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A synthetic peptide analog of the putative substrate-binding motif activates protein kinase C

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A 29-residue synthetic peptide, Leu[530]-Leu-Tyr-Glu-Met-Leu-Ala-Gly-Gln-Ala-Pro-Phe-Glu-Gly-Glu-Asp-Glu-Asp-Glu-Leu-Phe-Gln-Ser-Ile-Met-Glu-His-Asn-Val-NH_2[558], corresponding to part of the catalytic domain of protein kinase C, is a potent activator of the enzyme, with a K_a of approx. 10 μM. Activation was 59±4% of that observed with phosphatidylserine, predominantly due to an increased V_{max}, partially calcium-dependent, observed with all three isoenzymes (α, β, γ), and resulted in autophosphorylation. It is proposed that the region between Gly[528] and Arg[583] is part of the protein substrate binding region of protein kinase C and synthetic peptide analogs of this region activate the enzyme by blocking the action of the enzyme's basic pseudosubstrate autoregulatory region.

Protein kinase C; Enzyme activator; Peptide analog, synthetic; Substrate-binding region

1. INTRODUCTION

Protein kinase C (PK-C) is an important regulatory enzyme involved in multiple cellular responses [1]. The protein substrates phosphorylated by PK-C contain basic residues in proximity to the phosphorylated residue [2,3] that act as important specificity determinants. The enzyme's regulatory domain also contains a basic residue-rich region between residues 19 and 31, which is thought to act as a pseudosubstrate regulator binding to the active site [4]. However, it is not known where protein substrates or the pseudosubstrate bind to the catalytic domain. Comparisons between other protein kinases [5] and nucleotide-binding proteins such as 3-phosphoglycerate kinase [6] provide a guide to the possible location of this protein substrate-binding region in PK-C. Taylor et al. [7] identified the ATP-binding motif in the cAMP-dependent protein kinase that consists of a glycine triad and a nearby lysine residue. PK-C contains an analogous motif beginning at Gly[349] and extending to Lys[371]. The entire ATP-binding domain in the crystal structure of 3-phosphoglycerate kinase contains approx. 180 residues [6]. In the case of PK-C such a domain would extend from the glycine triad beginning at residue 349 to approximately Gly[528] (180 residues). This region contains conserved acidic residues found in all members of the protein kinase family (fig.1). Given the bilobal structure of some sugar kinases [6] it seems reasonable that components of the protein substrate-binding site of PK-C would be found at one or other ends of the ATP-binding domain. It seems reasonable that the protein binding site is located on the carboxyl-terminal end of the ATP-binding domain because the amino-terminus of the calmodulin-dependent protein kinase II is within 12 residues of the glycine triad [5]. The carboxyl-terminal boundary for the protein substrate-binding region is likely to be Arg[583] in PK-C because this is the most distal residue conserved in all protein kinases [5] and truncations near this residue in either the myosin light chain kinase [8] or pp60[src] [9] retain their capacity to bind and phosphorylate substrates. Thus the ATP-binding and protein substrate-binding properties for all protein kinases would be expected in a 240 amino acid segment between the

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glycine triad and the distal conserved Arg. Here, we show that a synthetic peptide corresponding to part of the putative substrate-binding region is a potent activator of PK-C.

2. MATERIALS AND METHODS

[γ-^{32}P]ATP was purchased from New England Nuclear and all other reagents and methods were as described [2]. The enzyme was purified from rat brain and stored in buffer containing glycerol and Triton X-100 as described [2,10]. Phosphorylation assays were performed in a volume of 40 μl in 20 mM Tris (pH 7.5), 12.5 mM $MgCl_2$, 0.5 mM EGTA, 0 or 0.75 mM $CaCl_2$, 300 μM [λ-^{32}P]ATP (500 cpm/pmol), 50 μM [Ala^{9,10}Lys^{11,12}]GS (1-12) (synthetic peptide substrate from glycogen synthase [4]), 0.35 μg rat brain PK-C, plus activators as shown. PK-C isoenzymes were separated on hydroxyapatite [11]. Autophosphorylation was carried out for 5 min in a volume of 40 μl containing 30 mM Tris (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, 200 μM ATP, 2 μg PK-C, and the activators Ca (calcium, 200 μM), PK-C [peptide PK-C (530–558) 250 μM], PS (phosphatidylserine, 10 μg/ml) or PMA (1 μM). Reactions were terminated by addition of 2% SDS and the samples separated on a 20 cm polyacrylamide (7.5%) gel, dried and autoradiographed.

