Identification of a brain-specific protein kinase C ζ pseudogene (Ψ PKC ζ) transcript

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Protein kinase C (PKC), a widely-distributed enzyme implicated in the regulation of many physiological processes, consists of a family of at least twelve isoenzymes which differ in tissue distribution, subcellular localization, regulatory properties, etc. In addition to this heterogeneity at the protein level, we identify here for the first time a PKC ζ pseudogene (Ψ PKC ζ) transcript, specifically expressed in the brain, which is identical with PKC ζ except for sequence divergence within the first variable domain (V1). The authenticity of this unique V1 sequence (V1') in mRNA was confirmed by RNase protection and reverse transcriptase PCR (RT-PCR) analysis. When translated in-frame with PKC ζ , a stop codon is located 28 amino acids towards the N-terminus of the divergence point and the intervening sequence lacks an expected initiating methionine. Ψ PKC ζ is non-functional in terms of protein synthesis since Western blotting with an antibody directed against the C-terminus of PKC ζ failed to reveal a protein smaller than PKC ζ , and synthetic Ψ PKC ζ RNA failed to support protein synthesis in a translation system *in vitro*. PCR amplification of rat genomic DNA demonstrated lack of an intron at the junction between V1' and the first constant domain

(the V1'–C1 border), and genomic DNA Southern blot analysis using PKC ζ - and Ψ PKC ζ -specific probes indicated that they have different loci. Ψ PKC ζ , therefore, is not derived from the PKC ζ gene by alternative splicing, but rather is the product of a distinct gene. In Northern blot analysis, brain PKC ζ mRNA was identified as a low-abundance 3.1 kb transcript, while the abundant 2.5 and 4.7 kb mRNAs previously reported to encode PKC ζ are, in fact, Ψ PKC ζ transcripts. Analysis of rat brain, heart, lung, liver, kidney and skeletal muscle revealed Ψ PKC ζ mRNA only in brain. PKC ζ transcripts were most abundant in lung and kidney (2.7 and 4.7 kb mRNAs), correlating with the tissue profile of PKC ζ immunoreactivity in Western blots. Probes complementary to the common V5 and C1 domains detected both PKC ζ and Ψ PKC ζ transcripts. Interestingly, the C1 probe also detected an abundant novel 1.75 kb mRNA in brain and heart, suggesting the existence of an additional PKC ζ -related species. This work, therefore, also emphasizes the importance of careful choice of oligonucleotide and cDNA probes to study PKC ζ mRNA.

INTRODUCTION

Protein kinase C (PKC) consists of a widely-distributed family of structurally-related, phospholipid-dependent protein serine/threonine kinases which are implicated in diverse signal transduction pathways [1]. To date, twelve isoenzymes of PKC have been identified which differ in tissue distribution, subcellular localization, substrate specificity and regulation by effector molecules. Based on their activation requirements and domains of sequence similarity, these isoenzymes have been divided into three groups. Conventional PKCs (cPKCs: α , β_1 , β_2 and γ) are activated by Ca^{2+} , phospholipid and diacylglycerol or phorbol esters, and activation is enhanced by *cis*-unsaturated fatty acids and lysophosphatidylcholine. Comparison of the deduced amino acid sequences of cPKCs revealed four constant (C1–C4) and five variable regions (V1–V5). Domain C1 has been implicated in binding phospholipid, diacylglycerol and phorbol esters, while domain C2 confers Ca^{2+} sensitivity [2]. Domains C3 and C4 together constitute the catalytic domain. Reverse transcriptase PCR (RT-PCR) approaches, using degenerate oligonucleotides based on conserved regions or low-stringency cDNA library screening with restriction fragments of known PKCs, led to the isolation of cDNAs encoding additional PKC isoenzymes: δ , ϵ , ζ [3,4], η (or L) [5,6], θ [7], ι [8], μ [9] and λ [10]. All these isoenzymes lack a C2 domain and the expressed or tissue-purified proteins do not require Ca^{2+} for activity. The ζ , ι and λ isoenzymes contain a single cysteine-rich zinc finger-like motif in domain C1,

unlike all other PKC isoenzymes which contain a tandem repeat of this sequence. For this reason, PKCs δ , ϵ , η , θ and μ can be classified together as novel PKCs (nPKCs) and ζ , ι and λ as atypical PKCs (aPKCs) [1]. nPKCs are activated by phospholipid and diacylglycerol or phorbol esters but are independent of Ca^{2+} ; the ϵ , but not the δ , isoenzyme is activated by *cis*-unsaturated fatty acids. aPKCs are dependent on phospholipid (although this has not been consistently observed), are activated by *cis*-unsaturated fatty acids, but are unaffected by diacylglycerol, phorbol esters or Ca^{2+} . Although the physiological signal for activation of aPKCs has not been unequivocally identified, PKC ζ is activated by phosphatidylinositol 3,4,5-trisphosphate which is produced upon activation of phosphatidylinositol 3'-kinase by insulin and other growth factors [11]. PKC ζ has been implicated as a critical component of mitogenic signal transduction downstream of insulin-p21^{ras}-phosphatidylcholine-specific phospholipase C in *Xenopus laevis* oocytes and mammalian cells [12,13], and PKC ζ activates nuclear factor κ B causing its translocation to the nucleus and regulation of a number of genes [14,15]. Recent evidence suggests that PKC ζ phosphorylates an I κ B kinase [16] and also that this pathway can be activated via the production of ceramide following cytokine-induced activation of sphingomyelinase [17].

Northern blot analysis using a cDNA probe corresponding to the 3'-end of PKC ζ revealed two mRNAs of 4.2 and 2.2 kb in rat brain [3]. Similar mRNA species have been detected by others using different cDNA or oligonucleotide probes based on

Abbreviations used: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase PCR; TBS, Tris-buffered saline; pfu, plaque-forming unit.

