTRACT: A complementary DNA (cDNA) of 2559 bp which encode all 674 amino acids of mouse protein kinase C-δ (PKC-δ) has been isolated from a cDNA library prepared from ABPL-2, a mouse myeloid tumor. The library was screened with a partial PKC-δ cDNA clone that had been created by polymerase chain reaction (PCR) amplification of ABPL-2 RNA using primers that are conserved among all rat PKC isozymes. This approach proved to be a distinct improvement over screening with synthetic oligonucleotides. Similar sets of cDNAs prepared from other hemopoietic cell lines were screened with this PKC-δ cDNA and with probes for the other PKC isoforms. These experiments revealed that the major isoform of PKC expressed in hemopoietic cells is PKC-δ. PKC-δ protein was purified from ABPL-3, a mouse myeloid tumor which expressed principally the δ isoform of PKC. The protein eluted from a hydroxylapatite column in the same position as PKC-β and -ε would elute, if present. The kinase activity of purified PKC-δ showed strict dependence on the presence of phospholipids, but showed no activation by Ca^{2+}.

The protein kinase C (PKC) enzymes are a family of at least seven different serine–threonine protein kinases that are central in a variety of signal transduction pathways (Nishizuka, 1988). Although each member of the family is encoded by a separate gene, sequence analysis of cDNA clones has revealed very closely related structures for all these isoforms (Knopf et al., 1986; Ono et al., 1988; Osada et al., 1990). High homology among isozymes can be observed in three of the four "constant domains", the phospholipid-binding domain, C_1, as well as in the kinase domains, C_3 and C_4 (Nishizuka, 1988). All members of the family require phospholipid for activation of their kinase activity, while requirement for Ca^{2+} could only be shown for PKC-α, -β, and -γ (Nishizuka, 1988). Analysis of deletion mutants strongly suggests that the C_2 domain, which is only present in the latter three PKC's, is involved in activation by Ca^{2+} (Kaibuchi et al., 1989). Biochemical characterization of PKC-ε, -ζ, and -η in fact revealed no requirement for Ca^{2+} (Schaap et al., 1989; Ono et al., 1989; Osada et al., 1990), and the same can be expected, but is not yet proven, for PKC-δ. This class of PKC's which lack the C_2 domain has been called novel or nPKC's (Ohno et al., 1988).

Activation of PKC is thought to fulfill several very different roles in hemopoietic cells. In T lymphocytes, PKC induces IL-2 and IL-2 receptor expression (Berry et al., 1989). In B lymphocytes, PKC is involved in proliferation and the secretion of immunoglobulins (Bijsterbosch et al., 1988, Shirakawa & Mizel, 1989). In myeloid cells, activation of PKC by phorbol ester (TPA) leads to secretion of IL-1 and IL-6 (Strulovici et al., 1989). This treatment, in many cases, also leads to differentiation of early myeloid cells. Many of these experiments rely on TPA-induced cellular responses as evidence of PKC involvement. Even though PKC is known to consist of at least seven different isoforms expressed by at least as many genes, many studies on hemopoietic cells are done without any knowledge of which of the different isozymes are involved.

To learn which PKC isozymes are expressed in T lymphocytes, B lymphocytes, and myeloid cells, we probed Northern blots of RNAs prepared from cell lines and tumors representing a wide variety of hemopoietic cell types. As we describe in detail in a different manuscript (Mischak et al., submitted for publication), we were able to show that PKC-δ is the dominant isozyme expressed in all mouse hemopoietic cells. We therefore decided to clone and further characterize mouse PKC-δ.

Experimental Procedures

PCR Amplification. Two oligonucleotides, "505" [TTGTC(AC)AG(TC)TT(GC)AG(AG)TCAC(GT)GTA-(AG)ATGA] and "507" [GG(GC)AG(TC)TTTGG(GC)-AAGGT(GC)(AC)TGCT], which are particularly conserved stretches within C_3 among all the known PKC isozymes (Figure 1) were used as primers in PCR reactions to amplify cDNAs for PKC isozymes generated by reverse transcription of 2 μg of mRNA from several hemopoietic cell lines. After 30 cycles, the resulting PCR fragments of ca. 380 bp were gel-purified and cloned into the *Sma*I site of pUC-19. Bacterial colonies were screened by colony hybridization with the PCR fragment. Several positive clones were sequenced from the universal primer in order to determine which PKC isozyme sequence they contain.

DNA Sequencing. Sequencing of inserts in pUC-19 was performed by primed DNA synthesis on a denatured DNA template in the presence of dideoxy nucleotide triphosphates using a Sequenase 2.0 kit.

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number M69042.

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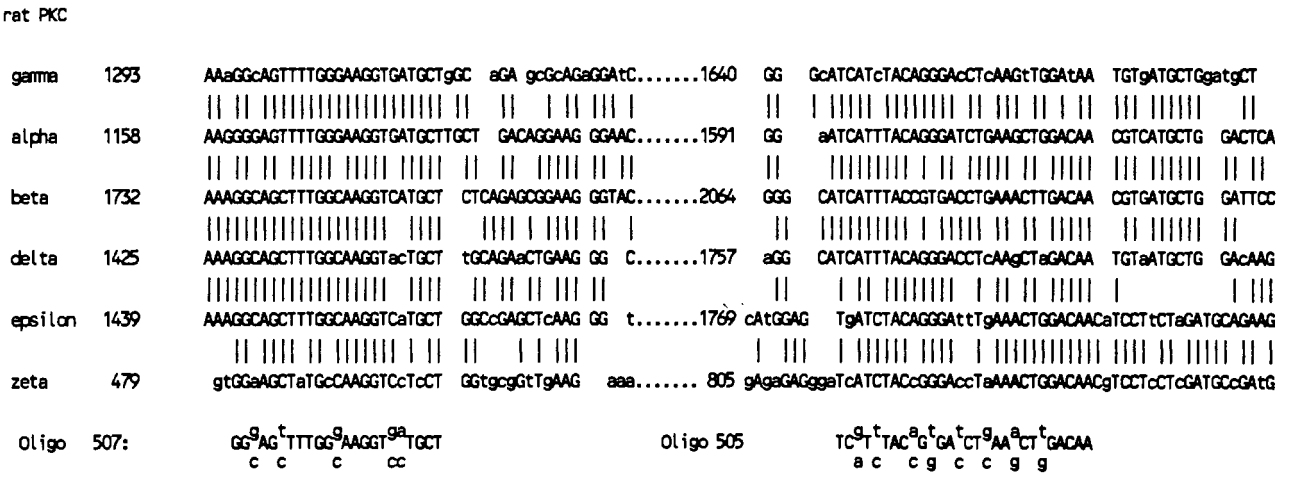