3. RESULTS AND DISCUSSION

The peptide, Leu^{530}-Leu-Tyr-Glu-Met-Leu-Ala-Gly-Gln-Ala-Pro-Phe-Glu-Gly-Asp-Glu-Asp-Glu-Leu-Phe-Gln-Ser-Ile-Met-Glu-His-Asn-Val-NH_2^{558}, corresponding to more than half the sequence between Gly^{528} and Arg^{583} (fig.1), was a potent activator of PK-C (fig.2A) with a K_a of approx. 10 μM and maximum activation of 59 ± 4% (n = 12) compared with phosphatidylserine. Although phosphatidylserine-dependent activation of the enzyme requires calcium, activation by the acidic peptide was only partially calcium-dependent [37 ± 6% (n = 8)] (fig.2A). The acidic peptide primarily increased the V_{max} with modest ef-

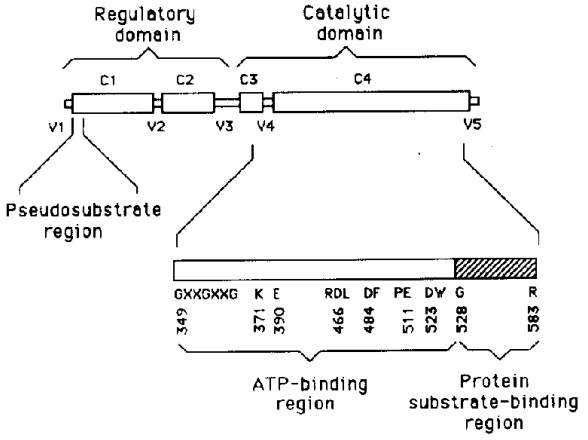

Fig.1. Summary of the location of the catalytic and regulatory features of PK-C. The overall structure corresponds to that proposed by Parker et al. [12]. The residues are numbered according to Knopf et al. [18]. The residues from the catalytic domain that share homology with all known serine, threonine and tyrosine protein kinases are shown in relation to the ATP-and protein substrate-binding regions.

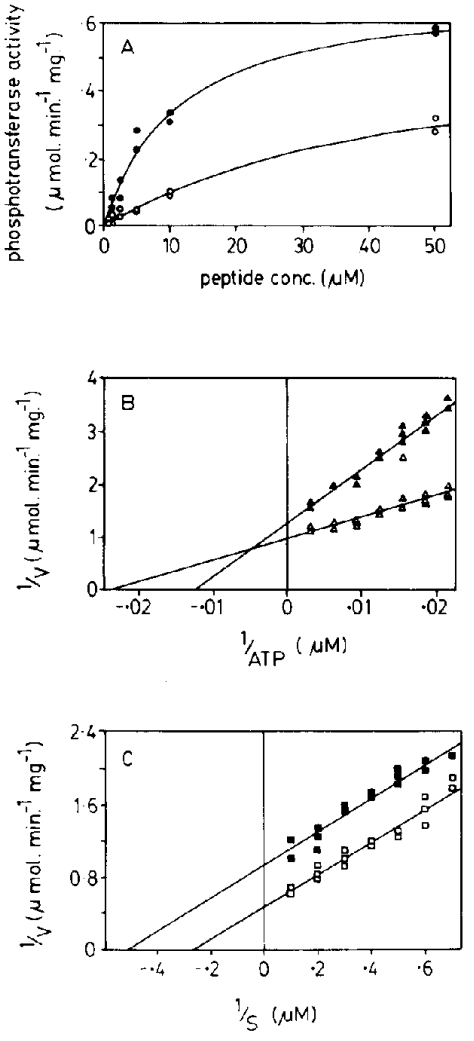

Fig.2. Activation of PK-C by the peptide PK-C(530–558). (A) Dose response for the peptide PK-C(530–558) in the absence (○) and presence (●) of calcium. (B) Dependence on ATP in the presence of 2.5 μg/ml phosphatidylserine (△) or 50 μM peptide PK-C(530–558) (▲). (C) Dependence on substrate kinetics in the presence of 2.5 μg/ml phosphatidylserine (□) or 50 μM peptide PK-C(530–558) (■).