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the PKC ζ sequence, e.g. [4,18,19]. We report here that rat brain PKC ζ is in fact encoded by a lower-abundance mRNA of 3.1 kb and that the two mRNAs previously described are derived from a PKC ζ pseudogene, which we propose to refer to as Ψ PKC ζ . (Pseudogenes bear a close resemblance to known genes at different loci, but have mutations which prevent transcription or translation). While Ψ PKC ζ is transcribed in brain tissue, it is unable to support protein synthesis due to the lack of an initiating methionine. It is unknown at this time whether the Ψ PKC ζ mRNA serves a regulatory role. Finally, PKC ζ transcripts are shown to be most abundant in lung and kidney and there is a good correlation between PKC ζ mRNA and protein levels in different tissues.

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP (< 5000 Ci/mmol), [α - 32 P]dCTP ($\approx$ 3000 Ci/mmol), [α - 35 S]UTP (< 1000 Ci/mmol) and L-[35 S]methionine (cell labelling *in vivo* grade; 50 mCi/mmol) were purchased from Amersham (Oakville, Ontario, Canada). The following oligonucleotides were synthesized in the Regional DNA Synthesis Laboratory, University of Calgary, Alberta, Canada: 5'-CACGGACTCCTCAGCAGACAGCAGAAGCGGGTTGAT-3' [probe 1 complementary to the V5 domain (nt 1888–1923) of PKC ζ and equivalent to part of the C-terminal peptide used to raise antibodies to PKC ζ] [4]; 5'-CTTGTCTTCTCCAGGAC-AAGGCATGCCCGGTTGTT-3' [probe 2 complementary to the C-terminus of the V1 domain (nt 449–483) of full-length PKC ζ] [4]; 5'-CTCGGCTTTCTCTGCTGCCTCTTCAGCAGGAATTC-3' [probe 3 complementary to the unique V1 (referred to herein as V1') sequence (nt 1–36) of the partial PKC ζ cDNA sequence originally reported by Ono et al. [3]; 5'-TTCCTCCATCTTCTGGCTCCACGGCGGTAGATGGA-3' [probe 4 complementary to the N-terminus of the C1 domain (nt 484–518) of PKC ζ] [4]; 5'-CCGCGTTCGCCGCTCTGCCC-CGCCTGGCAA-3' (corresponding to nt 40–69 of the 5' untranslated region of full-length PKC ζ) [4]. Three other oligonucleotide probes, boxed sequence 1 in Figure 3 and its complement and the complement of boxed sequence 2 in Figure 3, were also synthesized. A polyclonal antibody raised against a synthetic peptide corresponding to the C-terminus of PKC ζ (GFHEYINPLLSAEESV) was purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). A rat brain cDNA library in the λ ZAP II vector was purchased from Stratagene (La Jolla, CA, U.S.A.). Kodak X-OMAT-AR film was purchased from Picker International (Brampton, Ontario, Canada).

Hybridizations

In general, oligonucleotide probes (10 pmol) were labelled with T4 polynucleotide kinase (Pharmacia, Baie d'Urfe, Québec, Canada) in the presence of [γ - 32 P]ATP (150 μ Ci), and cDNA probes (25 ng) were labelled using a Random Primed DNA Labelling Kit (Boehringer Mannheim, Laval, Québec, Canada) in the presence of [α - 32 P]dCTP (50 μ Ci). Membranes were prehybridized in 5 $\times$ SSPE [1 $\times$ SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 5 $\times$ Denhardt's solution, 50% formamide, 0.1% SDS and 0.25 mg/ml sonicated denatured salmon testis DNA for 3 h at 37 or 42 $^{\circ}$ C for use with oligonucleotide or cDNA probes respectively. Hybridization (2 $\times$ 10⁶ c.p.m./ml) was performed overnight at the same temperature used for prehybridization. Membranes probed with oligonucleotides or cDNAs were washed with 1 $\times$ SSPE/0.1% SDS at 55 $^{\circ}$ C or with 0.1 $\times$ SSPE/0.1% SDS at 65 $^{\circ}$ C respectively

Figure 1 Northern blot analysis of mRNA from rat brain

Rat brain mRNA (6 μ g) was denatured, electrophoresed, transferred to nylon membranes and hybridized with 32 P-labelled oligonucleotide or cDNA probes. A schematic representation of the coding region of PKC ζ is shown in (a) with variable (V) and constant (C) regions. V1 represents the N-terminus of the full-length PKC ζ sequence [4] and V1' the distinct, partial sequence published earlier [3]. Oligonucleotide probes complementary to the four sites indicated by bars were used for Northern blot analysis as shown in (b). Lanes: 1, probe 1 complementary to the C-terminus of PKC ζ (V5); 2, probe 2 complementary to the C-terminal end of the first variable region (V1) of the full-length sequence; 3, probe 3 complementary to the partial sequence (V1') which differs from the V1 sequence; 4, probe 4 complementary to the N-terminus of the C1 domain of PKC ζ ; 5, ζ D.1 cDNA probe; 6, an oligonucleotide probe derived from the sequence of ζ F.1 cDNA which is complementary to the region upstream of the internal *Eco*RI site and which contains a stop codon (see Figure 3); 7, ζ F.1 cDNA (5' *Eco*RI fragment; see Figures 2 and 3). The sizes of the four hybridizing mRNAs (in kb) are shown on the left and RNA size markers on the right. Lanes 1, 3, 4 and 6 were exposed to X-ray film for 2 days at -80° C, lane 2 for 5 days, lane 5 for 18 h, and lane 7 for 3 days. After hybridization with probe 2, the membrane was stripped by boiling in 0.1 $\times$ SSPE/0.1% SDS and re-probed with probe 3: the same result as shown in lane 3 was obtained.

