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Identification of multiple, novel, protein kinase C-related gene products

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Abstract A PCR approach has been employed to screen two cDNA libraries for PKC(-related) sequences. In each case multiple cDNAs were identified, including known PKC isotypes, a previously unknown PKC-η related sequence and three members of what appears to be a protein kinase C related kinase (PRK 1–3) family. The origin and relationships of these predicted proteins is discussed.

Key words: Protein kinase; PCR; Protein kinase C

1. Introduction

Protein kinase C (PKC) is an area of intense investigation as evidenced by the weight of publications, including two books on the subject [1,2]. This family of proteins act both as 'receptors' for the second messengers diacylglycerol (DAG) (reviewed in [3,4]) and possibly phosphatidylinositol-3,4,5-trisphosphate [5,6], as well as being major targets for the phorbol ester class of tumour promoters [7]. Despite the attention provoked by PKCs involvement in many cell signalling processes, the actual extent of this kinase gene family remains poorly defined. While extensive screening of cDNA libraries from the CNS has led to the identification of six distinct PKC gene products (α–ζ [8]) subsequent cloning from peripheral tissues has added significantly to this number (η, θ, ι, μ [9–13]). Analysis at the genomic level would be one approach to the problem of defining the PKC gene family. In principle this should permit an exhaustive survey, however, attempts to use PCR with redundant oligonucleotide probes from within conserved exons has proved fruitless to date (J.R. and P.J.P., unpublished). However the lack of exon constraints, imposed by genomic analysis, does permit a PCR screen from cDNA libraries employing oligonucleotide probes covering highly conserved motifs present in known PKC gene products. This type of approach has revealed recently two PKC-related genes in *Schizosaccharomyces pombe* [14]. The work here describes such a screen of two human cDNA libraries and reports the identification of known as well as a series of novel PKC-related cDNA sequences.

2. Materials and methods

2.1. Materials

Tetramethylammoniumchloride (TMAC) was obtained from BDH Limited. [^{35}S]dATP was from Amersham International. Taq XL DNA Polymerase and restriction enzymes were obtained from NBL gene sciences. Sequenase and other sequencing reagents were from United States Biochemical Corporation.

2.2. PCR probes, PCR conditions and subcloning

Degenerate oligonucleotides used for PCR were 5′ oligonucleotide: 5′-GCGAATTCAMIGGIGGIGAYYTIATG-3′ (Kin5′*Eco*RI) and 3′ oligonucleotide: 5′-GCGCGGCCGCISYCCACCARTCIAC-3′ (Kin3′ *Not*I), where I = inosine, R = A + G, Y = T + C, M = A + C and S = G + C. The underlined 5′ sequence indicates the *Eco*RI or *Not*I restriction sites which were included for use in subcloning. Kin5′*Eco*RI corresponds to the consensus peptide Gly-Gly-Asp-Leu-Met within the kinase domain of PKC family members. Kin3′*Not*I corresponds to the consensus peptide of Trp-Trp-Asp-Val also within the PKC kinase domain. PCR amplification was performed using Taq XL DNA polymerase and a DNA thermal cycler (TECHNE PHC-2). Thirty cycles at 94°C for 1 min, 50°C for 2 min and 72°C for 1 min were performed after an initial 94°C for 10 min followed by 72°C for 5 min during which time *Taq* XL DNA polymerase was added. 120 pmol of each oligonucleotide were used in each reaction and 1 μg of cDNA was used for PCR. PCR reactions were carried out in the presence of 10 μg/ml TMAC. The resulting 340 bp PCR products were subcloned into Bluescript (Stratagene, CA, USA) after digestion with *Eco*RI and *Not*I and gel-purifying the corresponding bands.

2.3. DNA manipulation and sequence analysis of clones

Standard molecular biology techniques were employed [15]. Enzymes were used as recommended by their suppliers. Nucleotide sequence was determined by the dideoxy chain termination method [16].

