Protein Kinase C Contains a Pseudosubstrate Prototope in Its Regulatory Domain

COLIN HOUSE AND BRUCE E. KEMP*

The regulatory domain of protein kinase C contains an amino acid sequence between residues 19 and 36 that resembles a substrate phosphorylation site in its distribution of basic residue recognition determinants. The corresponding synthetic peptide (Arg^{19}-Phe-Ala-Arg-Lys-Gly-Ala^{25}-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn^{36}) acts as a potent substrate antagonist with an inhibitory constant of 147 ± 9 n*M*. It is a specific inhibitor of protein kinase C and inhibits both autophosphorylation and protein substrate phosphorylation. Substitution of Ala^{25} with serine transforms the pseudosubstrate into a potent substrate. These results demonstrate that the conserved region of the regulatory domain (residues 19 to 36) of protein kinase C has the secondary structural features of a pseudosubstrate and may be responsible for maintaining the enzyme in the inactive form in the absence of allosteric activators such as phospholipids.

PROTEIN KINASE C (PKC) IS ONE OF the major regulatory enzymes involved in the control of a wide variety of physiological processes (*1*) including differentiation, tumor promotion, and membrane receptor function. This enzyme was first identified as a latent protein kinase activity that was seen after proteolysis (*2*). Early studies led to the concepts that PKC contained both regulatory and catalytic domains (*1*) and that the enzyme could be activated by phospholipids or proteolytic removal of the inhibitory regulatory domain. The recent cloning and determination of the DNA-derived primary structure of PKC from a variety of species (*3, 4*) has provided direct structural support for this concept. Parker *et al.* (*3*) proposed that the regulatory domain extends over the first 300 residues. The catalytic domain shares sequence homologies with other members of the protein kinase gene family (*3, 4*). It has not been clear precisely how the regulatory domain inhibits catalytic activity.

There are now a number of examples of protein interactions where small segments of protein secondary structure play crucial roles in the interaction. These functional units, or prototopes (*5*), consist of segments of the primary structure whose function can be mimicked by synthetic peptides. Examples of this phenomenon include the interaction of calmodulin with a segment of the carboxyl-terminal region of the myosin light chain kinase (*6*), the interaction of the subunits of ribonucleotide reductase via a nonapeptide (*7*), the hepatitis B virus envelope protein with its hepatic receptor (*8*), many linear peptide epitopes with antibodies (*9*), and the cAMP (adenosine 3′,5′-monophosphate) dependent protein kinase with the heat-stable inhibitor protein (*10*). In the last case the inhibitor contains a pseudosubstrate sequence Arg-Arg-Asn-Ala-Ile bearing structural similarities to the substrate recognition sequence for this enzyme Arg-Arg-X-Ser(P)-X. Indeed, synthetic peptides corresponding to the pseudosubstrate region of the inhibitor protein act as potent inhibitors of the cAMP-dependent protein kinase (*10*). In this study we investigated whether PKC contained a pseudosubstrate prototope in its regulatory domain that may be responsible for maintaining the enzyme in its inactive form.

The substrate specificity of PKC has been studied in a number of laboratories (*11, 12*). Although there is considerable diversity in the local phosphorylation site sequences for this enzyme, evidence obtained from structure-function studies with synthetic peptide substrates indicates that the enzyme has a requirement for basic residue determinants in common with a number of other serine and threonine protein kinases (*13*). Some of the most potent synthetic substrates contained basic residues on both sides of the phosphorylation site (*12*). In the case of the peptide Ala^{229}-Lys-Arg-Arg-Arg-Leu-Ser-Ser(P)-Leu-Arg-Ala^{239}, corresponding to residues 229 to 239 in the ribosomal protein S6, Arg^{238} was found to be important, as were those basic residues on the amino-terminal side of Ser^{236} (*12*). With these specificity requirements in mind, we examined the amino acid sequence of the regulatory domain of PKC for the possible presence of pseudosubstrate sequences. The region of the regulatory domain corresponding to residues 19 to 31, Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val, contained a juxtaposition of basic residues around Ala^{25} similar to the substrate phosphorylation site in protein S6 including the carboxyl-terminal arginine and the multiple basic residues on the amino-terminal side (underlined). This segment of the sequence is contained in the first conserved region C1 (*3*) present in the three genes of all species of PKC for which DNA-derived sequence information is available (*3, 4*).