and exposed to X-ray film at -80° C for the times indicated in the Figure legends.

mRNA Isolation and Northern blot analysis

Total RNA was isolated from rat brain and other tissues using the Promega (Madison, WI, U.S.A.) RNeasy Total RNA Isolation Kit, and poly(A)⁺ mRNAs were isolated from the total RNA using the Promega PolyAtract mRNA Isolation System as described by the manufacturer. Poly(A)⁺ RNAs were denatured with 2.2 M formaldehyde and 50% formamide, electrophoresed on 1% agarose gels containing 0.5 M formaldehyde and transferred to Zeta Probe nylon membrane (BioRad Laboratories, Mississauga, Ontario, Canada) by capillary action overnight in 20 $\times$ SSPE. The blots were fixed by baking at 80 $^{\circ}$ C for 2 h and hybridized with the oligonucleotide or cDNA probes described in Figure 1.

Figure 2 Characterization of cDNA clones

The full-length sequence of PKC ζ (2661 bp) [4] is shown schematically at the top, with the coding region divided into constant (C) and variable (V) domains. The nucleotide sequences of the indicated cDNA clones are depicted relative to PKC ζ , where solid lines represent sequences identical with full-length PKC ζ and broken lines represent divergence from the full-length PKC ζ sequence. The location of an internal *Eco*RI site 35 nt upstream of the divergence point (the V1'–C1 border) is indicated as well as a *Bam*HI site used to generate a full-length construct from ζ F.1 and ζ N.1.

Isolation of cDNA clones

A rat brain cDNA expression library was initially screened using a polyclonal antibody raised against a synthetic peptide corresponding to the C-terminus (V5 domain) of PKC ζ (Figure 1a). Plaques were lifted onto BA-S supported nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U. S. A.) containing isopropyl β -D-thiogalactoside. Membranes were blocked by treatment with 3% low-fat milk in TBS (20 mM Tris-HCl (pH 7.5)/0.5 M NaCl) for 2 h at room temperature, incubated overnight at room temperature with anti-PKC ζ (5 μ g/ml in 1% BSA in TBS) pre-equilibrated with XL1-Blue *Escherichia coli* lysate, then washed three times with 0.05% Tween 20 in TBS. Following incubation for 1 h at room temperature with goat anti-(rabbit IgG) alkaline phosphatase conjugate (Sigma, St. Louis, MO, U.S.A.) at a dilution of 1:1000 in TBS containing 1% BSA, the membrane was washed three times with 0.05% Tween 20 in TBS and colour development was achieved with 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium. From a screen of $\approx 300\,000$ plaque-forming units (pfus), one positive clone (denoted ζ D.1) was obtained. ζ D.1 was plaque-purified, the plasmid DNA recovered by excision *in vivo* and the insert sized by 1% agarose gel electrophoresis after *Eco*RI digestion of the plasmid. The nucleotide sequence of the insert was obtained using the Perkin-Elmer/Applied Biosystems, Inc. (Mississauga, Ontario, Canada) automated fluorescent sequencing methodology and Genetics Computer Group, Inc. (Madison, WI, U.S.A.) sequence analysis software and was identified as a 1266 bp partial PKC ζ cDNA which lacked the 5' sequence (Figure 2). The ζ D.1 insert was labelled with 32 P and used to rescreen the library. Plaque lifts were denatured in 0.5 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris-HCl (pH 8.0)/1.5 M NaCl, fixed by baking at 80 °C for 2 h and hybridized as described in the previous section. From a screen of $\approx 300\,000$ pfus, one more positive clone was obtained (ζ F.1). Using an alternative strategy, the library was also screened with a 32 P-labelled C1 oligonucleotide probe (probe 4 in Figure 1a) using standard hybridization conditions. From a screen of $\approx 500\,000$ pfus, four more clones were obtained (ζ L.1, ζ N.1, ζ Q.1 and ζ X.1). The additional clones were processed as described for ζ D.1.

RNAse protection assay

DNA templates for RNA probe synthesis containing ζ F.1 nt 213–521 and nt 615–914 were obtained by digesting ζ F.1 cDNA with *Eco*RI-*Acc*I and *Cl*aI-*H*incII respectively. Restriction fragments were gel-purified, subcloned into pBluescript SK⁺ and the inserts confirmed by sequencing. Plasmids were linearized with *Not*I and single-stranded antisense riboprobes were generated using a Stratagene RNA transcription kit with T7 polymerase and [α - 35 S]UTP. The DNA template was removed with RQ1 RNase-free DNase (Promega) and probes (1 $\times 10^6$ c.p.m.) were incubated with 10 μ g of rat brain or yeast total RNA in 20 μ l of hybridization buffer [40 mM Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA and 80% formamide] for 18 h at 45 °C. Unhybridized RNA was digested by dilution of the reaction mixtures in 0.35 ml of 10 mM Tris-HCl (pH 7.6), 0.3 M NaCl and 5 mM EDTA and treatment with RNase A (40 μ g/ml; Boehringer) and RNase T1 (10 U/ml; Boehringer) for 30 min at 28 °C. Protected fragments were sized on a denaturing polyacrylamide gel (6% acrylamide/7 M urea) along with a 100 nt ladder (Gibco BRL) which was radiolabelled by exonuclease/resynthesis reaction with T4 DNA polymerase (Gibco BRL) in the presence of [α - 35 S]dCTP. The gel was dried and exposed to X-ray film at room temperature for 3 weeks.