3. Results and discussion

3.1. Characterisation of PCR products

In order to carry out a broad screen of two cDNA libraries, redundant oligonucleotides with flanking restriction sites were designed to two regions of the PKC kinase domain that are invariant (GGDLM and DWWA). These primers were found to direct the synthesis of a 340 bp PCR fragment from each of six distinct PKC cDNAs (α–ζ; data not shown). Using cDNA derived from a human fetal brain library and in parallel from the human monoblastoid U937 cell, PCR was carried out with these redundant oligonucleotides. Ethidium bromide staining 340 bp fragments could be detected in both situations. These fragments were subcloned into the Bluescript plasmid as *Eco*RI–*Not*I fragments utilising the restriction sites incorporated within the PCR primers. Colonies were randomly picked and plasmid inserts sequenced. As data accumulated, certain frequently occurring sequences (e.g. PRK1) were eliminated by T-tracking. The U937 cell screen yielded three novel sequences referred to as PKC-ηb, PKC-related-kinase 1 (PRK 1) and PRK 2. In addition, known sequences were also obtained. A summary is shown in Fig. 1A. The PKC-δ sequence consistently (10/10) contained one mismatch compared to the published human-δ sequence [17]. In view of this frequency this is likely to represent a polymorphism rather than a PCR error.

From the human fetal brain library three novel sequences were identified, PRK 1 (identical to the U937 sequence), PRK 3 and PKC-ηb (identical to the U937 sequence). PKC-δ was also identified and found to be identical to the U937 cell library sequence. This supports the notion that the single mismatch to

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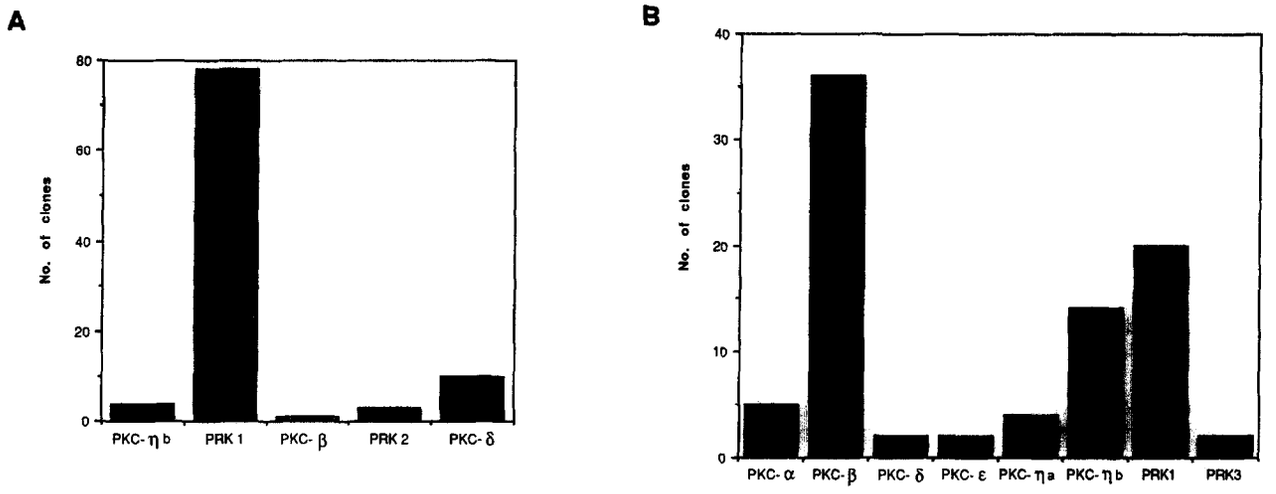


Fig. 1. PCR sequences identified in cDNA library screens. (A) The numbers of clones from the analysis of a U937 cell cDNA library identifying various known kinase domain fragments (as indicated) and novel kinase related sequences are shown. The nomenclature for the novel sequences is discussed and defined in the text. (B) Analysis of clones as for (A) but corresponding to the human fetal brain cDNA library screen.

the published sequence is due to a polymorphism. Again, for this brain library a number of other known sequences were identified as summarised in Fig. 1B.