We have synthesized the PKC pseudosubstrate sequence corresponding to residues 19 to 36 and found that it inhibited the glycogen synthase synthetic peptide substrate phosphorylation with an IC_{50} (inhibitor concentration required to give 50% inhibition of peptide substrate phosphorylation at the K_m concentration) of approximately 0.18 ± 0.02 μ*M* (n = 5). The inhibition pattern was competitive and gave a linear secondary plot of K_m/V_{max} (concentration required to give half-maximal velocity/maxi-

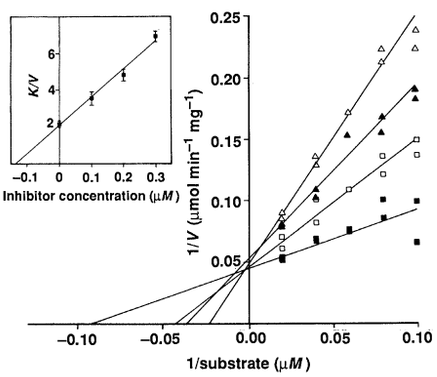

Fig. 1. Lineweaver-Burk plot for PKC(19–36)–dependent inhibition of synthetic peptide phosphorylation by PKC. Rat brain PKC was purified as described in (*12*), modified by additional anion-exchange chromatography in the presence of ATP and magnesium acetate as described in (*18*). PKC activity was determined as in (*12*) with [$Ala^{9,10}$,$Lys^{11,12}$]GS(1–12) peptide (Pro^1-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys^{12}) as substrate. Briefly, the reaction mixture (80 μl) containing 20 m*M* tris-HCl (*p*H 7.5), 12.5 m*M* $MgCl_2$, 0.75 m*M* $CaCl_2$, 0.5 m*M* EGTA, 600 μ*M* [γ-^{32}P]ATP (500 to 1000 cpm/pmol), phosphatidylserine (3.75 μg/ml) and synthetic peptides as indicated was incubated at 30°C. PKC (12 μl addition) was diluted in 20 m*M* tris-HCl (*p*H 7.5) buffer containing 0.005% (v/v) Triton X-100. Aliquots (60 μl) were removed after 5 minutes and added to 20 μl of 24% (w/v) trichloroacetic acid. The mixture was centrifuged, and a 50-μl aliquot of the supernatant was added to P81 paper discs. [γ-^{32}P]ATP was removed by washing in 75 m*M* H_3PO_4 (*13*). Peptide inhibitors were diluted in Triton X-100 (0.005% v/v) to prevent loss on the plastic surfaces at high dilution. The kinetic parameters of K_m and V_{max} were determined for the above peptide in the presence of varying concentrations of inhibitor peptide PKC(19–36). The double-reciprocal plots at each inhibitor concentration are shown. No inhibitor (■); 0.1 μ*M* inhibitor (□); 0.2 μ*M* inhibitor (▲); 0.3 μ*M* inhibitor (△). The K_i value for PKC was determined from the secondary plot of K_m/V_{max} ± SEM versus inhibitor concentration (insert).

Department of Medicine, University of Melbourne, Repatriation General Hospital, West Heidelberg, Victoria 3081 Australia.