FIGURE 1: Sequence comparison of conserved stretches of cDNA encoding the six different rat PKC isozymes described in the literature (Knopf et al., 1986; Ono et al., 1989). The two most conserved stretches, both in the C_3 domain, were chosen as template for primers 505 and 507. While Oligo 507 was used as shown, the complement of primer 505 shown in the figure was used for amplification by PCR. The numbers to the left of each sequence indicate the nucleotide position of the first base shown, using the numbering system in the published reports.

cDNA Library Screening. One 0.38-kb PCR subclone with 98% sequence identity with rat PKC-δ was used to screen a λ gt10 cDNA library of mRNA from the mouse myeloid tumor ABPL-2 (Cofano et al., 1990). Plaque lift filters were hybridized overnight as described and washed with a final wash in 0.1× SSC and 0.1% SDS at 65 °C. A clone with a 2559 bp insert was identified as a nearly full-length copy of mouse PKC-δ mRNA. It was subcloned in pUC-19, sequenced, and used as a probe in RNA expression studies.

Northern Blots. Poly(A)$^+$ RNA from different tumors and cell lines was prepared, and 5 μg of each was electrophoretically separated on 1% agarose/formaldehyde gels and blotted onto Hybond-N (Amersham) nylon membranes. The blots were hybridized overnight with 10^6 cpm/mL nick-translated DNA probe, washed with 2× and 0.2× SSC containing 0.1% SDS at 65 °C, and exposed to Kodak XAR-5 film at −80 °C for 16 h. In order to normalize the lanes with respect to their mRNA content, the blots were subsequently probed with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; Fort et al., 1985). Full-length cDNA probes were used to detect PKC-α, -δ and -ϵ mRNA. Subcloned PCR fragments obtained as described above were used to detect expression of PKC-β, -γ, -ζ, and -η.

Generation of Isozyme-Specific Antibodies. Antisera were obtained from rabbits hyperimmunized with synthetic peptides corresponding to unique sequences in rat PKC-α, -β, -γ, and -δ coupled to bovine serum albumin. The antibodies were shown to be isozyme-specific by using purified PKC isozymes obtained from NIH 3T3 (PKC-α), brain (PKC-α, -β, and -γ), and ABPL-3 (PKC-δ). The isozymes were purified by using Mono Q and hydroxylapatite columns as described below.

Protein Purification. PKC-δ was purified from different cell lines according to established protocols (Konno et al., 1989). Cell pellets were extracted with Tris-buffered saline containing 1% Triton X-100 and 1 mM phenylmethanesulfonyl fluoride (PMSF). Crude extracts were brought to 20 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol (DTT), pH 7.5 (buffer A), by gel filtration, applied onto a Mono Q column (Pharmacia), and eluted with a continuous gradient from 0 to 500 mM NaCl in buffer A. Fractions were collected and assayed for PKC-δ by Western blotting using a rabbit antiserum directed against the C_1 domain of PKC-δ. Positive fractions were desalted on Sephadex G-50 columns (Pharmacia) equilibrated with 20 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol, pH 7.5 (buffer B). These partially purified fractions were fractionated by using a 1 × 10 cm column filled with hydroxylapatite (Bio-Gel HTP, Bio-Rad) connected to an FPLC system, applying a continuous gradient from 20 to 320 mM potassium phosphate.

Western Blotting. Proteins were separated on 10% SDS–polyacrylamide gels and electrophoretically transferred onto nitrocellulose. Nonspecific binding to the nitrocellulose was blocked by using 2% Tween 20. The filters were then incubated for 1 h with anti-PKC-δ antiserum and subsequently with anti-rabbit Ig coupled to alkaline phosphatase (BRL). After being extensively washed, immunoreactive bands were developed by using BCIP/NBT (BRL).

Immunoprecipitations. Crude cell lysates obtained as described above were incubated for 2 h with 5 μL of antiserum and 100 μL of 10% (w/v) protein A–Sepharose (Pharmacia) per 10^8 cells. The Sepharose beads were washed 5 times with TBS containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS and once with TBS and resuspended in 0.1 mL of 20 mM Tris, pH 7.5.

In Vitro Kinase Assays. In vitro kinase reactions were performed in 20 mM Tris, pH 7.5, containing 5 mM $MgCl_2$, 10 μM ATP, and 1 μCi of [γ-^{32}P]ATP with varying concentrations of Ca^{2+}, diolein, and phosphatidylserine as specified under Results. PKC-δ had been purified either by immunoprecipitation with the rabbit antiserum as described above or by the multiple column chromatography steps described above. Histone H1 (Sigma) was used as a substrate. The kinase reaction was performed for 30 min at room temperature and subsequently loaded onto a 12.5% SDS–polyacrylamide gel. To remove unincorporated [^{32}P]ATP and to be able to identify PKC-δ, the gel was electroblotted and subsequently probed for PKC-δ with specific antiserum as described above and exposed to Kodak XAR-5 film.

