Volume 329, number 1,2, 129–133 FEBS 12877 August 1993

Altered substrate selectivity of PKC-η pseudosubstrate site mutants

Lodewijk V. Dekker[a], Peter McIntyre[b] and Peter J. Parker[a]

[a]*Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, UK* and [b]*Sandoz Institute for Medical Research, 5 Gower Place, London, WC1E 6BN, UK*

Received 5 July 1993

Three protein kinase C (PKC)-η mutants were constructed in which the whole or part of the pseudosubstrate site was replaced with corresponding parts of the PKC-α pseudosubstrate site. The resulting chimaeric kinases were compared with wild-type PKC-η in their ability to phosphorylate a PKC-η peptide substrate or histone. Changes in the pseudosubstrate site of PKC-η are accompanied by changes in substrate selectivity, indicating that the substrate selectivity observed for PKC-η is at least in part due to its pseudosubstrate site.

Protein kinase C; Protein kinase C-η; Phosphorylation; Pseudosubstrate

1. INTRODUCTION

Protein kinase C (PKC) is a serine/threonine kinase implicated in a variety of processes such as growth, differentiation, exocytosis and modulation of ion channels and receptors [1–3]. Many PKC isotypes exist (indicated by the Greek symbols α, β, γ etc.) which have a related structure but differ in amino acid composition [4–7]. Each PKC isotype shows a different tissue distribution, responsiveness to extracellular signals and biochemical properties. Thus, it is conceived that the various PKC isotypes have separate functions in vivo.

Previously, we reported that PKC-δ, ε and η are poor histone kinases in vitro, whereas PKC-α, β and γ are known to be potent histone kinases [8–13]. This difference is due to a difference in apparent affinity of the various PKC isotypes for histone. It appears that a sequence element in the regulatory domain, the pseudosubstrate site, is of critical importance to this substrate selectivity [13]. The pseudosubstrate site has an amino acid sequence similar to a consensus PKC phosphorylation site and is thought to interact with the substrate binding site on the catalytic domain of PKC, thus keeping the kinase in an inactive configuration under resting conditions [14–17]. Upon activation by cofactors, such as Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (or phorbol ester), this interaction weakens, allowing substrates to compete with the pseudosubstrate site to gain access to the catalytic domain [13]. Deletion of the pseudosubstrate site from PKC-η generates a cofactor-independent kinase in which the restriction on histone kinase activity is no longer present [13].

A possible consequence of these observations is that the amino acid sequence of the pseudosubstrate site of a PKC isotype determines the choice of the substrate. We have investigated this possibility by replacing the whole or parts of the PKC-η pseudosubstrate site with corresponding parts of the pseudosubstrate site of PKC-α and testing whether the histone kinase activity of PKCα could thus be conferred upon PKC-η. Some of the chimaeric kinases show alterations in effector dependence and histone kinase activity in comparison to wild-type PKC-η.

Correspondence address: L.V. Dekker, Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC1A 3PX, UK. Fax: (44) (71) 269 3092.

Abbreviations: PKC, protein kinase C; PS, phosphatidyl serine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

2. EXPERIMENTAL

The rat PKC-η expression plasmid (pKS1-PKC-η), the PKC-η polyclonal antiserum and pep-η, a synthetic peptide based on the pseudosubstrate site of PKC-η with a serine-for-alanine substitution, have been described before [12]. Synthetic oligonucleotides were synthesized on an Applied Biosystems PCR-Mate [13]. PKC-η pseusosubstrate site mutants were created by polymerase chain reaction following a previously described strategy [13]. A 300 bp *Msc*1–*Msc*1 fragment carrying the appropriate mutation was ligated into pKS1-PKC-η expression construct. Orientation and sequence of the fragment were then confirmed by restriction analysis and sequencing using an oligonucleotide priming outside of the cloned fragment. PKC-η (or mutant) expression plasmids were transfected into COS cells by the $CaPO_4$ precipitation method [12,18]. At day 3 PKC-η (or mutant) protein was partially purified by extracting the cells in 1% Triton X-100 and 0–15% ammonium sulphate fractionation of the Triton-soluble material. The 0–15% ammonium sulphate precipitate was dissolved in buffer A (20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 10 mM benzamidine, 0.3% (v/v) β-mercaptoethanol, 0.02% Triton X-100) and further purified over a 1 ml HiTrap heparin–Sepharose column. Fractions were collected at 0.5 ml/min with a 2.5 min isocratic wash followed by a gradient of NaCl in buffer A (0–2 M in 10 min). For biochemical analysis, freshly isolated enzyme was used in each experiment. Routinely, 5 μl of enzyme was assayed for 6 min in 40 μl as described in [10,12,13]. Since PKC-η has been shown to be Ca^{2+}-