*To whom correspondence should be addressed.

mal velocity) versus inhibitor peptide concentration. The apparent inhibitory constant (K_i) obtained from a double-reciprocal plot and secondary plot of K_m/V_{max} versus inhibitor peptide gave a value of 147 ± 9 nM (Fig. 1). The inhibitor peptide gave an uncompetitive inhibition plot with varying adenosine triphosphate (ATP) concentration, indicating that inhibition was not due to inhibition at the ATP-binding site. The inhibitor peptide has no effect on the dependence of PKC on phosphatidylserine or the enzyme's diacylglycerol-dependent shift in calcium dependence. The shorter peptide PKC(19–31) was more potent (IC_{50} = 92 ± 5 nM, n = 5) than the peptide PKC(19–36) indicating that increasing the length beyond residue 31 did not increase the inhibitory potency. The importance of the carboxyl-terminal basic residues was examined with the shortened analog PKC(19–27) and the peptide [Ala^{27}]PKC(19–31) containing alanine in place of Arg^{27}. In both cases these changes lead to a more than 20-fold increase in the IC_{50} for inhibition of peptide substrate phosphorylation, 6.2 ± 0.9 μM (n = 5) and 3.1 ± 0.6 μM (n = 3), respectively. These results are consistent with our earlier findings where the corresponding carboxyl-terminal arginine (Arg^{238}) in the peptide substrate analog of protein S6 was important for the phosphorylation of Ser^{236} in the sequence Lys-Arg-Arg-Arg-Leu-Ser-Ser(P)^{236}-Leu-$\underline{Arg}^{238}$ (*12*).

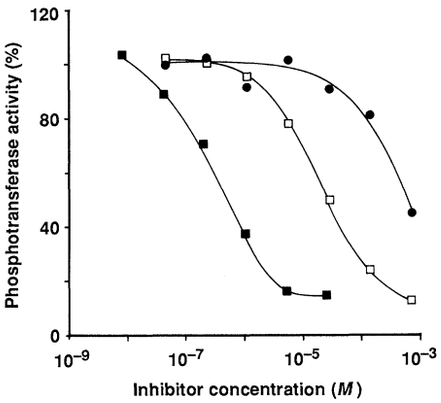

Fig. 2. PKC(19–36) as an inhibitor of protein kinases. The peptide PKC(19–36) was tested as an inhibitor of peptide phosphorylation catalyzed by three protein kinases known to recognize basic residues as specificity determinants. The phosphorylated peptide substrates were used at the respective K_m concentrations in the presence of increasing concentrations of PKC(19–36). Enzyme activity is expressed as a percentage of maximal activity. The activity of the catalytic subunit of cAMP-dependent protein kinase, prepared from bovine heart, was measured by using Leu-Arg-Arg-Ala-Ser-Leu-Gly as substrate (●). The activity of myosin light chain kinase [prepared from chicken gizzards (*17*)] was measured with [$Ala^{14,15}$]MLC(11–23) peptide (Lys^{11}-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ala^{23}) as substrate (□). PKC activity was determined as in (*12*) with [$Ala^{9,10}$,$Lys^{11,12}$]GS(1–12) peptide as substrate (■).

We were interested in testing the effect of substituting Ala^{25} in the PKC pseudosubstrate sequence with serine. The peptide [Ser^{25}]PKC(19–31) was found to act as a substrate with a K_m of approximately 0.2 μM and a V_{max} of 8 μmol min^{-1} mg^{-1}. This serine analog of the pseudosubstrate is comparable to the most potent peptide substrate previously reported for PKC (*12*). These results confirm that the region of the regulatory domain between residues 19 and 31 contains the necessary specificity determinants expected of a pseudosubstrate.

A number of serine and threonine protein kinases utilize basic residues, particularly arginine, as specificity determinants (*13*). Accordingly, it was of interest to test the specificity of the PKC pseudosubstrate peptide with other arginine-requiring protein kinases. The peptide PKC(19–36) was found to be a poor inhibitor of the cAMP-dependent protein kinase (IC_{50} = 423 ± 67 μM), and a moderate inhibitor of the myosin light chain kinase (IC_{50} = 24 ± 2 μM) (Fig. 2). These concentrations are more than 100 times as high as that required to inhibit PKC: these results indicate that the pseudosubstrate peptide has a high degree of specificity.