RESULTS

In order to identify which member of the PKC gene family is most abundantly expressed in hemopoietic cells, we used PCR to amplify cDNA from three different hemopoietic cell lines, ABPL-2, ABPL-109, and ABPC-103 (Mushinski et al., 1983, 1987) as well as brain (as control) using primers conserved among all the known members of the PKC family (Figure 1). Given the high homology between mouse and rat, the primers used should be able to prime the synthesis of all known PKC's by PCR, and the relative number of clones of each isoform is expected to correspond to the relative abun-

A

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1    GAATTCGGCACGAGCCGCACGAGCAGGAGGCGGCGGCCGCGGGGATCCCGCGAGCGGCCCGTGGACATCTACCCTTCGAGCCGGGACCCGGGAGGTCCCCACTGGCCTCCGGACCCGTAC
121  GGATCAGACTTGTGCCTACCTCCCCGTCCACGCGCATCCGGGAGAGCCGCGCCACGAGACGGACCCCGGCTCGCCGACCCCTGGCGCCTGGCCCTGCGTGGGAGGCTCCATCATGGCACC
                                                                                                                    M  A  P   3

241  CTTCCTGCGCATCTCCTTCAATTCCTATGAGCTGGGCTCCCTGCAAGTTGAGGACGAAGCAAGCCAGCCTTTCTGTGCTGTGAAGATGAAGGAGGCACTCAGCACAGAGCGAGGGAAGAC
     F  L  R  I  S  F  N  S  Y  E  L  G  S  L  Q  V  E  D  E  A  S  Q  P  F  C  A  V  K  M  K  E  A  L  S  T  E  R  G  K  T  43

361  ACTGGTACAGAAGAAGCCCACCATGTATCCTGAGTGGAAGACAACGTTCGACGCCCACATCTATGAAGGCCGTGTTATCCAGATTGTGCTGATGCGGGCAGCTGAAGACCCCGTGTCTGA
     L  V  Q  K  K  P  T  M  Y  P  E  W  K  T  T  F  D  A  H  I  Y  E  G  R  V  I  Q  I  V  L  M  R  A  A  E  D  P  V  S  E  83

481  GGTCACGGTGGGCGTGTCAGTACTGGCTGAGCGCTGCAAGAAGAACAACGGCAAGGCTGAGTTCTGGCTGGATCTGCAGCCTCAGGCCAAGGTGCTGATGTGTGTGCAGTATTTCCTGGA
     V  T  V  G  V  S  V  L  A  E  R  C  K  K  N  N  G  K  A  E  F  W  L  D  L  Q  P  Q  A  K  V  L  M  C  V  Q  Y  F  L  E  123

601  GGATGGGGATTGCAAACAGTCTATGCGTAGTGAGGAGGAGGCAAAGTTTCCAACCATGAACCGTCGTGGAGCCATTAAACAGGCCAAGATCCACTACATCAAGAACCACGAGTTTATCGC
     D  G  D  C  K  Q  S  M  R  S  E  E  E  A  K  F  P  T  M  N  R  R  G  A  I  K  Q  A  K  I  H  Y  I  K  N  H  E  F  I  A  163

721  CACCTTCTTCGGGCAGCCCACCTTCTGTTCTGTGTGCAAAGAGTTTGTCTGGGGCCTCAACAAGCAAGGCTACAAATGCAGGCAATGCAACGCTGCCATCCACAAGAAATGCATTGACAA
     T  F  F  G  Q  P  T  F  C  S  V  C  K  E  F  V  W  G  L  N  K  Q  G  Y  K  C  R  Q  C  N  A  A  I  H  K  K  C  I  D  K  203

841  GATTATCGGCCGCTGCACTGGCACTGCCACCAATAGCCGGGACACCATCTTCCAGAAAGAACGCTTCAACATCGACATGCCTCACCGATTCAAGGTTTATAACTACATGAGCCCCACCTT
     I  I  G  R  C  T  G  T  A  T  N  S  R  D  T  I  F  Q  K  E  R  F  N  I  D  M  P  H  R  F  K  V  Y  N  Y  M  S  P  T  F  243

961  CTGTGACCACTGTGGCAGTTTGCTCTGGGGACTGGTGAAGCAGGGATTAAAGTGTGAAGATTGTGGCATGAATGTGCACCACAAATGCCGGGAGAAGGTGGCCAACCTGTGTGGTATCAA
     C  D  H  C  G  S  L  L  W  G  L  V  K  Q  G  L  K  C  E  D  C  G  M  N  V  H  H  K  C  R  E  K  V  A  N  L  C  G  I  N  283

1081 CCAAAAGCTCTTGGCTGAGGCCTTGAACCAAGTGACCCAGAGATCTTCCCGGAAGCTGGACACAACAGAGTCTGTCGGAATATACCAGGGATTTGAGAAGAAGCCAGATGTCTCTGGGAG
     Q  K  L  L  A  E  A  L  N  Q  V  T  Q  R  S  S  R  K  L  D  T  T  E  S  V  G  I  Y  Q  G  F  E  K  K  P  D  V  S  G  S  323

1201 TGACATCCTAGACAACAACTGGACCTATGGCAAGATCTGGGTGGGGAGCACCCGGTGCACCCTTGAGAACTTCACCTTCCAAAAAGTACTTGGCAAAGGCAGCTTTGGCAAGGTGCTGCT
     D  I  L  D  N  N  W  T  Y  G  K  I  W  V  G  S  T  R  C  T  L  E  N  F  T  F  Q  K  V  L  G  K  G  S  F  G  K  V  L  L  363