independent [12,13], all assays were performed in the absence of Ca^{2+}-ions. Pep-η (10 μg per assay) or histone III-S (Sigma, concentrations as indicated in the text or figure legends) were used as substrate. The amount of histone protein was determined as described in [19]. Incorporation was assessed by Cerenkov counting. Kinetic parameters were fitted to the Michaelis–Menten equation using the Enzfitter programme. SDS-PAGE was performed as described in [20]. For Western analysis, proteins were transferred to nitrocellulose according to [21] and incubated with the PKC-η polyclonal antiserum. Immunoblots were processed employing the ECL system (Amersham UK). Standard molecular biological procedures were performed as described in [18].

3. RESULTS AND DISCUSSION

The pseudosubstrate site of PKC-η is defined as the region surrounding Ala^{161} [12,22], whilst the PKC-α pseudosubstrate site is the region surrounding residue Ala^{25} [23,24]. Three PKC-η pseudosubstrate site mutants were constructed containing either the whole, the left or the right part of the pseudosubstrate sequence of PKC-α (Fig. 1A). The central alanine residue in the pseudosubstrate site was not altered in these studies. In PKC-η-Lα, amino acids 155–160 of PKC-η were replaced with amino acids 19–24 of PKC-α. Similarly in PKC-η-Rα, amino acids 162–167 of PKC-η were replaced with the amino acids 26–31 of PKC-α. In PKC-η-L/Rα the amino acids 155–167 of PKC-η were replaced with residues 19–31 of PKC-α.

COS cells were transfected with wild-type PKC-η or pseudosubstrate site mutants and after 3 days they were extracted in standard Triton-containing extraction buffer [12,13]. Fig. 1B shows that all three chimaeric PKC-η proteins were readily extractable in Triton X-100. However, around 30% of PKC-η and PKC-η-L/Rα protein remained in the Triton-insoluble fraction, whilst less than 10% of PKC-η-Lα or wild-type PKC-η was Triton-insoluble (Fig. 1B, lanes 1). The increase in Triton-insoluble (cytoskeleton-associated) PKC-η-Rα and PKC-η-L/Rα correlates with the activated status of these mutants (see below) and suggests that the effector dependence observed in vitro is a reflection of their dependence in vivo. A similar correlation has been observed previously for activating mutants of PKC-α [24].

Subsequently, Triton-soluble material was fractionated by ammonium sulphate precipitation. Like wild-type PKC-η, the three pseudosubstrate mutants described in the present study precipitate in 15% ammonium sulphate (Fig. 1B, lanes 2 and 3). Previously it has been shown that PKC-α (which is endogenously present in COS cells) is not present in this 0–15% ammonium sulphate fraction [12]. Thus the ammonium sulphate fractionation step provides a convenient way of separating (mutant) recombinant PKC-η from endogenous PKC-α and makes the protein suitable for biochemical analysis.

Ammonium sulphate precipitates were further purified by heparin–Sepharose chromatography. Each fraction was tested for its ability to phosphorylate pep-η (the synthetic peptide based on the pseudosubstrate site of PKC-η [12]) either in the presence or in the absence of the cofactors, PS and 12-*O*-tetradecanoylphorbol 13-acetate (TPA; Fig. 2). Like PKC-η itself, PKC-η-Lα, PKC-η-Rα and PKC-η-L/Rα were potent pep-η kinases. PKC-η and PKC-η-Lα were both dependent on PS and TPA for pep-η kinase activity. However, PKC-η-Lα showed slightly higher cofactor-independent pep-η kinase activity than the wild-type protein. By contrast PKC-η-Rα and PKC-η-L/Rα were largely PS- and TPA-independent (Fig. 2). These characteristics of PKC-η-Rα and PKC-η-L/Rα are similar to those observed before for a PKC-η pseudosubstrate site deletion mutant and for the free catalytic domain of PKC-η [13].