Table 1

Comparison of activation of protein kinase C by synthetic peptides

Sequence	Concentration	Activation (%)
Phosphatidylserine	10 μg/ml	100
PK-C(530–558)	50 μM	47.5
PK-C(530–549)	50 μM	0.7
PK-C(539–558)	50 μM	0.6
PK-C(539–549)	50 μM	0.6
Poly(Asp) (M_r 14000)	50 μM	20.6
$(Asp)_{30}$	50 μM	6.0
$(Asp)_{21}$	50 μM	14.7
$(Asp)_{15}$	50 μM	5.3
$(Asp)_{10}$	50 μM	0.9
Poly(Glu) (M_r 50000)	32.5 μM	2.3

Leu^{530}-Leu-Tyr-Glu-Met-Leu-Ala-Gly-Gln-Ala^{539}-Pro-Phe-Glu-Gly-Glu-Asp-Glu-Asp-Glu^{549}-Leu-Phe-Gln-Ser-Ile-Met-Glu-His-Asn-Val-NH_2^{558} [polymers of Asp are shown as $(Asp)_n$ where n is the length of the polymer]

fects on the substrate K_m for ATP (increasing from 41 to 80 μM; fig.2B) and the peptide substrate K_m (decreasing from 3.8 to 2.0 μM; fig.2C). Activation by the peptide PK-C(530–558) was also observed with synthetic peptide substrates corresponding to phosphorylation sites in the epidermal growth factor receptor and ribosomal protein S6 [2] and to a lesser extent with histone. Since the peptide PK-C(530–558) did not increase the K_m for peptide substrate or alter inhibition by the pseudosubstrate inhibitor PK-C(19–31) it is unlikely that the peptide activator PK-C(530–558) exerts its effects by binding to the peptide substrate. Furthermore, at saturating concentrations of phosphatidylserine there was no further increase in PK-C activity with the peptide activator PK-C(530–558).

The structure-function requirements for activation by the peptide PK-C(530–558) was investigated by preparing a series of shorter peptides (table 1); all three peptides were poor activators, indicating that the full length of the peptide was important and that higher orders of structure may be important. Since the peptide PK-C(530–558) contains acidic residues the effect of polyaspartic and polyglutamic acid peptides were tested. Polyaspartic acid (M_r = 14000) was the most effective activator. In the range $(Asp)_{10}$ to $(Asp)_{30}$ there was an optimum at $(Asp)_{21}$ (table 1). Since polyaspartic acid can partially mimic the peptide PK-C (530–558) it seems reasonable that activation depends on acidic amino acids but it is not yet clear as to which of the acidic residues present in the peptide PK-C(530–558) are required for activation.

Rat brain is known to contain multiple PK-C isoenzymes [11] like other tissues [12,13]. The three major peaks of PK-C resolved by hydroxyapatite