1321 GGCAGAGCTGAAGGGCAAAGACAAGTACTTTGCAATCAAGTGTCTGAAGAAGGACGTGGTGTTGATTGACGATGATGTAGAGTGTACCATGGTGGAGAAGCGGGTGCTGGCGCTCGCCTG
     A  E  L  K  G  K  D  K  Y  F  A  I  K  C  L  K  K  D  V  V  L  I  D  D  D  V  E  C  T  M  V  E  K  R  V  L  A  L  A  W  403

1441 GGAGAGTCCCTTCCTCACCCACCTCATCTGTACCTTCCAGACCAAGGACCACCTGTTCTTCGTGATGGAGTTTCTCAATGGGGGTGACCTGATGTTCCACATTCAGGACAAAGGCCGCTT
     E  S  P  F  L  T  H  L  I  C  T  F  Q  T  K  D  H  L  F  F  V  M  E  F  L  N  G  G  D  L  M  F  H  I  Q  D  K  G  R  F  443

1561 CGAACTCTACCGGGCTACGTTTTATGCAGCTGAGATCATCTGCGGACTGCAGTTTCTACACAGCAAAGGCATTATTTACAGGGACCTCAAGCTGGACAATGTGATGCTAGACAGGGACGG
     E  L  Y  R  A  T  F  Y  A  A  E  I  I  C  G  L  Q  F  L  H  S  K  G  I  I  Y  R  D  L  K  L  D  N  V  M  L  D  R  D  G  483

1681 CCACATCAAGATCGCTGACTTTGGGATGTGCAAAGAGAATATATTTGGGGAGGACCGCCCCAGCACATTCTGCGGCACTCCTGACTACTCCGCCCCTGAGATCCTGCAGGGCCTGAAGTA
     H  I  K  I  A  D  F  G  M  C  K  E  N  I  F  G  E  D  R  P  S  T  F  C  G  T  P  D  Y  S  A  P  E  I  L  Q  G  L  K  Y  523

1801 CTCCTTCTCGGTGGACTGGTGGTCTTTCGGGGTCCTCCTGTACGAAATGCTCATCGGCCAGTCCCCCTTCCACGGCGACGATGAGGACGAGCTCTTCGAGTCCATCCGGGTGGACACACC
     S  F  S  V  D  W  W  S  F  G  V  L  L  Y  E  M  L  I  G  Q  S  P  F  H  G  D  D  E  D  E  L  F  E  S  I  R  V  D  T  P  563

1921 ACACTATCCCCGTTGGATCACCAAGGAATCCAAGGACATCATGGAGAAGCTATTCGAGAGGGACCCTGACAAGAGGCTGGGAGTAACAGGAAACATCAGGATTCACCCCTTTTTCAAGAC
     H  Y  P  R  W  I  T  K  E  S  K  D  I  M  E  K  L  F  E  R  D  P  D  K  R  L  G  V  T  G  N  I  R  I  H  P  F  F  K  T  603

2041 TATCAACTGGTCCCTCCTGGAGAAGCGGAAGGTGGAGCCGCCCTTTAAGCCCAAAGTGAAATCCCCTTCAGACTACAGCAACTTTGACCCAGAGTTCCTGAATGAGAAACCTCAGCTTTC
     I  N  W  S  L  L  E  K  R  K  V  E  P  P  F  K  P  K  V  K  S  P  S  D  Y  S  N  F  D  P  E  F  L  N  E  K  P  Q  L  S  643

2161 CTTCAGTGACAAGAACCTCATCGACTCTATGGACCAGGAAGCCTTCCATGGCTTCTCCTTTGTGAATCCCAAGTTTGAGCAATTCCTGGACATTTAATTAAGCTCCCAGACCTGCTTTAA
     F  S  D  K  N  L  I  D  S  M  D  Q  E  A  F  H  G  F  S  F  V  N  P  K  F  E  Q  F  L  D  I  *                          674

2281 ACGCCCTGGCAGAGTAGGCCCGTCTGCCCTGGTTTGCTCCGCATTGCCCGTGGAGAACAGTGGGTGACTGGCTGATTTCTGCTGCCGCCCCCTCTTCCTTGGAGAGTCCGGCTCCTGCTG
2401 GCTGGGCTCACAGTACTTCCTCTGTGAACTGTTTGTGAATTTGCCTTCCCTTTGCCATCAGAGGGAAACTGTAAATCCTGTGTTTCATTACTTGAATGTAGTTATTGAAATATATATTAT
2521 ATATATGCACATATAAAAAAAACGCACGAGCTCGTGCCGAATTC
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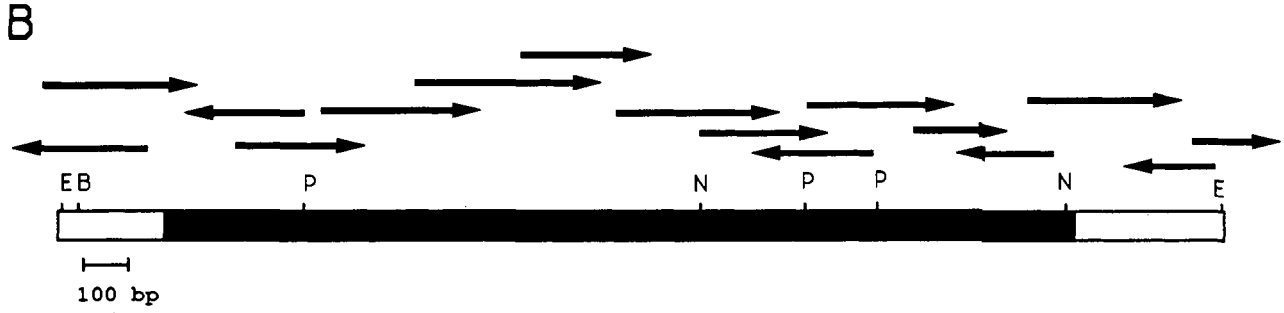

