Complete Activation of Protein Kinase C by an Antipeptide Antibody Directed against the Pseudosubstrate Prototope*

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It has been proposed that the regulatory domain of protein kinase C contains a pseudosubstrate site between amino acid residues 19 and 36 (House, C., and Kemp, B. E. (1987) Science 238, 1726-1728). Antiserum raised against this peptide sequence has now been shown to completely activate protein kinase C in the absence of calcium and phospholipids. Pre-clearing the antiserum with resin-immobilized pseudosubstrate peptide eliminates the ability of the serum to activate protein kinase C. Activation is not the result of degradation of the enzyme to a calcium- and phospholipid-independent fragment; the activated protein kinase remains intact. Although there are minor sequence differences in the pseudosubstrate region, the three principal protein kinase C isoforms (α, β, and γ) are recognized and apparently activated by the same pseudosubstrate antiserum. These results provide strong evidence that the pseudosubstrate region, presumably by interacting with the substrate binding site, is responsible for maintaining the catalytic domain in an inactive state. We propose that incubation of protein kinase C with the pseudosubstrate antiserum renders the catalytic domain accessible to protein substrates in a manner analogous to the conformational changes induced by physiological activators such as phospholipids.

An understanding of the mechanism of activation of protein kinase C (PKC) is important because of the central role of this enzyme in tumor promotion, cellular differentiation, transformation, and other signal transduction pathways (1). The enzyme can be cleaved with trypsin to generate a 32-kDa regulatory domain that retains the lipid binding activity of the intact enzyme (2) and a 50-kDa catalytic domain (3) that is active in the absence of calcium and phospholipids.

Although the mechanism by which the regulatory region inhibits catalytic activity remains unclear, it has been proposed that amino acid residues 19–36 (RFARKGALRQKN-VHEVKN) near the amino terminus of the regulatory domain encode a pseudosubstrate prototope (4). In the absence of exogenous activators, the pseudosubstrate site may inhibit the enzyme by limiting access of the catalytic domain to substrates through direct interaction with the substrate binding site. The term prototope, as defined previously (4), refers to a functional region of protein primary sequence that can be mimicked by a short synthetic peptide. In fact, the pseudosubstrate peptide has been shown to be a potent inhibitor of PKC activity with a $K_i$ of 0.15 μM (4). If alanine 25 is replaced by serine, however, the peptide serves as an excellent substrate for PKC (4).

Molecular cloning studies have shown that PKC consists of a group of homologous protein kinases referred to as PKCa, PKCb, and PKCγ (5–8). The sequences of these proteins differ extensively in the readily proteolyzed hinge region which separates the catalytic and regulatory domains. These differences in sequence have been exploited to generate antipeptide antisera which can specifically recognize the individual isoforms (9). The isoforms share, however, the pseudosubstrate sequence presented above with only minor differences, none of which involve the critical basic residues adjacent to alanine 25.

The cloning of two additional members of the PKC family, PKCδ and PKCe, has recently been reported (10, 11). Although little information is presently available concerning the substrate specificity and the requirements for the activation of these more distantly related kinases, both enzymes have amino acid sequences which are readily identifiable as pseudosubstrate sites by their distributions of basic residues and their locations within the molecules (TMNRRGAIKQKAK and PRKRGQAVRRRV). The presence of unique pseudosubstrate sites and the extensive sequence homologies of these kinases to PKC suggest that they will also be regulated by a mechanism similar to that proposed for PKCa, -β, and -γ.

To investigate further this potentially general mechanism of protein kinase regulation, we have prepared antipeptide antiserum directed against the pseudosubstrate sequence of PKC. The present study shows that the antiserum can completely activate PKC in the absence of calcium and phospholipids. The use of site-directed antibodies described here provides a novel approach to understanding the nature and specificity of proteinsubstrate interaction.

EXPERIMENTAL PROCEDURES

Materials—Protein A, protein A-Sepharose CL-4B, bovine serum albumin (fraction V), phosphatidylerine, 1,2-diolein, histones (type III), CAMP-dependent protein kinase catalytic subunit (bovine heart), and protein kinase inhibitor (bovine heart) were all from Sigma. Leupeptin and apropin were purchased from Boehringer Mannheim, and soybean trypsin inhibitor was from Behring Diagnostics. Nitrocellulose sheets (BA-85, 0.45 μm) were obtained from Schleicher & Schuell. All radionucleotides were from Amerham Corp.