3.2. *Comparison of PCR clones with existing PKC family members*

The overall similarity of the four novel sequences (PRK 1–3 and PKC-ηb) to equivalent predicted protein sequences of established members of the PKC gene family is shown in Fig. 2A(i). It is evident that beyond those residues conserved in all protein kinases, the PKCs and those novel sequences determined here retain a number of conserved residues that clearly marks them as being on at least a separate 'branch' of the kinase superfamily. This is wel illustrated by Fig. 2B which provides an indication of evolutionary relatedness for all these sequences alongside representatives of the two most closely related protein kinase families (PKA and rac). This dendrogram also reveals the separation that exists between the PKCs including PKC-ηb and PRK 1–3, suggesting that PRK 1–3 may in fact form part of a distinct subfamily. In particular there are two features that seem to distinguish PKC from PRK. Firstly, in all cases to date PKC retains a methionine within the sequence FGMCK while PRKs have a leucine at this position. Secondly, the PRKs uniformly contain the sequence LDNLLLD where in PKC the three underlined leucine residues are variably replaced by one or other residue with a hydrophobic sidechain. At least within the context of the sequence information available to date these two features distinguish PKC and PRK. An alignment of the predicted amino acid sequences for the PRKs reveals how similar they are, with most differences being conservative changes (Fig. 2A(iii)).

The assignment of PKC-ηb reflects the very close relationship to the established human PKC-η (PKC-ηa) sequence relative to other PKC isotypes. This relationship is illustrated above (Fig. 2a(ii)) but is clearly demonstrated in comparing just these two η sequences directly (Fig. 2C). There is only 1 amino

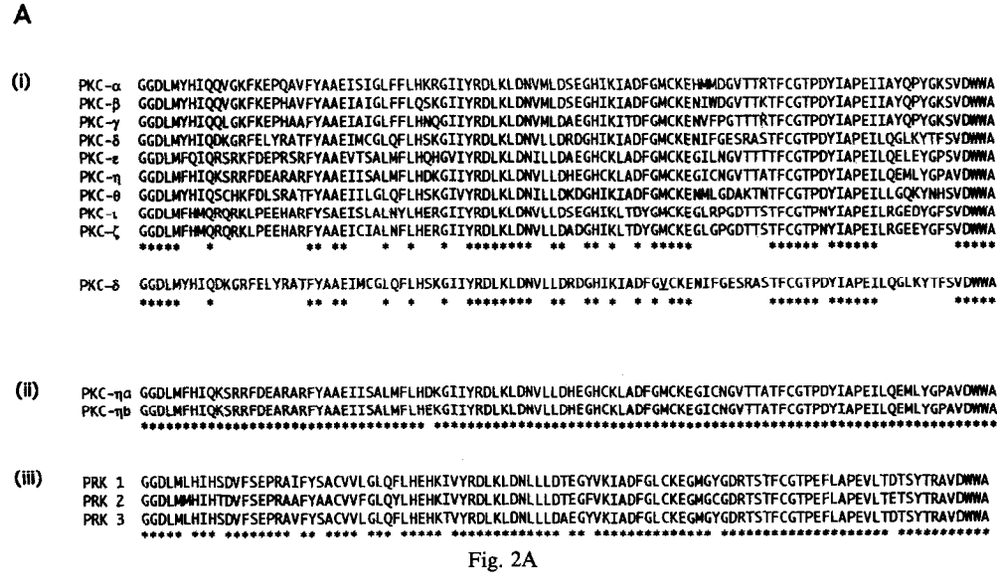
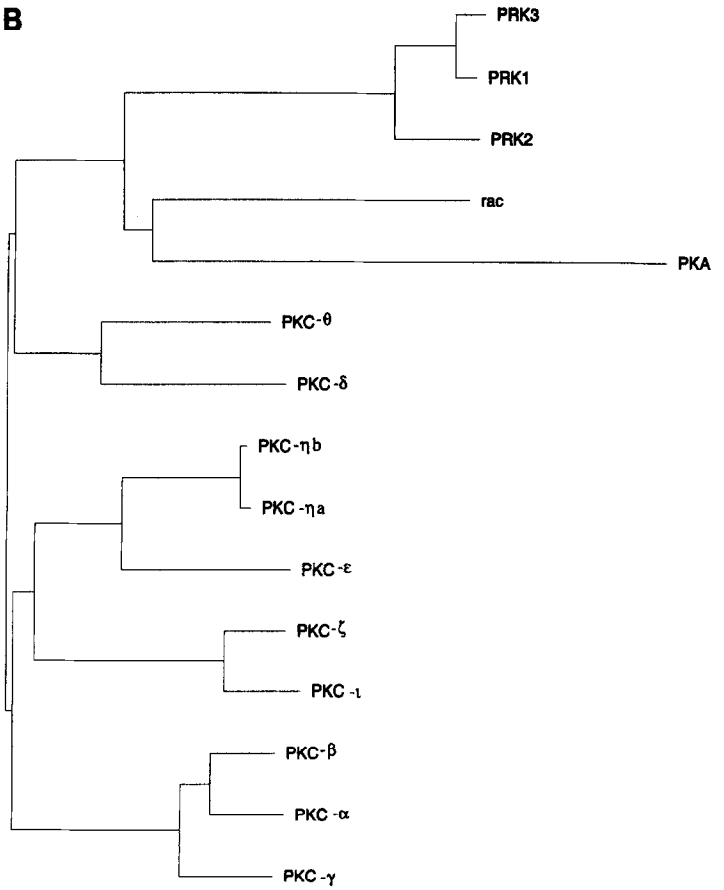