FIGURE 2: (A) Composite sequence of mouse PKC-δ cDNA and the deduced amino acid sequence. (B) Sequencing strategy of PKC-δ cDNA. Arrows indicate the extent and direction of sequencing. The restriction sites for *Bam*HI (B), *Eco*RI (E), *Nco*I (N), and *Pst*I (P) are indicated.

dance of the mRNA for that particular isoform. Sequencing of 12 PCR subclones from each of the tumors revealed that between 8 and 11 subclones from each cell line had identical sequences which were 98% homologous to rat PKC-δ. In contrast, no subclone containing a PKC-δ-derived insert could be obtained from brain mRNA. This is in accordance with the lower expression of PKC-δ mRNA in brain than in most hemopoietic cells when compared on Northern blots (see below and Figure 4). The sequences of the PCR fragments that were not PKC-δ were determined to encode examples of mouse PKC-α, -β, -γ, -ε, and -η.

In order to further characterize the PKC-δ transcripts, we

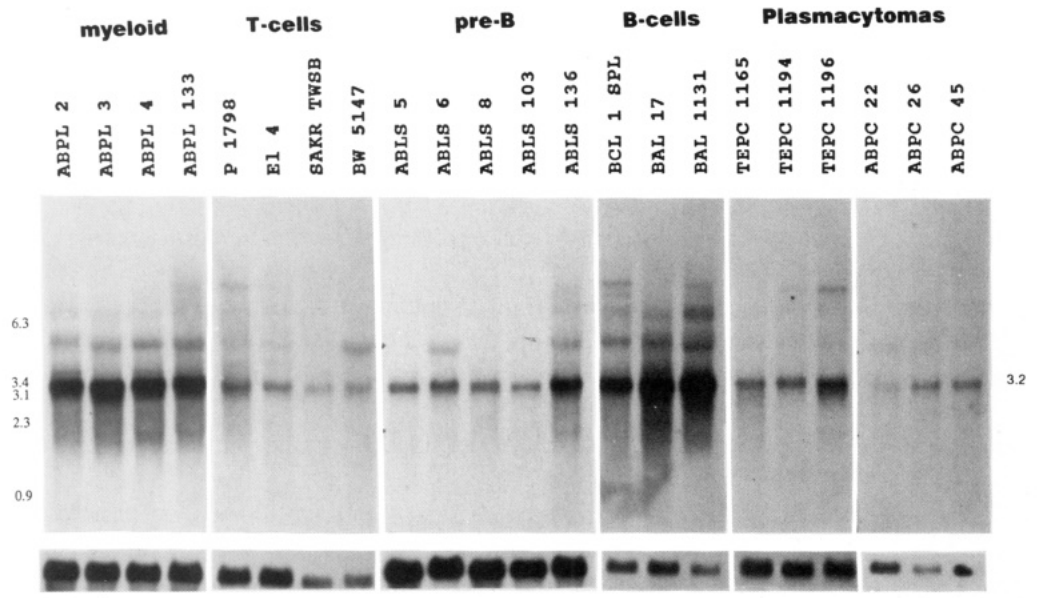

FIGURE 3: Northern blots representing different hemopoietic lineages and B-cell development. The sizes ($\times 10^{-3}$) and positions of RNA markers are indicated on the left. The predominant 3.2-kb PKC-δ mRNA is clearly visible in all lanes. Additional bands of higher molecular weight, a phenomenon often observed with highly expressed mRNAs, are probably due to partially spliced nuclear RNA. The blots were subsequently probed with glyceraldehydephosphate dehydrogenase (GAPDH) for normalization, and these are shown in the lower panels.

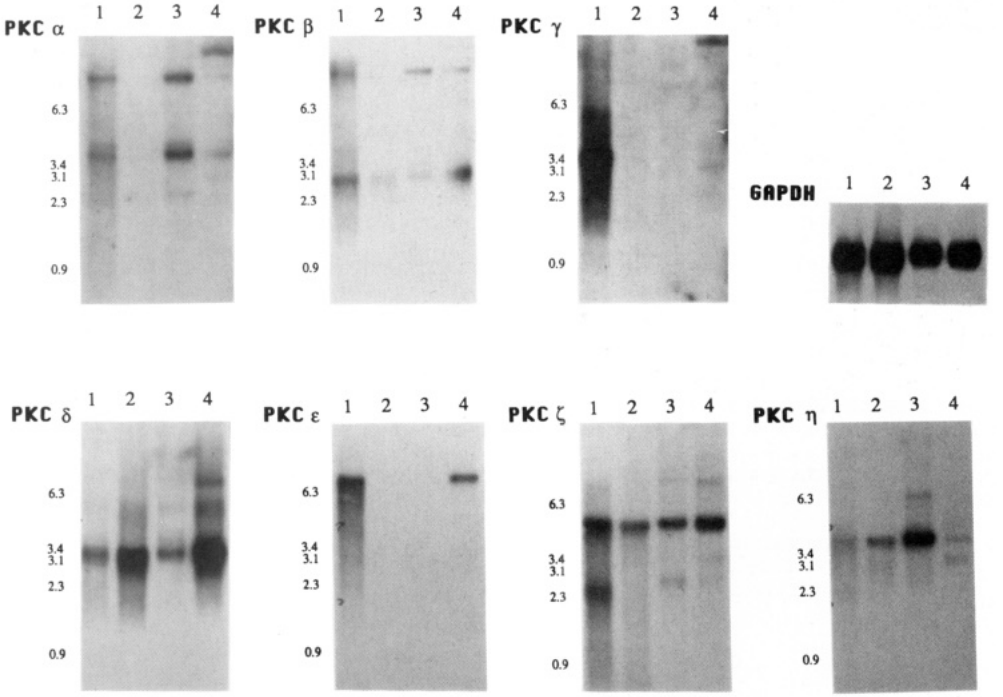

FIGURE 4: Northern blots of brain (lane 1) and three different hemopoietic cell lines representing myeloid cells (ABPL-3, lane 2), T cells (SAKRTLS 13, lane 3), and B cells (BAL 1131, lane 4) probed for the expression of the seven different PKC isozymes. The size ($\times 10^{-3}$) and position of molecular weight standards are indicated on the left. All autoradiograms were exposed overnight, except those for PKC-δ (3 h), PKC-ϵ (2 h), and GAPDH (30 min).