Histone is known to be a poor substrate for PKC-η

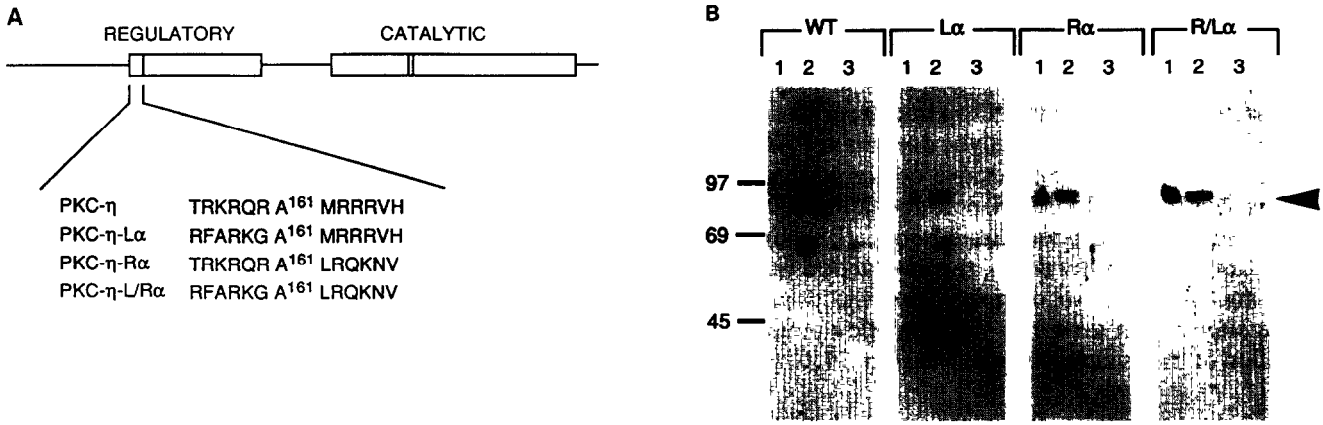

Fig. 1. (A) Structure of PKC-η and PKC-η pseudosubstrate site mutants. The variable and conserved domains in the PKC structure are indicated according to [7]. The amino acid sequence of the pseudosubstrate sites in each protein is expanded. (B) SDS-PAGE analysis of PKC-η (WT) and PKC-η pseudosubstrate site mutants. The Triton-insoluble fraction (lanes 1), 0–15% ammonium sulphate precipitate of the Triton-soluble fraction (lanes 2) and the 15–45% ammonium sulphate precipitate of the Triton-soluble fraction (lanes 3) were analyzed by 10% SDS-PAGE and Western blotting. For details see [12]. The position of molecular weight markers (Rainbow markers, Amersham) is shown on the left (kDa). The arrowhead indicates the position of PKC-η or PKC-η pseudosubstrate site mutants. The 66 kDa immunoreactive protein in the WT and Lα lanes is aspecific.

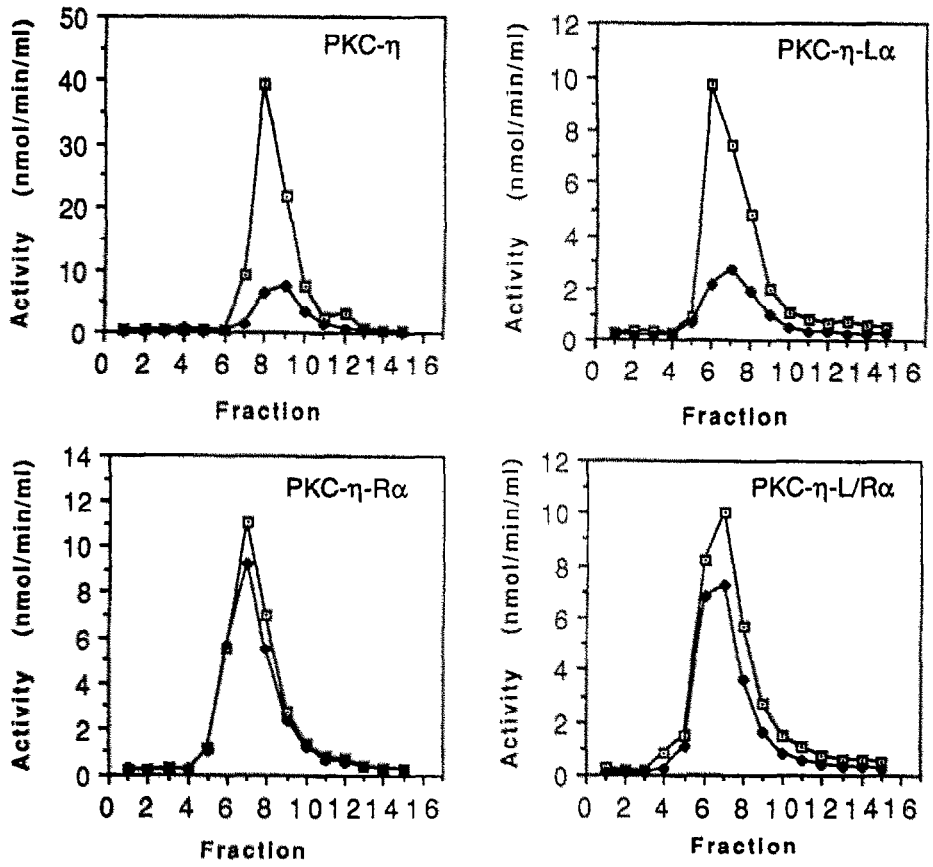

Fig. 2. Pep-η kinase activity of PKC-η and PKC-η pseudosubstrate mutants after heparin–Sepharose chromatography. COS cells were transfected with the indicated constructs and after 3 days PKC-η or mutant protein was partially purified by extraction in 1% Triton X-100, 0–15% ammonium sulphate fractionation and heparin–Sepharose chromatography. 5 μl of each fraction was tested for pep-η kinase activity in the absence (■) or in the presence (□) of PS and TPA. Comparable results were obtained in a second experiment using distinct enzyme preparations.