Preparation of Peptides and Antipeptide Antibodies—Peptides were
synthesized on an automated Biosearch peptide synthesizer, and the quality of each peptide was verified by high pressure liquid chromatography and amino acid analysis. The peptides were coupled to keyhole limpet hemocyanin (Behring Diagnostics) through a cysteine residue added to the carboxyl-terminal amino acid of each peptide. m-Maleimidobenzoyl-N-hydroxysuccinimide (Pierce Chemical Co.) was used as the coupling agent (12). Each synthesized peptide was injected subcutaneously into two (psub) or three (reg) rabbits and the specificity of the antisera for their respective peptide antigens was checked by solid-phase radioimmunoassay with 125I-protein A as described previously (9). The pseudosubstrate and regulatory domain antipeptide antisera were able to recognize their respective peptide antigens at dilutions of 1:10,000. The titer of the pseudosubstrate antiserum for the pseudosubstrate peptide was 3-fold higher than the titer of the regulatory domain antiserum for its peptide antigen when assayed at a 1:10,000 dilution. Similar titers were obtained with all rabbits immunized against a given peptide; antisera from a single bleed of one rabbit in each set was used throughout the following studies.

PKCo, -a, and -g isomeric specific polyclonal antiserum and PKC-specific polyclonal antiserum have been described previously (9, 13).

Purification and Activation of Protein Kinase C—Partial purification of rat brain PKC was performed by sequentially subjecting a cytosolic extract obtained by centrifugation at 100,000 x g for 1 h to DEAE-cellulose chromatography (Whatman), Ultrogel AcA-34 gel filtration (LKB), and DEAE-Trisacryl ion exchange chromatography (LKB), as described previously (9, 14). The resulting PKC was approximately 10-fold purified. The PKC used in Fig. 2A was further purified to >50% homogeneity by subjecting the DEAE-cellulose eluate to phenyl-Sepharose (Pharmacia LKB Biotechnology Inc.) and phospholipid affinity chromatography (15).

PKC activity was assayed by incubation for 2 h at room temperature with the indicated antibody in a buffer containing 20 mM Hepes (pH 7.5), 10 mM MgCl2, 2 mM dithiothreitol, 0.2% bovine serum albumin, and a mixture of antiproteases (soybean trypsin inhibitor, leupeptin, and aprotinin, 10 μg/ml each) in a final volume of 62.5 μl. The IgG used in these experiments had been collected by ammonium sulfate precipitation, reassembled in its original volume, and diluted as indicated into the activation mixture. In Fig. 2B, the IgG was further purified by DEAE-cellulose chromatography (16).

PKC activity was assayed in a mixture (125 μl) containing 20 mM Hepes (pH 7.5), 10 mM MgCl2, 2 mM dithiothreitol, 1 mg/ml histone (type IIa), 100 μM [γ-32P]ATP (1000 cpm/μmol; Amersham Corp.), and either 5 mM EGTA or 2 mM CaCl2, 80 μg/ml phosphatidylserine, and 12 μg/ml diolein (14). Aliquots were removed at timed intervals, added to P-81 paper disks (Whatman), and the disks were washed and counted. In each experiment, kinase activity was determined under conditions in which the transfer of 32P from [γ-32P]ATP into histone was in the linear range. Each value represents the result of four or more independent assays. The kinase activity measured in duplicate experiments varied by less than 10%.

Electrophoresis and Immunoblotting—Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper (17). Nonspecific binding was blocked with 5% bovine serum albumin. Immunoblots were performed as described previously (9) except that, unless otherwise indicated, the nitrocellulose was incubated with 6 M urea for 45 min and reblocked with bovine serum albumin for 30 min immediately prior to incubation with the antisera. All antisera were used at dilutions of 1:100 and they were incubated with the nitrocellulose for 90 min at 37°C. Following incubation with 125I-protein A for 30 min, the blots were dried and subjected to autoradiography at -70°C.

The molecular weight standards were myosin (M, 200,000), phosphorylase b (M, 97,400), bovine serum albumin (M, 68,000), ovalbumin (M, 44,000), and α-chymotrypsinogen (M, 26,000).

RESULTS AND DISCUSSION

Polyclonal antisera were prepared against two synthetic peptides whose sequences were derived from the regulatory domain of rat brain PKC. The peptide corresponding to the pseudosubstrate region (psub, RFARKGALRQKNVHEVKN) has previously been synthesized (4); the sequence of the second peptide (reg, PGADKGPATDPPRS) begins 51 amino acid residues carboxy-terminal to the end of the pseudosubstrate region. Fig. 1 shows that both antipeptide antisera recognize a single immunoreactive species of 80-kDa on an immunoblot of cytosolic rat brain PKC which has been purified 10-fold (9). This species is absent on immunoblots performed with preimmune sera. The ability of both antisera to recognize PKC is significantly enhanced by a brief incubation of the immunoblot in 6 M urea prior to addition of the antisera.