screened a cDNA library from ABPL-2, a myeloid tumor and cell line. Screening of about 5×10^5 clones resulted in five positive plaques. Further characterization indicated that all five contained a 2.55-kb cDNA insert. Sequencing of one of these cDNAs revealed a 2022-nucleotide open reading frame encoding 674 amino acids (Figure 2). The protein sequence shows 96% identity with rat PKC-δ and between 55% (rat PKC-ϵ) and 48% (rat PKC-α) identity with the sequence of other members of the PKC gene family.

Northern blots of RNA from different hemopoietic cell lines were probed with PKC-δ cDNA to learn how extensively this member of the PKC gene family is expressed in hemopoietic cells. All hemopoietic cells we examined contained high amounts of a 3.2-kb PKC-δ mRNA. A composite blot of a representative selection of these RNAs is shown in Figure 3.

To compare the levels of all PKC isozymes expressed in T and B as well as myeloid cells to the level of expression found in brain, Northern blots from brain, ABPL-3, SAKRTLS 13 (a T-cell lymphoma), and BAL 1131 (a mature B-cell lymphoma), were probed for the expression of all seven isozymes (Figure 4). As reported in a more detailed study (Mischak et al., submitted for publication), PKC-δ is, in general, the most abundantly expressed PKC isoform found in hemopoietic cells, with the possible exception of T-cell lines, where similar levels of PKC-η expression can be observed. Note, that with the exception of the exposures done for detection of PKC-δ (3 h)

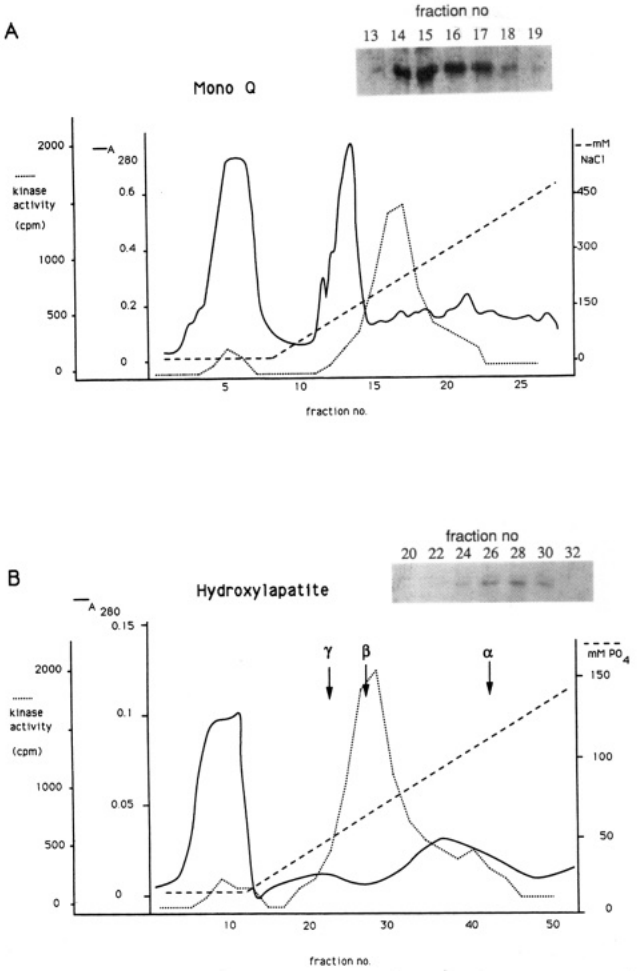

FIGURE 5: Purification of PKC-δ from ABPL-3. A total of 3×10^8 cells were lysed and applied onto a Mono-Q FPLC column. Panel A shows the absorbance profile (—) as well as the kinase activity (···) and the salt gradient (---). The column was eluted at 1 mL/min; 1-mL fractions were collected, and the kinase activity of 1 μL/fraction was measured as the incorporation of ^{32}P in 1 μg of histone H1 in 20 min in the presence of phospholipid, but in the absence of Ca^{2+}. Incorporation was measured by counting the histone bands resolved on a 12.5% SDS gel. The inset shows a Western blot of 10 μL of fractions 13–19 stained with the anti-PKC-δ antiserum. Fractions 14–17 were pooled and applied onto a 100 × 10 mm hydroxylapatite column. The absorbance (—), the salt gradient (---), and the kinase activity (···) are shown in panel B. The column was eluted at 0.3 mL/min, and 4-mL fractions were collected; 10 μL/fraction was used for Western blotting. The inset shows fractions 20–32 stained with the anti-PKC-δ antiserum. For determination of the kinase activity, 10 μL/fraction was used as described above. α, β, and γ indicate the elution positions of PKC-α, -β, and -γ purified from brain in a parallel experiment.

and PKC-ε (2 h) expression, all blots were exposed overnight.

To characterize the PKC-δ protein and also to compare its biochemical properties to those of the already described PKC's, the enzyme was purified from the myeloid cell line ABPL-3, which does not express PKC-α, -β, -γ, or -ε, and only low amounts of PKC-ζ and -η (Figure 4). Western blotting performed on crude cell extracts from ABPL-3 using antibodies against PKC-α, -β, -γ, and -δ revealed the presence of only PKC-δ protein.

