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DNA-BINDING PROTEINS IN PROTEIN KINASE C PREPARATIONS

Alessandro Testori, Charles S.T.Hii, Agnes Fournier, Leigh A.Burgoyne and Andrew W. Murray

School of Biological Sciences, Flinders University, Bedford Park, South Australia, 5042

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Summary: Human DNA enriched in repetitive sequences specifically bound to a component(s) in purified preparations of rat brain protein kinase C (PKC). DNA which bound to protein was cloned in pUC-19 and one clone characterized as containing an approx. 140 bp insert. The band containing this insert (separated by acrylamide gel electrophoresis) was lost when the DNA was incubated with purified PKC preparations. Thus a protein in relatively pure PKC preparations is a sequence-selective DNA-binding protein. The results raise the possibility that PKC or a fragment of PKC binds selectively to specific DNA sequences.

Identification of protein kinase C (PKC) as a binding protein for the tumor-promoting phorbol esters was a major advance in understanding promotion at the molecular level (1,2). Although the question of whether PKC activation is the only mechanism mediating the effects of the phorbol esters is still controversial (3,4), there is considerable interest in PKC as a target for phorbol esters because of its role in transmembrane signaling systems associated with the turnover of inositol phospholipids (2,5). Phorbol esters such as 12-0-tetradecanoylphorbol-13-acetate (TPA) act as structural analogs of 1,2-diacylglycerol and cause a massive translocation of PKC to membrane and the subsequent phosphorylation of specific proteins.

Although many of the early responses to TPA occur at the cell surface, these are followed by a programmed series of changes in gene expression (6,7). While it is generally assumed that the role of PKC in these changes is indirect (i.e. via phosphorylation), the enzyme contains a conserved cysteine-rich

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tandem repeat near its amino terminus characteristic of some metalloproteins and DNA-binding proteins (8,9). In the present report we show that a component(s) in highly purified rat brain PKC preparations binds specifically to human DNA enriched in repetitive sequences.

Materials and Methods

Preparation of PKC. Rat brain PKC was purified by adsorption to and elution from inside-out red cell vesicles essentially as described (10). The eluted enzyme was further purified by DE-52 cellulose chromatography (11). Peak fractions of Ca^{2+}/phosphatidylserine-dependent activity were pooled and stored at 4^{o}. Such preparations were stable for about 2 weeks. Typically, 18 g of rat brain yielded about 300 μg of protein with a specific activity of about 600 pmoles/min/μg protein when assayed as described (11). Protein was determined by the method of Bradford (12).Following electrophoresis on 12% SDS-polyacrylamide gels, the preparations showed a protein doublet of approx. 80kD and minor bands between 50-67 kD after Coomassie Blue (Fig.1) or silver staining (not shown). Both doublet bands and the more rapidly migrating material were detected by immunoblotting after electrophoretic transfer to nitrocellulose and probing with a rabbit antiserum to a peptide corresponding to amino acids 279-292 of bovine α-PKC (Fig.1). This antiserum detects all of the three major PKC isozymes present in rat brain (unpublished). Sheep anti-rabbit was used as the secondary antibody and detection was carried out with rabbit anti-sheep coupled to alkaline phosphatase. The bands were also detected by a sheep polyclonal antibody to mouse brain PKC (13) and a monoclonal antibody to rat brain PKC (Amersham). A similar doublet has previously been observed in purified preparations of rat brain PKC (14,15), and it has been concluded that the two bands are not a consequence of proteolysis or phosphorylation. The bands presumably represent partial resolution of the three major, closely related, isozymes of PKC known to be present in purified rat brain PKC preparations (16).
Human repetitive DNA. Human DNA was sonicated, denatured, renatured under slowly decreasing stringency and digested with S1 nuclease. After phenol extraction, those fragments larger than approx. 100 bp were selected by gel filtration. The final product represented approx. 10% of the original DNA, and on agarose gel electrophoresis it showed a near-random smear ranging from 0.1kb to 0.4kb, except for a pronounced band in the region of 0.3-0.4kb that was presumably the small interspersed repeat, ALU. Repeated cloning from this material has demonstrated that all of the common repetitive species of human DNA are well represented. The DNA was labelled using Klenow polymerase and [α-^{32}P]ATP.
Cloning of sequences binding to PKC preparations. PKC (4 μg) was dot-blotted onto nitrocellulose, hybridised with repetitive DNA (200 ng) and washed with binding buffer (20mM Tris,pH 7.6, 50mM KCl,0.05% Nonidet P40, 5% glycerol, 50μg/ml BSA, 10mM 2-mercaptoethanol, 1mM EDTA). The nitrocellulose was incubated for 5 h in 0.2% tris dodecyl sulphate- 5mM EDTA containing proteinase K (6 μg/ml). The mixture was phenol extracted, the DNA precipitated with alcohol and resuspended in 10mM Tris,pH 8, 1mM EDTA. The DNA was ligated to dephosphorylated pUC-19 (100ng) cut