Rat genomic DNA isolation

High-molecular-mass genomic DNA was prepared by removing the spleen (1 g) from a freshly-killed rat and immediately freezing it in liquid nitrogen. After crushing the tissue to a fine powder, it was incubated in 20 ml of digestion buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinase K] for 6 h at 50 °C, extracted with phenol-Sevag and dialysed against 2 $\times$ 1 litre of 50 mM Tris-HCl (pH 7.5)/1 mM EDTA for 20 h at 4 °C. RNA was digested by the addition of NaCl (10 mM) and RNase A (0.1 μ g/ml) and incubation at 37 °C for 2 h. After extraction with phenol-Sevag, the final DNA preparation was dialysed against 3 $\times$ 1 litre of 10 mM Tris-HCl (pH 7.5)/0.1 mM EDTA for 48 h at 4 °C. The final DNA sample was at least 100 kb in size, as determined by 0.5% agarose gel electrophoresis.

Figure 3 Nucleotide sequence of ζ F.1 cDNA and deduced amino acid sequence

The *Eco*RI, *Acc*I, *Cl*aI and *Hinc*II restriction sites used later for production of riboprobe templates (Figure 4) are indicated. The location of sequence divergence from the full-length PKC ζ sequence is indicated by the solid triangle (at nt 248), where the upstream region corresponds to V1'. The boxed regions indicate the locations of primers used later for PCR (Figure 5). Also shown is the deduced amino acid sequence obtained by translation of the nucleotide sequence in-frame with the PKC ζ sequence. Stop codons are represented by an asterisk. Each of the first $\approx$ 400 nucleotides at the 5'-end was sequenced in both directions a total of six times. The remaining nucleotides, which were identical with PKC ζ , were each sequenced 1–3 times.

PCR analysis of rat brain reverse-transcribed mRNA and genomic DNA

Rat brain mRNA (2 μ g) treated with RQ1 RNase-free DNase (Promega) was reverse transcribed at 52 °C for 30 min with Superscript RNase H– Reverse Transcriptase (Gibco BRL) using the V5 oligonucleotide as a primer (probe 1 in Figure 1a). PCR was performed in a Perkin-Elmer System 2400 using the primers indicated in Figure 3 (boxed sequence 1 and the complement of boxed sequence 2). The 50 μ l reaction mixtures contained 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.2 μ M of each primer, 5 U Taq DNA polymerase (Gibco BRL) and either 5% of the RT sample, 10 ng of genomic DNA or 10 ng of ζ F.1 plasmid DNA. After preheating for 1 min before the addition of enzyme, the samples were denatured at 95 °C for 5 min followed by 35 cycles of 1 min denaturing at 95 °C, 30 s annealing at 65 °C and 1 min extension at 72 °C, with a final extension of 10 min. Volumes of 10 μ l of the PCR reactions were

analysed by electrophoresis on a 1.5% agarose gel and staining with ethidium bromide. Since no PCR product was visible with the genomic DNA sample, 1 μ l of the PCR reaction was re-amplified as previously.

Southern blot analysis of rat genomic DNA

Genomic DNA (10 μ g) was digested with *Bam*HI, *Hinc*II, *Kpn*I or *Pst*II for a total of 6 h at 37 °C with the addition of 40–50 U of enzyme at the beginning of the incubation and again after 3 h. Digests were electrophoresed on a 0.8% agarose gel, the gel was washed with 0.4 M NaOH/1.5 M NaCl for 2 $\times$ 20 min and transferred onto Hybond N+ (Amersham) overnight by capillary action in the same buffer. The membrane was neutralized in 1.5 M Tris-HCl (pH 7.5)/1 M NaCl, fixed by baking at 80 °C for 2 h and hybridized with a ³²P-labelled *Eco*RI fragment of ζ F.1 (nt 1–212). After washing in 0.05 $\times$ SSPE/0.1% SDS at 65 °C,

the blot was exposed to X-ray film at -80°C . The probe was then stripped by boiling 2×20 min in $0.05 \times \text{SSPE}/0.1\%$ SDS and the membrane rehybridized with a PKC ζ cDNA (nt 80–353) obtained by RT-PCR exactly as described in the previous section except a forward primer corresponding to PKC ζ nt 40–69 was used. The 524 bp PCR product was digested with *Ava*I and *Rsa*I and the resulting 274 bp fragment was gel-purified and labelled with ^{32}P . The membrane was washed and exposed to X-ray film as previously.

Tissue distribution of PKC ζ

The brain, aorta, skeletal muscle, heart, lung, liver, spleen and kidney were removed from a freshly-killed rat, washed in ice-cold saline, trimmed and homogenized for 2×30 s at 4°C with a Polytron in 10 vol. of 50 mM Tris-HCl (pH 7.5)/5 mM EGTA/2 mM EDTA/50 mM 2-mercaptoethanol/0.2% Triton X-100/10% glycerol/1 mg/l leupeptin/1 mg/l pepstatin/1 mM phenylmethanesulphonyl fluoride. Homogenates (750 μl) were added to $4 \times$ SDS gel sample buffer (250 μl) and boiled for 5 min. The remainder of each homogenate was used for protein concentration determination by the Coomassie Blue dye-binding assay [20]. Protein samples (200 μg /lane) were electrophoresed in a 7.5–20% acrylamide gradient slab gel as previously described [21] and transferred to a nitrocellulose membrane (BioRad) for 3 h at 80 V and 4°C in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% methanol and 0.1% SDS using a BioRad Trans-Blot Cell. Membranes were probed with anti-PKC ζ as described for 'Isolation of cDNA clones'.