We have tested the capacity of the pseudosubstrate peptide to inhibit PKC autophosphorylation and protein substrate phosphorylation. The pseudosubstrate peptide inhibited autophosphorylation of PKC but not autophosphorylation of the myosin light chain kinase consistent with the latter enzyme's different substrate specificity requirements. Phosphorylation of the myosin light chain kinase catalyzed by PKC was inhibited by the pseudosubstrate peptide with an IC_{50} = 0.21 ± 0.05 μM (n = 3) (Fig. 3), but phosphorylation of histone III-S, the commonly used substrate for PKC, was not inhibited. The interactions between histone III-S and PKC are complex. Histone III-S was a potent inhibitor of PKC-catalyzed phosphorylation of glycogen synthase peptide with an IC_{50} of approximately 20 nM. Histone had previously been reported to inhibit PKC autophosphorylation (*14*). The concentration of histone III-S required for inhibition is less than 1/30th of the apparent K_m for histone phosphorylation (*11*). Because histone acts both as an inhibitor at nanomolar concentrations and as a substrate at micromolar concentrations, the pseudosubstrate peptide is not sufficiently potent to inhibit phosphorylation of micromolar concentrations of histone. If, however, the histone concentration is reduced well below the K_m to 0.1 μM, then the pseudosubstrate peptide at 5 to 100 μM inhibits histone phosphorylation. Under these limiting conditions it is necessary to measure the low level of histone phosphorylation autoradiographically after polyacrylamide gel electrophoresis. Polylysine and polyarginine were also potent inhibitors (IC_{50} = 10 nM) of peptide substrate phosphorylation by PKC, suggesting that the polybasic structure may be important for inhibition.

It has been reported that the regulatory domain derived by tryptic digestion of the enzyme does not inhibit the corresponding catalytic fragment (*15*). Because of the presence of critical arginine residues in the amino-terminal pseudosubstrate region it is possible that proteolytic removal of the enzyme's regulatory domain by trypsin would also cause cleavage at one or more of the basic residues and destroy its capacity to act as a pseudosubstrate inhibitor.

Our results demonstrate that the regulatory domain of PKC contains a pseudosubstrate sequence. Synthetic peptide analogs of this sequence act as potent and specific substrate antagonists by virtue of the presence of basic residue determinants necessary for substrate recognition. It is therefore proposed that the pseudosubstrate region,

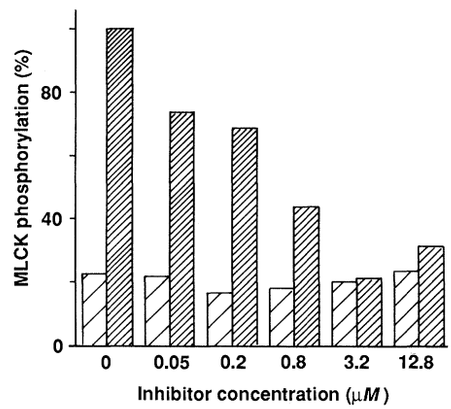

Fig. 3. PKC(19–36) inhibition of PKC-dependent phosphorylation of myosin light chain kinase (MLCK). Myosin light chain kinase [prepared from chicken gizzards (*17*)] was incubated in the presence of [γ-^{32}P]ATP with or without PKC under the conditions outlined in (*12*), with the exception that the reaction was carried out at 0°C for 24 minutes, and terminated by the addition of 2% SDS and 100 μM β-mercaptoethanol followed by boiling for 3 minutes. The samples were subjected to SDS–polyacrylamide gel electrophoresis on a 7 to 20% gradient gel. The gel was stained for protein with Coomassie blue and autoradiographed with Kodak X-Omat RP film. Phosphorylated bands were quantitated by densitometry of the autoradiograph. Myosin light chain kinase autophosphorylation is indicated by the wide hatching and PKC-catalyzed phosphorylation by the narrow hatching. Values given are for a single polyacrylamide gel. Similar inhibition of PKC-catalyzed phosphorylation of the myosin light chain kinase with the pseudosubstrate peptide was observed in each of three independent experiments (IC_{50} = 0.21 ± 0.05 μM).