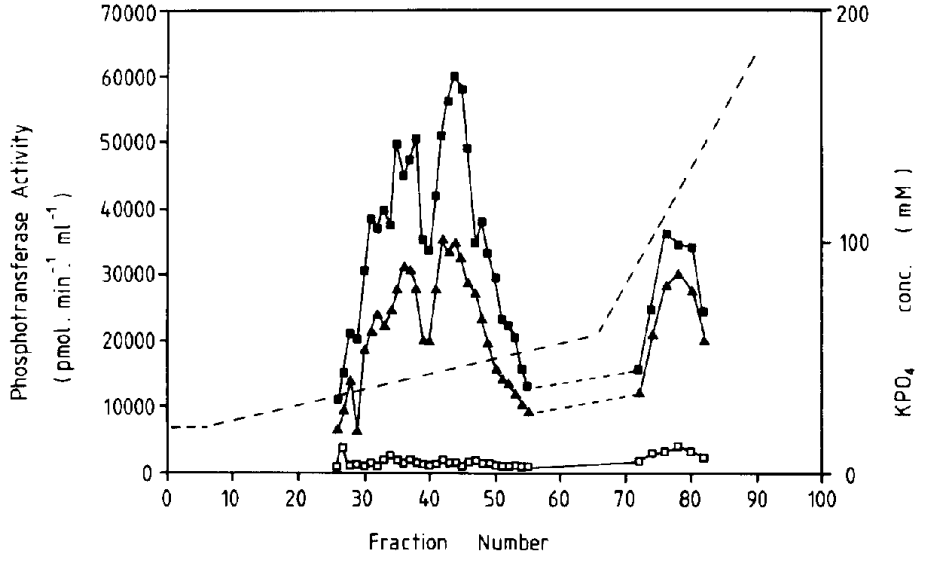

Fig.3. Hydroxyapatite separation of PK-C isoenzymes. Results shown are for reactions with calcium (□); calcium, plus 5 μg/ml phosphatidylserine (■) and calcium, plus 50 μM peptide PK-C(530–558) (▲).

chromatography [11], corresponding to the α, β and γ forms, were all activated by the peptide PK-C(530–558) (fig.3).

The peptide PK-C(530–558) stimulated autophosphorylation of both PK-C forms (80–83 kDa) resolved on 7.5% polyacrylamide gels (fig.4A). Autophosphorylation without calcium stimulated by the peptide PK-C(530–558) mimicked that by phorbol 1-myristate 2-acetate (PMA), although to a much lower extent (fig.4A). Autophosphorylation in the presence of calcium plus peptide or calcium plus phosphatidylserine resulted in phosphorylation of the 80 and 81 kDa forms (fig.4A) at the same sites (fig.4B). The peptide PK-C(530–558) therefore stimulates autophosphorylation in the same way as the previously described activators with calcium modulating autophosphorylation of one of the two sets of autophosphorylation sites.

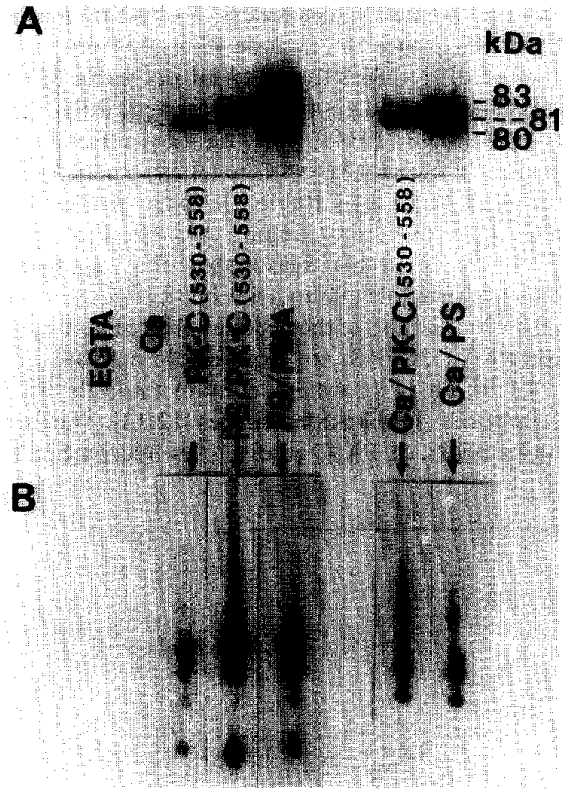

Fig.4. Autophosphorylation of PK-C. (A) Incubation in the presence of EGTA, Ca, or PS alone did not produce significant autophosphorylation. In the presence of the activators shown, an 80 kDa form of PK-C was always autophosphorylated. Higher molecular mass forms were also autophosphorylated with sizes ranging from 81 to 83 kDa depending on the activator. (B) Phosphopeptide mapping of the 80 kDa form with *Staphylococcus aureus* V8 protease [19] separated on a 15% polyacrylamide gel. The autoradiograph of the phosphopeptide maps for PK-C(530–558) and PS/PK-C(530–558) is overexposed 5-fold relative to the remaining phosphopeptide maps to facilitate comparison. Similar results were obtained in 4 separate experiments on different PK-C preparations.