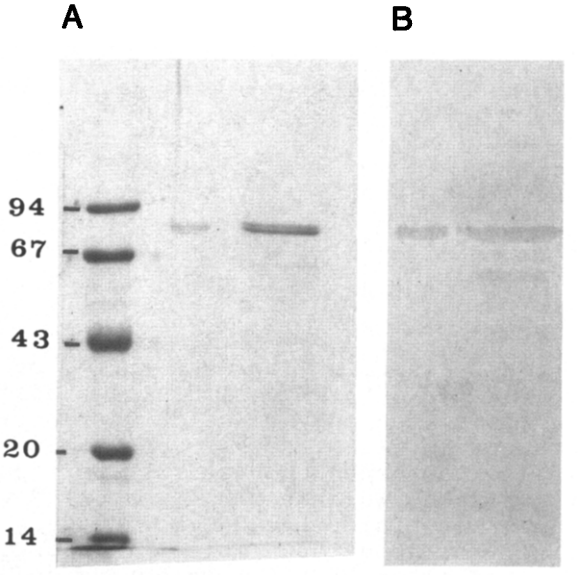

Figure 1. Characterization of purified PKC.
A. SDS-acrylamide gel of PKC stained with Coomassie blue. Left lane, molecular weight markers; centre lane, 0.35 μg protein; right lane, 3.5 μg protein. B. Western blot of parallel gel stained with anti-PKC (see Materials and Methods).

with Sma I, and the ligation mixture used to transform competent JM-101 cells. The inserts were cut out with EcoR1 and Sal 1, Klenow-labelled and used as probes in mobility-shift assays.
DNA-binding assay. Purified PKC (0.1-1 μg protein) was mixed with the probe (approx.0.5μC_i) in binding buffer and incubated for 30 min in the presence of up to 1μg of poly(d[I-C]). Reaction mixtures were loaded onto 5% polyacrylamide gels and electrophoresed for 3 h in 40mM Tris-borate, pH 7.8, 1mM EDTA. Gels were dried and autoradiographed.

Results and Discussion

Incubation of PKC preparations (see Materials for characterization) with labelled DNA enriched in repetitive sequences followed by gel electrophoresis, resulted in the appearance of labelled bands with slower electrophoretic mobility (see arrows in Fig.2). These anomalous bands were not abolished by an excess of poly(d[I-C]), indicating that a component(s) of the DNA specifically bound proteins in the PKC preparation. In these preliminary experiments it was noted that the majority of the PKC preparation protein did not enter the gel. This was established by both Coomassie Blue staining and by loading gels with autophosphorylated PKC. However, a minor proportion of intact PKC, of a PKC fragment or of a non-PKC contaminant did enter the gel resulting in poly (d[I-C]) competition-resistent DNA-binding bands.