Fig. 2A



C

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                G  G  D  L  M  F  H  I  Q  K  S  R  R  F  D  E  A  R  A  R  F  Y  A  A  E  I
PKC-ηa  ggg ggt gac ttg atg ttc cac att cag aag tct cgt cgt ttt gat gaa gca cga gct cgc ttc tat gct gcn gaa atc
PKC-ηb  ggg ggg gac ttg atg ttc cac atc cag aag tcc agg cgt ttc gat gaa gcc cga gct cgt ttc tac gct gca gag atc
        *** ** *** *** *** *** *** ** ** *** **  *  *** ** *** ** *** *** ** ** *** *** ** ***

                I  S  A  L  M  F  L  H  D/E K  G  I  I  Y  R  D  L  K  L  D  N  V  L  L  D  H
PKC-ηa  att tcg gct ctc atg ttc ctc cat gat aaa gga atc atc tat aga gat ctg aaa ctg gac aat gtc ctg ttg gac cac
PKC-ηb  att tcc gca ctc atg ttc ctc cag gag aaa ggc atc atc tac aga gag ctg aaa ctg gac aac gtg ctg ctg gac cac
        *** ** ** *** *** *** ** ** ** *** *** ** *** ** ** ** *** *** *** ** ** ** ** *** ***

                E  G  H  C  K  L  A  D  F  G  M  C  K  E  G  I  C  N  G  V  T  T  A  T  F  C
PKC-ηa  gag ggt cac tgt aaa ctg gca gac ttc gga atg tgc aag gag ggg att tgc aat ggt gtc acc acg gcc aca ttc tgt
PKC-ηb  gaa ggc cac tgt aaa ctg gcc gac ttc gga atg tgc aag gag ggc att tgt aac ggg gtc acc acg gcc acc ttc tgc
        ** ** *** *** *** *** ** *** *** *** *** *** ** *** ** ** ** *** *** ** *** ** *** **

                G  T  P  D  Y  I  A  P  E  I  L  Q  E  M  L  Y  G  P  A  V  D  W  W  A
PKC-ηa  ggc acg cca gac tat atc gct cca gag atc ctc cag gaa atg ctg tac ggg cct gca gta gac tgg tgg gca
PKC-ηb  ggc acg cct gac tac atc gcc cca gag atc ctt cag gag atg ttg tat gga cct gca gtc gac tgg tgg gca
        *** *** ** *** ** ** ** *** *** *** ** *** ** *** ** ** *** *** ** *** *** *** ***
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Fig. 2. Multiple alignment and relationship of kinase domain fragments. (A) (i) Alignments comparing the amino acid sequence of existing PKC family members. Residues that are identical between all sequences are identified by an asterisk. The underlined valine residue (V) in the PKC-δ sequence determined here indicates the single difference with the published human PKC-δ sequence (see text). The alignments were obtained using the Clustal programme [19]. (ii) Human PKC-η (PKC-ηa) and the related sequence, here indicated as KC-ηb, are aligned. Identities are noted by asterisks. (iii) The three PRK predicted protein sequences are shown. Residues identical in all three are indicated by asterisks. (B) A dendrogram providing an indication of the evolutionary relationship of these kinases. For comparison the cAMP-dependent protein kinase (PKA) and rac (which is related to A and C kinase) is included [21]. (C) Comparison of the PKC-ηa and PKC-ηb sequences at both the nucleic acid and amino acid level.