Activation of PK-C by a fragment of its protein substrate-binding site is consistent with the pseudosubstrate regulatory model of this enzyme and may have important implications for other protein kinases. Inspection of the primary sequences [5] of the cAMP-dependent protein kinase, casein kinase-II, myosin light chain kinase, and the pp60^{src} family of tyrosine protein kinases within the region corresponding to Gly^{528} and Arg^{583} in PK-C reveals potential substrate-binding regions containing acidic or basic residues complementary to their known protein substrate specificity requirements. The overall model of a protein kinase catalytic domain indicated here (fig.1) is one of an ATP-binding core of approx. 180 residues and a carboxyl-terminal 60–80-residue flap responsible for binding the protein substrate in appropriate apposition to the ATP. Previous affinity labelling studies of the cAMP-dependent protein kinase with peptide substrate analogs [14,15] resulted in the labelling of residues within the ATP-binding region rather than the substrate-binding region identified here. However, this is readily explained, since the reactive groups were substituted in place of the serine phosphate acceptor site of the peptide substrate, these would be expected to face the ATP-binding region and therefore label it. Location of the primary protein substrate-binding site between Gly^{528} and Arg^{583} does not preclude contributions from residues on the ATP-binding region expected to be nearby in the three-dimensional structure. Indeed, the results of Levin et al. [16] with the cAMP-dependent protein kinase are an example of a point mutation in the ATP-binding domain modulating the interaction of the regulatory subunit, but not peptide substrates. The extent to which synthetic peptide analogs of the substrate-binding regions of other protein kinases can modulate their regulatory properties will depend on how well these peptides can mimic the structures present in the parent proteins in binding to pseudosubstrate structures. This may vary considerably just as synthetic peptides

corresponding to phosphorylation site sequences vary in their potency as substrates for particular protein kinases. The present results suggest that it may be possible to manipulate genetically protein kinase specificities by exchanging substrate-binding regions, analogous to the approaches taken to alter the hormone specificity of the tyrosine protein kinase receptor family [17].

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REFERENCES

[1] Nishizuka, Y. (1988) Nature, 334, 661–665.
[2] House, C., Wettenhall, R.E.H. and Kemp, B.E. (1987) J. Biol. Chem. 262, 772–777.
[3] Woodgett, J.R. and T. Hunter (1986) Eur. J. Biochem. 161, 177–184.
[4] House, C. and Kemp, B.E. (1987) Science 238, 1726–1728.
[5] Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42–52.
[6] Blake, C.C.F., Rice, D.F. and Cohen, F.E. (1988) Int. J. Peptide Protein Res. 27, 443–448.
[7] Taylor, S.S., Bubis, J., Toner-Webb, J., Saraswat, L.D., First, E.A., Buechler, J.A., Knighton, D.R. and Sowsdski, J. (1988) FASEB J. 2, 2677–2685.
[8] Pearson, R.B., Wettenhall, R.E.H. Means, A.R., Hartshorne, D.J. and Kemp, B.E. (1988) Science 241, 970–973.
[9] Yaciuk, P. and Shalloway (1986) D. Mol. Cell. Biol. 6, 2807–2819.
[10] Kikkawa U., Masayoshi, G., Koumoto, J. and Nishizuka, Y. (1986) Biochem. Biophys. Res. Commun. 135, 636–643.
[11] Huang, K.-P., Nakabayashi, H. and Huang, F.L. (1986) Proc. Natl Acad. Sci. USA 83, 8535–8539.
[12] Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233, 853–859.
[13] Ohno, S., Kawasaki, H., Imajoh, T., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T. and Hidaka, H. (1987) Nature 325, 161–166.
[14] Bramson, H.N., Thomas, N. Matsueda, R., Nelson, N.C., Taylor, S.S. and Kaiser, E.T. (1982) J. Biol. Chem. 257, 10575–10581.
[15] Miller, W.T. and Kaiser, E.T. (1988) Proc. Natl. Acad. Sci. USA 85, 5429–5433.
[16] Levin, L.R., Kuret, J., Johnson, K.E., Powers, S., Cameron, S., Michaeli, T., Wigler, . and Zoller, M.J. (1988) Science 240, 68–70.
[17] Riedel, H., Dull, T.J. Schlessinger, J. and Ullrich, A. (1986) Nature 324, 68–70.
[18] Knopf, J.L. Lee, M.H., Sultzman, L.A., Kriz, R.W., Loomis, C.R., Hewick, R.M. and Bell, R.M. (1986) Cell 46, 491–502.
[19] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.