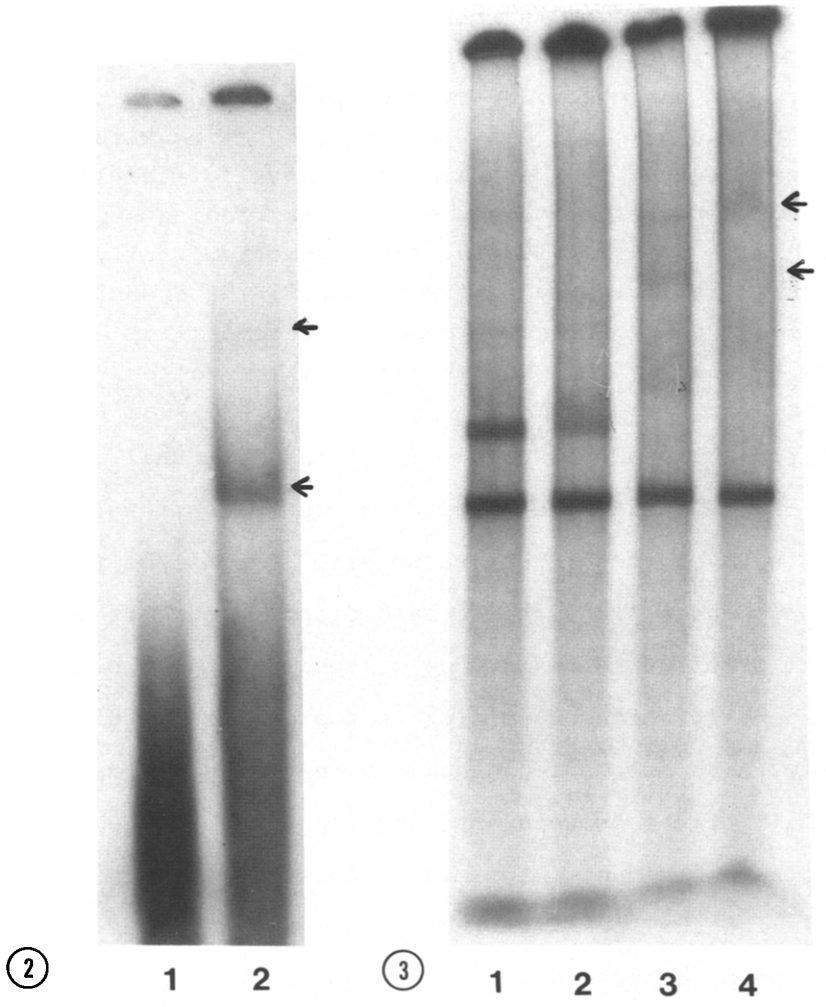

Figure 2. Binding of human repetitive DNA to PKC preparations. Unfractionated repetitive DNA (10 ng) was incubated in the absence (lane 1) or presence (lane 2) of PKC preparation (1 μg protein) and electrophoresed on an acrylamide gel.

Figure 3. Binding of cloned DNA to PKC preparations. Clone C-11 (5 ng DNA) was cut with EcoRI and Sal 1 and incubated in the absence (lane 1) and presence (lanes 2-4) of purified PKC (0.1, 0.5 and 1 μg protein respectively).

DNA which bound to the PKC preparations was extracted and the DNA fragments cloned in pUC-19. This gave 4 clones containing inserts which bound to PKC preparations in the presence of poly (d[I-C]). One of these clones (C-11) was used in further experiments. Incomplete cleavage of C-11 with EcoR1 and Sal gave two bands on acrylamide gels (Fig.3). The more slowly migrating band (approx. 170 bp, including 33 bp derived from the pUC polylinker) reacted with protein and had one uncleaved Sal 1

site within it.The faster, unreactive band (approx. 150 bp) is the same species, but with the internal Sal 1 site cleaved. Only this latter band was observed after complete cleavage with Sal 1 and EcoR1 or with Sal 1 alone. Clearly, incubation with PKC preparations resulted in the selective removal of the 170 bp band (Fig.3), and we therefore conclude that the binding sequence is close to the internal Sal 1 site. The unreactive, fully cleaved, species served as a closely related control. The experiment shown in Fig.3 was carried out in the absence of competitor except for the high molecular weight DNA derived from the vector. Similar results were obtained if up to 1 μg poly (d[I-C]) was included in the incubations (data not shown). Faint mobility shift bands were routinely observed in these experiments (eg see arrows in Fig 3). It is not, however, clear that these were responsible for the band disappearance. Although the band disappearance is an unequivocal proof of the existence of specificity in binding, it does not identify which protein(s) is the source of this specificity. A number of technical ambiguities unavoidably arise from the bulk of the protein not entering the gel. The results are compatible with one of the minor mobile species being responsible for the specific binding, but can not rule out the participation of other species that have not entered the gel.

Despite the high purity of the PKC preparations, we recognise the difficulties in unambiguously attributing the DNA-binding observed in these experiments to PKC or a fragment derived from PKC (17), and are currently using DNA-affinity chromatography to resolve this question. It will, however, be of great interest to determine whether the DNA sequence we have identified is associated with the regulatory elements of genes known to be regulated by phorbol esters and therefore with a suspected involvement of PKC.

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