Translation *in vitro*

A full-length clone was constructed by digesting $\zeta\text{F.1}$ and $\zeta\text{N.1}$ cDNAs at a common *Bam*HI site (see Figure 2a). The 5' $\zeta\text{F.1}$ fragment was ligated with the 3' $\zeta\text{N.1}$ fragment in pBluescript SK $^{+}$ and the final construct was confirmed by restriction enzyme analysis. The vector was linearized with *Xho*I and sense-strand RNA synthesized using Promega's RiboMAX system with T3 RNA polymerase. RNA (2 μg) was analysed by denaturing agarose gel electrophoresis, as described for 'Northern blot analysis'. Construct and control RNAs (0.5–2 μg) were included in 25 μl translation reactions *in vitro* using Promega's Rabbit Reticulocyte Lysate system in the presence of 20 μCi of [^{35}S]methionine. After incubation for 1 h at 30°C , RNase A was added at 0.2 mg/ml and the reaction incubated for an additional 5 min. SDS sample buffer (100 μl) was added, the samples were heated at 100°C for 5 min and 10 μl was electrophoresed on a 15% SDS-polyacrylamide mini-gel. The gel was dried and exposed to X-ray film for 0.5 h at room temperature.

RESULTS

Identification of authentic PKC ζ and PKC ζ -related mRNAs

The full-length cDNA encoding PKC ζ [4] is shown schematically in Figure 1(a) along with a partial cDNA sequence published earlier [3] which differed in sequence at the 5'-end (denoted V1'). Four oligonucleotide probes, based on these published sequences, were synthesized (Figure 1a): two (probes 1 and 4) corresponded to sequences found in both cDNAs and the other two (probes 2 and 3) were unique to the full-length and partial cDNA sequences respectively. These oligonucleotide probes were used in high-stringency Northern blot analysis of rat brain mRNA (Figure 1b, lanes 1–4). The V5 probe hybridized to two mRNA species of 4.7 and 2.5 kb (Figure 1b, lane 1) which correspond to the previously reported 4.2 and 2.2 kb mRNAs detected using a 3' PKC ζ -

Figure 4 RNase protection

^{35}S -labelled antisense RNA probes were generated from templates containing $\zeta\text{F.1}$ *Eco*RI-*Acc*I (nt 213–521) and *Clal*-*Hinc*II (nt 615–914) restriction fragments which span the V1'–C1 border and which contain a control downstream sequence respectively (see Figure 3). Probes protected from RNase digestion after hybridization to rat brain mRNA or yeast total RNA were sized by 6% acrylamide/7 M urea gel electrophoresis. Lanes: M, ^{35}S -labelled 100 nt ladder; 1, undigested control probe; 2, RNase digest of control probe with rat brain total RNA; 3, RNase digest of control probe with yeast total RNA; 4, undigested V1'–C1 probe; 5, RNase digest of V1'–C1 probe with rat brain total RNA; 6, RNase digest of V1'–C1 probe with yeast total RNA.

specific cDNA probe [3] (see below). Northern blot analysis using $\zeta\text{D.1}$ cDNA, corresponding to nt 1036–2301 of PKC ζ (see Figure 2), showed that this cDNA also hybridized to the 4.7 and 2.5 kb mRNAs, confirming their identity with the 4.2 and 2.2 kb mRNAs previously identified (Figure 1b, lane 5) [3]. The V1 probe, which is derived from the full-length PKC ζ cDNA sequence, however, did not hybridize to either of these mRNAs, but rather to a low-abundance 3.1 kb message (Figure 1b, lane 2). On the other hand, the V1' probe, which is derived from the partial cDNA sequence, hybridized to the more abundant 4.7 and 2.5 kb mRNAs (Figure 1b, lane 3). The C1 probe detected the same two mRNA species (4.7 and 2.5 kb) and a third of 1.75 kb (Figure 1b, lane 4). The low-abundance 3.1 kb mRNA encoding PKC ζ presumably also hybridized to the C1 and V5 probes but was not detected due to the intensity of the other mRNAs.

Figure 5 PCR analysis of reverse-transcribed rat brain mRNA and rat genomic DNA

PCR was performed using primers which span the V1'–C1 border of ζ F.1 cDNA, as shown in Figure 3. Lanes: 1, DNA size markers (Gibco BRL); 2, ζ F.1 plasmid PCR control; 3, reverse transcribed mRNA; 4, genomic DNA (first amplification); 5, genomic DNA (second amplification); 6, 7 and 8, *Eco*RI digests of lanes 2, 3 and 5 respectively. (a) Ethidium bromide-stained agarose gel. (b) Southern blot of the gel shown in (a) using the procedure described for genomic DNA Southern blot analysis described in the Materials and methods section and hybridization with the 32 P-labelled V1' synthetic oligonucleotide (probe 3 in Figure 1a). The blot was exposed to X-ray film for 0.5 h at room temperature.

Characterization of PKC ζ -related cDNAs

Upon rescreening the library with ζ D.1 cDNA, a second positive clone, ζ F.1, was obtained and sequenced. As depicted in Figure 2, the 3'-end of ζ F.1 corresponds to nt 1671 in domain C4 of PKC ζ [4]. Going towards the 5'-end, the sequence is identical with that of PKC ζ up to the V1–C1 junction and then diverges abruptly, where the V1' sequence described in the previous section was found as well as an additional 212 bp. An internal *Eco*RI site is located at a position corresponding to the first nucleotide of the previously reported partial sequence. The complete nucleotide sequence of ζ F.1 and its deduced amino acid sequence when translated in-frame with the known PKC ζ sequence is shown in Figure 3, where the divergence point (the V1'–C1 junction) is indicated by the solid triangle. A short distance upstream of the *Eco*RI site is a stop codon. An oligonucleotide probe spanning this stop codon as well as the 5' *Eco*RI fragment of ζ F.1 cDNA hybridized to the 4.7 and 2.5 kb mRNAs described in the previous section (Figure 1b, lanes 6 and 7). Notably, the V1' region does not contain a methionine codon downstream of the last stop codon, analogous to that found in PKC ζ .