Incubation of PKC (purified to >50% homogeneity, (15)) with anti-pseudosubstrate IgG activates the kinase as completely as do calcium and phospholipids (Fig. 2A). IgG purified

Fig. 1. Immunoblot of rat brain PKC with antipeptide antisera. Partially purified PKC (10 μg) was immunoblotted with antipeptide antiserum (1:100 dilution) directed against either the pseudosubstrate peptide (Ab psub) or a second peptide derived from the regulatory domain of protein kinase C (Ab reg). Where indicated, the nitrocellulose was incubated with 6 M urea immediately prior to addition of the antisera, as described under "Experimental Procedures." An autoradiograph (18 h exposure at -70°C) is shown. Molecular weight standards (M, ×10-5) are shown at left. PI, preimmune; I, immune.

Fig. 2. Activation of PKC by the pseudosubstrate antibody. A shows PKC activity following incubation for 2 h at room temperature in the absence of IgG (A), or in the presence of either preimmune IgG (B), pseudosubstrate IgG (C), or control regulatory domain IgG (D). Activity in the presence of EGTA is indicated by the solid bars; activity in the presence of calcium and phospholipids is indicated by the open bars. The PKC used in this experiment was purified to >50% homogeneity, and the IgG had been collected by ammonium sulfate precipitation, resuspended in its original volume, and used at a 1:10 final dilution. Pseudosubstrate and regulatory domain antibodies prepared in this manner contained similar amounts of total IgG; the preimmune control contained approximately one-half as much total IgG as shown by subsequent purification of the IgG to homogeneity by DEAE-cellulose chromatography. In B, partially purified PKC (10 μg) was similarly incubated with the indicated amounts of either pseudosubstrate IgG (●) or regulatory domain IgG (○) that had been further purified to homogeneity by DEAE-cellulose chromatography. Data obtained from assays containing 5 mM EGTA are expressed as the percentage of maximal activation seen in the presence of calcium and phospholipids after subtracting the basal activity in the absence of calcium and phospholipids with preimmune IgG.
from either preimmune serum or from the control regulatory domain antiserum fails to activate under identical conditions. Complete activation with pseudosubstrate IgG is also observed with the less purified PKC preparation (9) used in Fig. 1 (data not shown). Partial activation of PKC was also observed with a 1:50 dilution of whole pseudosubstrate antipeptide antiserum from the same rabbit and with whole antiserum from a second rabbit similarly immunized with the pseudosubstrate peptide (data not shown).

The extent of kinase activation is dependent upon the concentration of IgG (Fig. 2B). Pre-clearing the pseudosubstrate IgG with resin-immobilized pseudosubstrate peptide to remove the antibodies that recognize the peptide reduces the ability of this antiserum to activate PKC by more than 95% (data not shown). In contrast, pseudosubstrate IgG aliquots pre-cleared with either resin alone or with resin-immobilized heterologous peptides from the regulatory and catalytic domains (ILKKDVIVQDDDVD) retained the ability to activate PKC. These experiments were necessary since activation of PKC by pseudosubstrate IgG could not be inhibited directly with the pseudosubstrate peptide because the peptide itself is a potent inhibitor of PKC.

When PKC is partially activated with limiting amounts of pseudosubstrate IgG and immunoprecipitated with protein A coupled to Sepharose CL-4B (Sigma), the activity which remains in the supernatant continues to demonstrate calcium and phospholipid dependence (Fig. 3). The activity associated with the pellet has, however, become calcium- and phospholipid-independent. PKC incubated instead with either preimmune IgG or regulatory domain IgG continues to demonstrate calcium and phospholipid dependence after immunoprecipitation, and virtually all of the activity is recovered in the supernatant fraction (not shown).

We were not able, however, to reverse the activation and restore calcium and phospholipid dependence in a series of dilution experiments. Conditions were chosen so that antibody dissociation, if it were to occur, would most likely be accompanied by intramolecular pseudosubstrate/substrate site reassociation and the resulting calcium and phospholipid-dependent PKC activity could be assayed. PKC was first activated in a small volume (6.25 μl) with pseudosubstrate IgG and then immunoprecipitated with protein A coupled to Sepharose CL-4B to remove activated kinase molecules. The immunoprecipitated pellet was diluted 5-, 10-, 20-, 64-, or 160-fold with activation buffer, incubated 2 h at room temperature, re-immunoprecipitated, and the supernatant and pellet fractions were assayed for PKC activity. At all dilutions tested, the PKC activity remained associated with the pellet (IgG-protein A) fraction and continued to display calcium and phospholipid independence.

Because degradation of PKC could also generate a calcium- and phospholipid-independent activity, we examined the integrity of activated PKC by immunoblotting. PKC was partially activated with the pseudosubstrate IgG, immunoprecipitated and immunoblotted with either the pseudosubstrate IgG or with a polyclonal antiserum which recognizes PKC (13). No fragments of PKC were detected with either antibody on immunoblots of the supernatant and pellet fractions of immunoprecipitated PKC (data not shown). Immunoblots of total PKC activated in a parallel experiment showed that the recovery of intact PKC was quantitative (not shown).