residues 19 to 31, may bind to the active site of PKC and inhibit its activity. Activation of the enzyme by phospholipids would be expected to cause a conformational change that removed the pseudosubstrate sequence from the active site and allowed access to protein substrates. There are now several precedents for the regulation of protein kinases by pseudosubstrate structures. The inhibitor protein of the cAMP-dependent protein kinase is the most thoroughly studied example (see above). The "hinge" regions in the regulatory subunits of cAMP-dependent protein kinase are also believed to inhibit by binding to the active site of the catalytic subunit (*16*). Recently, we reported that the calmodulin-binding domain of the smooth muscle myosin light chain kinase contains a pseudosubstrate structure that may be responsible for maintaining the enzyme in the inactive form in the absence of calmodulin (*17*). Synthetic peptides corresponding to this region were shown to act as potent substrate antagonists.

The proposed role of the pseudosubstrate prototope present in the PKC regulatory domain provides an important focus for protein engineering studies to test the consequences of deletions and point mutations in this region of the enzyme. In view of the results obtained here and those with the cAMP-dependent protein kinase and the myosin light chain kinase, it would appear that pseudosubstrate prototopes may occur widely in the regulation of protein kinases and perhaps other enzymes.

REFERENCES AND NOTES

1. Y. Nishizuka, *Science* **233**, 305 (1986).
2. Y. Takai *et al.*, *J. Biol. Chem.* **252**, 7603 (1977).
3. P. Parker *et al.*, *Science* **233**, 853 (1986).
4. L. Coussens *et al.*, *ibid.*, p. 859; J. L. Knopf *et al.*, *Cell* **46**, 491 (1986); Y. Ono *et al.*, *FEBS Lett.* **203**, 111 (1986); S. Ohno *et al.*, *Nature (London)* **325**, 161 (1987).
5. The term prototope is defined as a functional segment of a protein that can be mimicked with a synthetic peptide usually not more than 30 residues in length. It is a general term to cover ligand-protein, peptide-protein, or protein-protein interactions where the protein's functional segment is dependent on the structure dictated by a short segment of the primary sequence. Thus linear epitopes recognized by antibodies represent a specific subgroup of prototopes.
6. D. Blumenthal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3187 (1985).
7. B. M. Dutia, M. C. Frame, J. H. Subak-Sharpe, W. N. Clark, H. S. Marsden, *Nature (London)* **321**, 439 (1986); E. A. Cohen, P. Gandreau, P. Brazeau, Y. Langelier, *ibid.*, p. 441.
8. A. R. Neurath, S. B. H. Kent, N. Strick, K. Parker, *Cell* **46**, 429 (1986).
9. J. G. Sutcliffe, T. M. Shinnick, N. Green, R. A. Lerner, *Science* **219**, 660 (1983).
10. H. C. Cheng *et al.*, *Biochem. J.* **231**, 655 (1985); J. D. Scott, E. H. Fischer, J. G. Demaille, E. G. Krebs, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4379 (1985); H. C. Cheng *et al.*, *J. Biol. Chem.* **261**, 989 (1986).
11. C. A. O'Brian *et al.*, *Biochem. Biophys. Res. Commun.* **124**, 296 (1984); R. S. Turner *et al.*, *J. Biol. Chem.* **260**, 11503 (1985); S. Ferrari *et al.*, *FEBS Lett.* **184**, 72 (1985); J. R. Woodgett and T. Hunter, *Eur. J. Biochem.* **161**, 177 (1986).
12. C. House *et al.*, *J. Biol. Chem.* **262**, 772 (1987).
13. D. B. Glass and E. G. Krebs, *ibid.* **257**, 1196 (1982); B. E. Kemp *et al.*, *ibid.* **252**, 4888 (1977); T. K. Eckols *et al.*, *Eur. J. Biochem.* **124**, 249 (1983); B. E. Kemp, R. B. Pearson, C. House, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7471 (1983).
14. D. Mochly-Rosen and D. E. Koshland, Jr., *J. Biol. Chem.* **262**, 2291 (1987).
15. M. H. Lee and R. M. Bell, *ibid.* **261**, 14867 (1986).
16. K. Titani *et al.*, *Biochemistry* **23**, 4193 (1984); K. Takio *et al.*, *ibid.*, p. 4200; K. Takio *et al.*, *ibid.*, p. 4207.
17. B. E. Kemp *et al.*, *J. Biol. Chem.* **262**, 2542 (1987).
18. A. Y. Jeng *et al.*, *Cancer Res.* **46**, 1966 (1986).