The remaining four clones (ζ L.1, ζ N.1, ζ Q.1 and ζ X.1) obtained by screening the rat brain library with the C1 oligonucleotide probe (probe 4 in Figure 1a) were sequenced and are mapped relative to the PKC ζ sequence in Figure 2. The 5'-ends of ζ L.1 and ζ X.1 were identical with the 5' sequence of ζ F.1, and sequences 3' of the V1–C1 junction in all clones were identical with the corresponding region of PKC ζ [4]. In addition, ζ N.1, ζ Q.1 and ζ X.1 contained a poly(A)_{22–32} sequence beginning at a position corresponding to PKC ζ nt 2301. Using a cDNA probe containing PKC ζ nt 2300–2661, Ono et al. [4] detected only the 4.7 kb transcript; therefore, it appears that the 2.5 and 4.7 kb mRNAs are generated by alternative polyadenylation. It is also noteworthy that no clones containing the PKC ζ V1 sequence were obtained in any screening attempts, supporting the relative abundance of V1' and V1 sequences indicated by Northern blot analysis.

RNase protection

RNase protection analysis with a radiolabelled antisense RNA probe which spans the V1'–C1 junction (nt 213–521 of ζ F.1) was used to verify the contiguity of V1' sequence with C1 in mRNA. In addition, a probe containing downstream PKC ζ sequence (nt 615–914 of ζ F.1) was used as a control. Probes were hybridized to rat brain total RNA, treated with RNase A+T1 to digest single-stranded RNA, then subjected to high-resolution denaturing gel electrophoresis and autoradiography. The untreated probes (Figure 4, lanes 1 and 4), which contained insert and some plasmid sequence, had the expected sizes (385 and 377 nt for the control and V1'–C1 probes respectively). RNase digestion reduced the size of the control probe to 300 nt, as predicted (Figure 4, lane 2). The V1'–C1 probe was reduced to 309 nt (Figure 4, lane 5), corresponding to the full-length probe, thereby confirming that the V1' sequence is indeed contiguous with C1 in mRNA. An additional 276 nt product was also detected, as predicted for probe hybridized to PKC ζ in which the V1' region was digested. The much greater intensity of the 309 bp fragment compared with the 276 bp fragment is consistent with the relative abundance of the mRNA species containing V1' and V1 in Figure 1 (lanes 2 and 3). Yeast total RNA, used as a negative control in place of rat brain total RNA, was completely digested by RNase A+T1, indicating the absence of non-specific hybridization (Figure 4, lanes 3 and 6).

PCR analysis of reverse transcribed rat brain mRNA and genomic DNA

To examine the contiguity of V1' with C1 in genomic DNA, a PCR strategy was used in which the primer pair spanned the V1'–C1 junction (primers 1 and 2, Figure 3). It is noteworthy that the forward primer is identical with oligonucleotide F.1 which spans the stop codon upstream of the internal *Eco*RI site and which hybridized to the 4.7 and 2.5 kb mRNAs (Figure 1b, lane 6). After one round of amplification, a PCR product of the expected size (225 bp) was obtained with the ζ F.1 plasmid

Figure 6 Southern blot analysis of rat genomic DNA

High-molecular-mass genomic DNA (10 μ g) was digested with the following restriction enzymes: lane 1, *Bam*HI; lane 2, *Hinc*II; lane 3, *Kpn*I; lane 4, *Pst*I. Digests were electrophoresed, transferred to nylon membrane and hybridized with 32 P-labelled cDNA probes containing a sequence unique to ζ F.1 (a) or PKC ζ (b), and washed under high-stringency conditions. The blots were exposed to X-ray film for 2 days at -80°C .

control template (Figure 5a, lane 2) and with reverse-transcribed rat brain mRNA (Figure 5a, lane 3), thereby confirming the RNase protection results. After two rounds of amplification with genomic DNA, the same 225 bp product was observed (Figure

5a, lane 5), demonstrating that V1' is contiguous with C1 in genomic DNA. To verify the identity of the PCR products they were treated with *Eco*RI, resulting in the predicted 155 and 70 bp fragments (Figure 5a, lanes 6, 7 and 8). In addition, samples were subjected to Southern blot analysis in which a V1' oligonucleotide complementary to a sequence within the amplified region was used as a probe (probe 3, Figure 1a). As expected, the probe hybridized to the 225 bp product as well as its 155 bp *Eco*RI fragment (Figure 5b).

Southern blot analysis of rat genomic DNA

The genetic relationship of the V1'-containing PKC ζ -related sequence to that of authentic PKC ζ was examined by genomic Southern blot analysis. High-molecular-mass genomic DNA was digested with various restriction enzymes, electrophoresed, transferred to nylon membrane and hybridized with 32 P-labelled cDNA probes containing V1' sequence (nt 1–212 of ζ F.1) or V1 sequence (nt 80–353 of PKC ζ). The hybridization patterns of these two probes were clearly different (Figures 6a and 6b), indicating that they are derived from distinct genes.

Tissue distribution of PKC ζ mRNA and protein

Northern blot analysis of mRNAs isolated from a variety of rat tissues is shown in Figure 7. In Figure 7(a), using the PKC ζ V1 oligonucleotide probe, the 3.1 kb mRNA was detected in brain as before (lane 1). A strong signal of 2.7 kb was detected in lung and kidney (lanes 3 and 5 respectively). In addition, a 4.7 kb message, which we speculate is the result of alternative polyadenylation, was clearly detected in lung (lane 3) and also visible in other tissues examined. In contrast, when the membrane was stripped and reprobed with the V1' oligonucleotide, only the 4.7 and 2.5 kb mRNAs were detected, and only in brain (Figure 7b). Using the C1 oligonucleotide probe (Figure 7c), mRNAs of 4.7, 2.5 and 1.75 kb were detected in brain (lane 1) as before (Figure 1b, lane 4) and the 2.7 and 4.7 kb PKC ζ mRNAs were detected in lung and kidney (Figure 7c, lanes 3 and 5 respectively). The 1.75 kb mRNA was also found in rat heart in high abundance (Figure 7c, lane 2).