PKC-δ eluted from a Mono Q anion-exchange column between 160 and 185 mM NaCl under the conditions described (Figure 5A). PKC-α, -β, and -γ, purified from mouse brain in a parallel experiment, also eluted at the same position in the salt gradient (data not shown). Further purification using a hydroxylapatite column (Figure 5B) revealed that PKC-δ elutes at the same concentration of PO_4^{3-} as PKC-β (Huang et al., 1986) and -ε (Konno et al., 1989). Immunostaining of Western blots of the fractions obtained from the hydroxylapatite column revealed that the immunoreactivity for PKC-δ can be found in the same fractions as the kinase activity (Figure 5B), which was scored in the presence of phospholipid, but in the absence of Ca^{2+}. Antibodies directed against PKC-α, -β, and -γ did not detect any protein of these isoforms in the fractions where we found PKC-δ (data not shown). Furthermore, since only trace amounts of mRNA for PKC-β or -ε could be detected (Figure 4), we can rule out contamination of PKC-δ purified from ABPL-3 with these isoforms. Much to our surprise, efforts to purify PKC-δ from brain by using the same protocol failed. What is more, we could detect only very low amounts of PKC-δ protein in crude or fractionated brain extracts, although we found moderately high levels of mRNA in brain.

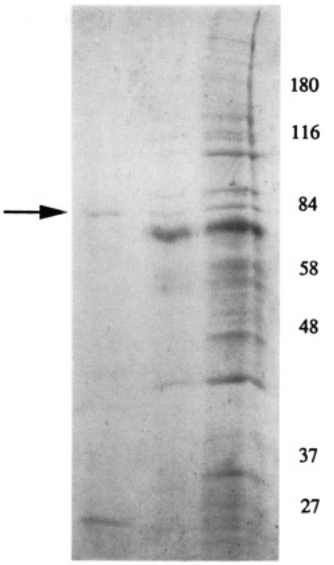

FIGURE 6: 10% SDS–polyacrylamide gel of PKC-δ following the different purification steps. The sizes of molecular weight standards ($\times 10^{-3}$) are indicated on the right. The arrow on the left indicates the immunoreactive band. Right lane, crude extract; middle lane, fraction 15 from Mono Q; left lane, fraction 28 from the hydroxylapatite column.

As shown in Figure 6, PKC-δ can be purified to a great extent by using sequential anion-exchange and hydroxylapatite chromatography.

In vitro kinase assays showed the strict dependence of the enzyme activity on the presence of phospholipid, a feature shared by all members of the PKC family. In the presence of 5 μg/mL phosphatidylserine, no enhancing effect of diolein could be observed. As expected from its sequence and its homology to PKC-ε, no Ca^{2+} dependence was observed (Figure 7). Although we always found slight differences in the intensity of the phosphorylated histone bands in the presence or absence of Ca^{2+}, these differences were minor and did not show any trend.

Discussion

This paper demonstrates that PCR amplification using primers of highly conserved regions can be used as a simple and powerful means to find additional members of a gene family. This method is an improvement on screening cDNA libraries with synthetic oligonucleotides or with related probes under lower stringency. Both of these methods gave only weak and very unreliable signals.

The oligonucleotides used as primers for the PCR reaction seemed to have no preference for one specific PKC isozyme.

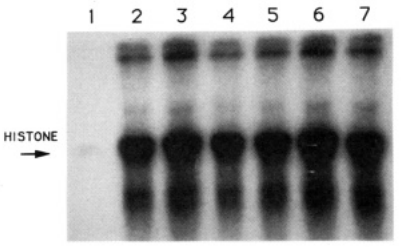

FIGURE 7: In vitro kinase assays of purified PKC-δ using 1 μg of histone as substrate. Samples were incubated in 20 mM Tris and 100 mM NaCl, pH 7.5, with 1 μCi of ATP and the following additives: lane 1: none; lane 2, 5 μg/mL phosphatidylserine; lane 3, 5 μg/mL phosphatidylserine + 2 μg/mL diolein; lane 4, 5 μg/mL phosphatidylserine + 0.1 mM $CaCl_2$; lane 5, 5 μg/mL phosphatidylserine + 2 μg/mL diolein + 0.1 mM $CaCl_2$; lane 6, 5 μg/mL phosphatidylserine + 1 mM $CaCl_2$; lane 7, 5 μg/mL phosphatidylserine + 2 μg/mL diolein + 1 mM $CaCl_2$. The arrow indicates the histone band.

The relative numbers of the various isoform subclones obtained and sequenced correspond to the levels of isozyme expression seen on Northern blots (Figure 4). PKC-δ shows the highest number of PCR-derived cDNAs and also the highest intensity of hybridization on Northern blots from hemopoietic cells (with the possible exception of T-cells, where PKC-η may be slightly higher). In brain, however, the abundance of PKC-δ is lower than PKC-γ, -ε, and -ζ; therefore, no PCR fragments corresponding to PKC-δ could be found in 12 randomly picked subclones.

Mouse PKC-δ shows a very high degree of homology (96%) to rat PKC-δ. This is as expected, since extreme conservation of sequence has been consistently observed among the same PKC isoforms isolated from different species. Other PKC isoforms that are closely related to PKC-δ are PKC-ε and PKC-η. All three of these isoforms lack the C_2 domain, which is most likely involved in the activation of PKC-α, -β, and -γ by Ca^{2+} (Kaibuchi et al., 1989).