19. This work was supported by grants from the Anti-Cancer Council of Victoria and the National Health and Medical Research Council. Equipment grants were provided by the Ian Potter Foundation, the Clive and Vera Ramaciotti Foundation, the Sunshine Trust, and the Buckland Foundation.

13 July 1987; accepted 22 October 1987

Technical Comments

The Cerebellum and Memory Storage

R. F. Thompson (*1*) presents an overview of the neurobiology of learning and memory and includes a discussion of the role of the cerebellum in motor learning. Specifically he argues that "recent evidence . . . overwhelmingly favors an essential role for the cerebellum in both learning and memory of discrete, adaptive behavioral responses to aversive events, thus supporting the. . . role of the cerebellum in motor learning." The climbing fiber projection to the cerebellum is described as playing a major role in this learning process. I would like to point out that these views were expressed without adequate discussion of either the experimental findings that are inconsistent with this hypothesis or the shortcomings of the existing data obtained by using the conditioning paradigms.

The work of Thompson and his colleagues, as well as the studies of Yeo *et al.* (*2*), indicates that pathways involving the cerebellum are necessary for the acquisition of the classically conditioned eye-blink reflex in the rabbit. However, this demonstration that the cerebellum is required for an adaptive or associative process is not unique, as it has been shown previously that portions of the cerebellum are required for adaptation of the vestibuloocular reflex (*3*). The most pertinent issue is whether these data prove that the learning process actually occurs within the cerebellum itself.

In arguing that the climbing fiber system plays a key role in establishing memory traces in the cerebellum, two lines of evidence are cited: (i) Thompson's own studies implicating the climbing fiber system as an unconditioned stimulus (UCS) in the conditioned eye-blink paradigm, and (ii) the studies of Ito *et al.* (*4*) illustrating that the climbing fiber input to Purkinje cells can produce a long-term depression of Purkinje cell excitability. In my view, these experiments provide only indirect support for his arguments, particularly in the context of published results favoring other views of climbing fiber function. I also have concerns about the interpretation of Thompson's data.

The studies supporting UCS and conditioned stimulus (CS) roles for the climbing and mossy fiber systems, respectively, are difficult to evaluate because some of the cited articles and abstracts are "in press." On the basis of information that is available, there are specific problems and controversies regarding these experimental data. For example, whether stimulation of the inferior olive can evoke motor behavior is still an open question (*5*), as other investigators have not been able to demonstrate this phenomenon. Regrettably information regarding stimulus parameters essential for the evaluation of these data is not included. Second, arguments favoring the mossy fiber input from the pons as the CS cite as supportive evidence the immediate transfer occurring when the stimulus location was changed from one side of the pontine nuclei to the other. This finding may have little to do with the conditioning paradigm; rather it may reflect the fact that both stimulus sites are activating pontine projections to both sides of the cerebellum, as pontine neurons from one side predominantly cross and project through the pontine nuclei on the opposite side on their way to the middle peduncle. However, even if one assumes that olivary stimuli can act as UCS, this observation is not sufficient to differentiate between two viable interpretations: (i) that the climbing fibers are establishing a memory trace in the cerebellum and (ii) that the climbing fibers are essential for a cerebellar