Western blotting with anti-PKC ζ (Figure 8) indicated that the tissue content of PKC ζ protein paralleled PKC ζ mRNA levels (3.1 kb in brain, 2.7 and 4.7 kb in lung and kidney respectively) (Figure 7). PKC ζ protein levels decreased in the order: lung >

Figure 7 Northern blot analysis of mRNA isolated from various rat tissues

mRNA (6 μ g) was isolated from various rat tissues, denatured, electrophoresed and transferred to nylon membrane. The same blot was successively hybridized with the 32 P-labelled V1 oligonucleotide (probe 2 in Figure 1a) (a), the 32 P-labelled V1' oligonucleotide (probe 3 in Figure 1a) (b), and the 32 P-labelled C1 oligonucleotide (probe 4 in Figure 1a) (c). The blot was stripped by boiling for 2 $\times$ 20 min in 0.1 $\times$ SSPE/0.1% SDS between hybridizations and verified by autoradiography. Lanes: 1, brain; 2, heart; 3, lung; 4, liver; 5, kidney; 6, skeletal muscle. Blot (a) was exposed for 5 days, (b) for 1 day and (c) for 2 days at -80°C .

Figure 8 Western blot analysis of PKC ζ in various rat tissues

Total tissue homogenates were subjected to SDS/PAGE (200 μ g of protein/lane), transferred to nitrocellulose membrane and probed with an antibody directed against the V5 domain of PKC ζ . Lanes: 1, brain; 2, aorta; 3, skeletal muscle; 4, heart; 5, lung; 6, liver; 7, spleen; 8, kidney.

Figure 9 Translation *in vitro*

(a) Ethidium bromide-stained 1% agarose gel of RNA templates. Lanes: 1, RNA size markers (Promega); 2, RNA (2 μ g) transcribed from the full-length ζ F.1/ ζ N.1 construct; 3, RNA (2 μ g) transcribed from the control template provided in the translation kit. (b) Autoradiogram of [35 S]methionine-labelled *in vitro* translation products using the RNAs shown in (a). Reactions contained no RNA (lane 1), 0.5, 1.0 or 2.0 μ g of construct RNA (lanes 2, 3 and 4 respectively), 2.0 μ g of control RNA (lane 5) and 2.0 μ g of control RNA + 2.0 μ g of construct RNA (lane 6). Protein size markers (Bio-Rad) are indicated on the left and the size of the control translation product on the right. The dried gel was exposed to X-ray film for 0.5 h at room temperature.

kidney > brain > liver > aorta = spleen. No PKC ζ was detected in skeletal or cardiac muscle. The immunoreactive band of higher M_r than PKC ζ , evident in lanes 1, 5 and 8 of Figure 8, is due to cross-reactivity of anti-PKC ζ with cPKC isoenzymes [22]. No brain-specific immunoreactivity was observed, supporting the conclusion based on the deduced amino acid sequence shown in Figure 3 that the novel brain-specific PKC ζ -related sequence does not contain an appropriate initiating methionine for protein synthesis.

Translation *in vitro*

To further investigate the translation potential of the novel sequence, an '*in vitro*' rabbit reticulocyte lysate system was used. RNA was synthesized from a full-length construct in pBSK+, resulting in a 2200 bp sense-strand RNA containing insert as well as some plasmid DNA (Figure 9a, lane 2). When various amounts of this RNA were subjected to translation *in vitro* in the presence of [35 S]methionine, no radiolabelled proteins were observed (Figure 9b, lanes 2, 3 and 4). On the other hand, radiolabelled protein of the expected size (61 kDa) was synthesized from control RNA (Figure 9b, lane 5). To eliminate the possibility that protein synthesis inhibitors were present in the construct RNA sample, it was included in a control RNA translation reaction. In this case, control protein was synthesized to the same extent as in the absence of construct RNA (Figure 9b, lane 6).

DISCUSSION

PKC ζ was originally discovered by molecular cloning and sequence analysis [3,4]. A partial cDNA sequence was initially reported which lacked the N-terminal part of the coding region [3]. Subsequently a full-length sequence was published [4] which was identical with the partial sequence except for the 5'-end, specifically nt 1–36 of the partial sequence, referred to here as the V1' domain. Ono et al. [4] suggested that the original cDNA was derived from an unspliced molecule and that its 5'-terminal sequence was encoded by an intron sequence. Our results, however, are not consistent with this conclusion. Using an oligonucleotide probe complementary to the V1' domain, we detected two messages in rat brain of 4.7 and 2.5 kb, identical in size and amount to the mRNAs detected with an oligonucleotide probe complementary to the V5 domain at the 3'-end of the coding region of PKC ζ . On the other hand, neither mRNA was detected with an oligonucleotide complementary to the sequence of the full-length cDNA in the V1 region corresponding to the location of nt 1–36 in the partial sequence (PKC ζ nt 449–483); rather, a low-abundance mRNA of 3.1 kb was detected. While the PKC ζ sequence predicts that the 3.1 kb mRNA also hybridizes to the V5 probe, it was undetectable, due to the intensity of the other 4.7 and 2.5 kb V5 probe-hybridizing mRNAs. The existence of a fourth PKC ζ -related mRNA in rat brain was indicated using an oligonucleotide complementary to the beginning of the C1 domain of PKC ζ (nt 484–518 of the full-length sequence; nt 37–71 of the partial sequence): this probe detected the 4.7 and 2.5 kb mRNAs and also hybridized to an abundant message of 1.75 kb. The 1.75 kb mRNA, which is also found in heart, does not correspond to the mRNA size or tissue distribution of any other known PKC isoenzymes and, therefore, appears to be a novel PKC ζ -related transcript.