Our preparation of rat brain PKC contains PKCa, PKCB, and PKCy (Fig. 4A). The pseudosubstrate peptide sequence is derived from PKCb; the minor sequence differences between the α, β, and γ isoforms are shown in Fig. 4. The α and β isoforms differ by a single amino acid at their carboxyl termini and would both be expected to be activated by the pseudosubstrate IgG. PKCy has four sequence differences, so we investigated whether this isoform can also be recognized by the pseudosubstrate IgG. PKC activated with pseudosubstrate IgG was immunoprecipitated to remove activated kinase molecules, washed extensively, subjected to electrophoresis, and immunoblotted with isof orm-specific antisera (Fig. 4B). Both PKCb and PKCy are present in the immunopre-
present in abundance in all lanes in Fig. 4B. The pseudosubstrate antibody could distinguish among the isoforms, this possibility seems unlikely. Fig. 4A shows that no residual Ca\(^{2+}\) and phospholipid-dependent PKC activity could be detected after activation with the pseudosubstrate antibody. Such a result would have been expected if the pseudosubstrate antibody could distinguish among the isoforms since it has been shown that PKCa, -\(\beta\), and -\(\gamma\) account for 25, 49, and 26%, respectively, of total PKC activity in whole rat brain cytosol (18). Furthermore, our partially purified rat brain PKC preparation contains all three isoforms (Fig. 4A) in relative amounts approximately equivalent to those observed in whole rat brain cytosol as shown by immunoblotting with the same isoform-specific antisera (see Fig. 3 in Ref. 9). Therefore, we have chosen a purification procedure that does not appear to enrich for any one of the isoforms. Thus, the presence of residual Ca\(^{2+}\) and phospholipid-dependent activity from an unactivated isoform following pseudosubstrate antibody incubation should have been readily apparent.

The model discussed here for the regulation of PKC by a pseudosubstrate site is not without precedent; similar regulation by inhibitory epitopes has also been proposed for other serine and threonine protein kinases (19–28). The regulatory domain of cAMP-dependent protein kinase Type I contains a pseudosubstrate site, and the regulatory domains of Type II cAMP-dependent and cGMP-dependent protein kinases contain sequences that resemble substrate sites and that become autophosphorylated in response to physiological activators. It is likely that these sequences will prove to be in close contact with the catalytic active site (19, 20). In addition, a potent, heat-stable inhibitor of cAMP-dependent protein kinase contains a pseudosubstrate site and appears to inhibit by competing with substrate for binding (21, 22). A pseudosubstrate sequence is also thought to inhibit both skeletal and smooth muscle myosin light chain kinases as well as calcium- and calmodulin-dependent protein kinase II by interacting with the substrate binding site (23–28).

To test the specificity of the pseudosubstrate antiserum for PKC, we examined its ability to interfere with the inhibition of the catalytic subunit of cAMP-dependent protein kinase by the heat-stable inhibitor protein (Fig. 5). Incubation of pseudosubstrate IgG with limiting amounts of the heat-stable inhibitor protein under conditions which activate PKC does not alter the concentration of the heat-stable inhibitor required to inactivate the catalytic subunit (Fig. 5). The concentration of catalytic subunit used in this experiment was approximately one-tenth that of PKC in the activation experiment in Fig. 2A. Thus, the activation by the pseudosubstrate IgG appears to be specific for PKC, as predicted by the differences of the two enzymes in substrate specificity and in pseudosubstrate sequence. We were unable to test directly whether the pseudosubstrate IgG could activate cAMP-dependent holoenzyme because the IgG itself interfered with the enzyme assay.

Our experiments with the pseudosubstrate antibody provide a new experimental approach to analyzing the mechanism of kinase activation. The results support the hypothesis that endogenous sequences can interfere directly with catalytic active sites. It is likely that the use of such site-specific antibodies can be extended to the study of other types of protein/substrate and protein/protein recognition.

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REFERENCES

Protein Kinase C Activation

15199
Acad. Sci. U. S. A. 80, 6858–6862
12314
147–155
Sci. U. S. A. 76, 4350–4354
18. Sekiguchi, K., Tsukuda, M., Ase, K., Kikkawa, U., and Nishizuka,
19. Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln,
Chem. 253, 6002–6009
22. Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi,
261, 989–992
23. Edelman, A. M., Takio, K., Blumenthal, D. K., Hansen, R. S.,
260, 11275–11285
24. Kennelly, P. J., Edelman, A. M., Blumenthal, D. K., and Krebs,
25. Kemp, B. E., Pearson, R. B., Guerriero, V., Jr., Bagchi, I. C., and
28. Colbran, R. J., Fong, Y.-L., Schworer, C. M., and Soderling, T.