PKC-δ coelutes with PKC-β and -ε on a hydroxylapatite column. Since ABPL-3, as well as several other hemopoietic cell lines, does not express PKC-β and -ε, PKC-δ can be purified from those cells without contamination of other PKC's. As shown in Figure 6, two column chromatographic steps are sufficient to purify the protein almost to homogeneity, provided that the cells used do not express PKC-β or -ε. Significant contamination of PKC-δ purified from ABPL-3 with PKC-α, -β, and -γ can be ruled out by the absence of any protein reacting with antibodies against those isozymes and also by the absence of a Ca^{2+}-dependent kinase activity. Since no antibodies against PKC-ε and -ζ were available, the absence of those isozymes could not be directly proven. Nevertheless, this seems to be the case, because PKC-ε expression could not be observed on Northern blots of ABPL-3 mRNA, and PKC-ε and -ζ have different molecular weights than PKC-δ, which would have appeared as additional bands in the 90- or 70-kDa range, respectively. Our failure to detect PKC-δ protein in brain is consistent with the reports from other laboratories (Huang et al., 1986) that PKC's purified from brain generally contain only Ca^{2+}-dependent PKC's. The presence of PKC-δ mRNA in brain (Figure 4) suggests that there may be an element of posttranscriptional control in PKC isozyme expression.

Biochemical characterization of purified PKC-δ clearly showed that this kinase can be activated by phospholipid, a hallmark of all PKC isozymes (Figure 7). It does not require Ca^{2+}, which is in accordance with the lack of the C_2 domain in this class of PKC's, called nPKC (Ohno et al., 1988). As shown in Figure 7, a major substrate for PKC-δ is histone, as it is for all the other PKCs described.

Northern blots hybridized with mouse PKC-δ (Figures 3 and 4) show that this is the most abundantly expressed isoform of PKC in hemopoietic cells. These data, together with the fact that the majority of PCR-amplified cDNAs from different hemopoietic cell lines corresponded to PKC-δ suggest that the non-Ca^{2+}-responsive PKC purified from porcine spleen reported by Leibersperger et al. (1990) is PKC-δ.

The strict regulation of the expression of different PKC isozymes observed in these and other cells makes it very likely that different PKC's act on different targets and probably also can be activated by different stimuli. It seems very questionable whether stimulation of cells with phorbol ester or other PKC activators, which cannot distinguish between the different isozymes, is an appropriate method to delineate the involvement of PKC in different signal transduction pathways. We would like to propose that the different PKC isozymes must be looked upon as clearly different enzymes, sometimes activated by different substances and also most likely acting on different targets.

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Evidence for Domain Organization within the 61-kDa Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase from Bovine Brain†

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ABSTRACT: The complete amino acid sequence of the 61-kDa calmodulin-dependent, cyclic nucleotide phosphodiesterase (CaM-PDE) from bovine brain has been determined. The native protein is a homodimer of N^{α}-acetylated, 529-residue polypeptide chains, each of which has a calculated molecular weight of 60755. The structural organization of this CaM-PDE has been investigated with use of limited proteolysis and synthetic peptide analogues. A site capable of interacting with CaM has been identified, and the position of the catalytic domain has been mapped. A fully active, CaM-independent fragment (M_r = 36000), produced by limited tryptic cleavage in the absence of CaM, represents a functional catalytic domain. N-Terminal sequence and size indicate that this 36-kDa fragment is comprised of residues 136 to ≈450 of the CaM-PDE. This catalytic domain encompasses a ≈250 residue sequence that is conserved among PDE isozymes of diverse size, phylogeny, and function. CaM-PDE and its PDE homologues comprise a unique family of proteins, each having a catalytic domain that evolved from a common progenitor. A search of the sequence for potential CaM-binding sites revealed only one 15-residue segment with both a net positive charge and the ability to form an amphiphilic α-helix. Peptide analogues that include this amphiphilic segment were synthesized. Each was found to inhibit the CaM-dependent activation of the enzyme and to bind directly to CaM with high affinity in a calcium-dependent manner. This site is among the sequences cleaved from a 45-kDa chymotryptic fragment that has the complete catalytic domain but no longer binds CaM. These results indicate that residues located between position 23 and 41 of the native enzyme contribute significantly to the binding of CaM although the involvement of residues from additional sites is not excluded.

Changes in the intracellular concentration of free calcium ions can alter cyclic nucleotide metabolism through the action of the calcium/calmodulin complex (CaM)[1] on calmodulin-stimulated cyclic nucleotide phosphodiesterases (CaM-PDEs). CaM binds with high affinity and greatly increases the rate of cAMP or cGMP hydrolysis, thus providing a mechanism whereby stimuli that alter intracellular calcium concentrations (e.g., via phosphoinositide turnover) can modulate cellular responses that are mediated by changes in cyclic nucleotide concentrations.

CaM-PDEs constitute one of the five different families of cyclic nucleotide phosphodiesterase (PDE) isozymes [for reviews, see Beavo and Reifsnyder, 1990; Wang et al., 1990]. In mammals, there are at least five distinct isozymes within the CaM-PDE family. These are distinguished by apparent size, immunoreactivity, ability to be phosphorylated in vitro by protein kinases, affinity for CaM, and in some cases kinetic parameters (Beavo, 1988). The two major isozymes found in bovine brain are homodimers with subunit molecular weights of 61000 and 63000 (Sharma et al., 1984; Hansen & Beavo, 1986). The two brain isozymes can be readily resolved from one another and purified by procedures employing isozyme and conformation-specific monoclonal antibodies (Hansen et al., 1988). This study utilizes the 61-kDa isozyme since it is generally obtained in higher yield.

The present study provides the complete amino acid sequence of the 61-kDa CaM-PDE. The structural relationship among the mammalian CaM-PDE isozymes has not been elucidated, and it is not known whether these isozymes are

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[1] Abbreviations: PDE, cyclic nucleotide phosphodiesterase; BNPS-skatole, an acronym for the reagent described by Fontana et al. (1973); CaM, calcium/calmodulin complex; CaM-PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase; CAP, catabolite activator protein, CM, S-carboxymethyl; HPLC, high-performance liquid chromatography; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; Pth, phenylthiohydantoin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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