cDNA clones containing the previously reported V1' sequence [3], as well as additional upstream sequence, were isolated from a rat brain cDNA library. When translated in-frame with the PKC ζ sequence, the V1' cDNA sequence was found to be unusual in that it contained two stop codons but no methionine downstream of the last stop codon. The existence of mRNA species containing this novel sequence contiguous with the C1 domain was confirmed by two independent methods: (1) an RNA probe spanning the V1'–C1 junction was protected from RNase digestion after hybridization with rat brain mRNA; and (2) RT-PCR using a forward primer spanning the second stop codon in V1' and a reverse primer complementary to a region within the C1 domain generated a PCR product of the predicted size. In addition, the same sequence was recently obtained by 5'-rapid amplification of cDNA ends (RACE) from rat brain using a downstream primer corresponding to the location of our C1

oligonucleotide probe [23]. Analogous to the RNA protection analysis described here, a full-length RNA probe containing PKC ζ V1–C1 (PKC ζ nt 340–683) and a fragment corresponding to the size of the probe's C1 component were protected with rat brain and rat Dunning G prostatic tumour cell RNAs [23]. While it was presumed that the C1 fragment was generated from hybridization to the novel V1'-containing mRNA, this was not verified.

Northern analysis of mRNA isolated from various rat tissues revealed that the abundant V1'-hybridizing PKC ζ -related mRNAs of 4.7 and 2.5 kb are expressed only in the brain and that authentic PKC ζ mRNA is most abundant in lung, followed by kidney, then brain. The size of the PKC ζ message in lung and kidney is 2.7 and 4.7 kb respectively and in brain 3.1 kb. Western blotting with a C-terminally-directed anti-PKC ζ antibody revealed that PKC ζ protein levels are highest in lung, then kidney, lower in brain and still lower in liver, spleen and aorta, and were not detected in skeletal or cardiac muscle, similar to the results reported by others [24,25]. The tissue distribution of PKC ζ , therefore, correlates with that of authentic PKC ζ mRNA. Although the novel brain-specific transcript does not contain an initiating methionine in V1', the possibility was considered that downstream AUGs or cryptic initiators may be used as translation start sites. Analysis of the full-length sequence reveals a total of nine methionines downstream of the last stop codon in V1', which would potentially result in proteins of M_r 14600–45000; however, no such brain-specific proteins were detected in the Western blot analysis described above or in other similar studies [8,24,25]. Furthermore, synthetic mRNA did not support protein synthesis in a translation system *in vitro*.

The novel PKC ζ -related sequence was also investigated at the level of genomic DNA in order to determine whether it is derived by alternative splicing or from a distinct gene. Using primers which span the V1'–C1 junction, the same PCR product was obtained with genomic DNA as that seen with reverse-transcribed mRNA, demonstrating that no intron is located within this region in genomic DNA. In a similar experiment using primers to amplify mouse PKC ζ nt 9–698 (spanning V1–V2/V3), Goodnight et al. [19] found the same 688 bp fragment using either reverse-transcribed mouse brain mRNA or genomic DNA. In addition, by genomic DNA Southern analysis with V1- and V1'-specific cDNA probes, we have provided evidence that these sequences are located at different loci. Based on these results, we conclude that the novel mRNA is the product of a PKC ζ pseudogene which we propose to refer to as Ψ PKC ζ . In general, pseudogenes arise by gene duplication and sequence drift or by retroposition. Consequently, they are similar to 'functional' genes but contain inactivating mutations with respect to mRNA or protein synthesis. While most pseudogenes are transcriptionally silent, a number of translationally-defective pseudogene transcripts have been identified (see for example [26–28]). Although the function of pseudogenes is generally considered in an evolutionary context where they provide for genetic diversity, it is an emerging view that some pseudogene transcripts may also serve a regulatory role by mechanisms such as antisense RNA control. Unfortunately, functional studies are presently lacking. It will, of course, be of interest to obtain the genomic DNA sequence of the complete Ψ PKC ζ gene so that we may have a better understanding of its origin, regulation and potential physiological function.

Overall, we conclude that: (1) rat brain PKC ζ is encoded by a 3.1 kb low-abundance mRNA and by more abundant 2.7 and

4.7 kb mRNAs in other rat tissues; (2) the levels of PKC ζ mRNA and protein correlate well in different rat tissues, with highest levels in lung, kidney and brain; (3) the abundant PKC ζ -related 2.5 and 4.7 kb mRNAs, expressed exclusively in the brain among several rat tissues examined, do not encode protein since they lack an appropriate initiating methionine; (4) these mRNAs are transcribed from a pseudogene which we refer to as Ψ PKC ζ ; (5) an additional novel 1.75 kb transcript, which is similar to the PKC ζ C1 domain, is present in rat brain and heart; (6) these results draw attention to the problems of using particular oligonucleotide and cDNA probes to study PKC ζ mRNA and necessitate a re-evaluation of the literature in this context.

This work was supported by a grant to M. P. W. from the Alberta Heart and Stroke Foundation. J. E. A. is the recipient of a Studentship from the Medical Research Council of Canada and M. P. W. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research. The authors are very grateful to Drs. Bruce G. Allen and Dylan Edwards for helpful discussions, and to Lenore Youngberg for expert secretarial assistance.

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