acid difference in spite of the 26 nucleotide differences observed between these two. That PKC-ηb is not a cloning/PCR artefact is demonstrated by the fact that it was isolated on multiple occasions from two distinct DNA libraries. It is possible that these sequences might represent alternative exons from within the human PKC-η gene. However it should be noted that the intron/exon boundaries in the Drosophila 53E gene and those defined to date in the human PKC-β gene [18] are conserved, if this were true over the entire length of the β gene and by inference the η then the sequences discussed here would encompass partially two consecutive exons. Interestingly two loci have recently been described for PKC-η [20]. It is likely that the PKC-ηa and PKC-ηb described here are transcripts from these two loci, although the precise relationship of the transcripts and evolution of the genes awaits further investigation.

3.3. Conclusions

The approach employed here clearly provides an effective strategy for the discovery of PKC-related genes expressed in particular cell/tissues of interest. This will clearly be of impor-

tance in light of the need to define cause and effect in the dissection of these pathways. Specifically the analysis presented here shows that there is a novel PKC-η related mRNA and defines a related but distinct family of kinases that comprises at least three members i.e. PRK 1, 2, 3. The anticipated divergence of the PKC and PRK gene products has been confirmed recently following the isolation of a full length cDNA that shows identity to PRK1 [22]; in the absence of typical PKC regulatory motifs, the regulation of these proteins and their functional relationship to the PKCs requires further investigation.

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References

[1] Protein Kinase C: Current Concepts and Future Perspectives (Lester, D.S. and Epand, R.M., Eds.) 1992, Ellis Horwood Ltd., UK.
[2] Protein Kinase C (Kuo, J.F., Ed.) 1994, Oxford University Press.
[3] Nishizuka, Y. (1984) Nature 308, 693–698.
[4] Kikkawa, U. and Nishizuka, Y. (1986) Annu. Rev. Cell Biol. 12, 149–178.
[5] Nakanishi, H., Brewer, K.A. and Exton, J.H. (1993) J. Biol. Chem. 268, 13–16.
[6] Singh, S.S., Chauhan, A., Brockerhoff, H. and Chauhan, V.P.S. (1993) Biochem. Biophys. Res. Commun. 195, 104–112.
[7] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[8] Stabel, S. and Parker, P.J. (1991) Pharmacol. Ther. 51, 71–95.
[9] Osada, S., Mizuno, K., Saido, T.C., Yoshiko, A., Suzuki, K., Kuroki, T. and Ohno, S. (1990) J. Biol. Chem. 265, 22434–22440.
[10] Dekker, L.V., Parker, P.J. and McIntyre, P. (1992) FEBS Lett. 312, 195–199.
[11] Baier, G., Telford, D., Giampa, L., Coggeshall, K.M., Baier-Bitterlich, G., Isakov, N. and Altman, A. (1993) J. Biol. Chem. 268, 4997–5004.
[12] Selbie, L.A., Schmitz-Pieffer, C., Sheng, Y. and Bidden, T.J. (1993) J. Biol. Chem. 268, 24296–24302.
[13] Johannes, F.J., Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994) J. Biol. Chem. 269, 6140–6148.
[14] Toda, T., Shimauki, M. and Yanagida, M. (1993) EMBO J. 12, 1987–1995.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Press, Cold Spring Harbor, NY.
[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[17] Aris, J.P., Basta, P.V., Holmes, W.D., Ballas, L.M., Moomaw, C., Rankl, N.B., Blobel, G., Loomis, C.R. and Burns, D.J. (1993) Biochim. Biophys. Acta 1174, 171–181.
[18] Obeid, L.M., Blobe, G.C., Karolak, L.A. and Hannun, Y.A. (1992) J. Biol. Chem. 267, 20804–20810.
[19] Devereux, J. (1990) Nucleic Acids Res. 12, 387–395.
[20] Chida, K., Sagara, H., Suzuki, Y., Murakami, A., Osada, S.I., Ohno, S., Hirosawa, K. and Kuroki, T. (1994) Mol. Cell. Biol. 14, 3782–3790.
[21] Jones, P., Jakubowicz, T., Pitossi, F., Maurer, F. and Hemmings, B. (1991) Proc. Natl. Acad. Sci. USA 88, 4171–4175.
[22] Mukai, H. and Ono, Y. (1994) Biochem. Biophys. Res. Commun. 199, 897–904.