in vitro (apparent K_m 50 μg/ml [13]). We investigated whether the present PKC-η chimaeric kinases differed from wild-type PKC-η in their ability to phosphorylase histone. Fig. 3 shows the histone kinase activity of the pseudosubstrate site mutants in the presence of PS and TPA at different concentrations of histone. It appears that PKC-η-Rα and PKC-η-L/Rα are efficient histone kinases in comparison with PKC-η-Lα. Histone kinase activity of PKC-η-Rα and PKC-η-L/Rα did not depend on the presence of PS and TPA (Table I). The apparent K_m value for histone of PKC-η-Lα is comparable to that of wild-type PKC-η (50 μg/ml [13]) and 17-fold higher than that of PKC-η-Rα or PKC-η-L/Rα (Table I). Previously we observed that the catalytic domain of PKC-η and a PKC-η pseudosubstrate site deletion mutant showed a similar increased (25-fold) affinity for histone in comparison to wild-type PKC-η [13]. V_{max} values of PKC-η- Lα, PKC-η-Rα and PKC-η-L/Rα are similar to each other (Table I).

The results described here indicate the importance of the pseudosubstrate site for substrate selectivity of PKC-η (see [13]). Changing the pseudosubstrate site alters the ability of the resulting mutant to phosphorylate histone by altering the apparent affinity for the

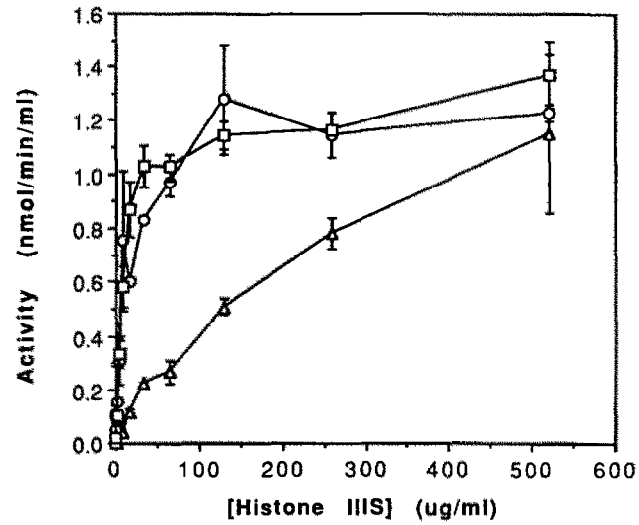

Fig. 3. Concentration dependence of histone phosphorylation by PKC-η pseudosubstrate mutants. 5 μl of the peak fraction of each enzyme from Fig. 2 was tested for histone kinase activity in the presence of PS and TPA. Mean and range of duplicate incubations is indicated. (△) PKC-η-Lα; (□) PKC-η-Rα; (○) PKC-η-L/Rα. Comparable results were obtained in a second experiment using distinct enzyme preparations.

substrate. Thus in wild-type PKC-η the pseudosubstrate site maintains the restriction on histone kinase activity even in the presence of PS and TPA. It is pertinent to note that the pseudosubstrate site mutants tested show both loss of substrate selectivity and enhanced cofactor-independent activity or do not show any change at all. We have not yet encountered a situation in which changes in substrate selectivity occur in the absence of changes in cofactor dependence. Therefore it is likely that both cofactor dependence and substrate selectivity are inseparable consequences of the same phenomenon, i.e. the interaction between the pseudosubstrate site and the substrate binding site on the catalytic domain (pseudosubstrate site inhibition).

The present findings allow a description of the residues relevant for the pseudosubstrate site inhibition and, by inference, of the residues interacting with the substrate binding site on the catalytic domain. Since replacing the 6 residues upstream of Ala^{161} with their PKC-α sequence did not affect histone kinase activity or cofactor dependence of PKC-η, it seems that these residues are not of critical importance. However, Arg^{158}, which did not change in the present study because it is the same in PKC-α and PKC-η, could still be important for pseudosubstrate inhibition. It was observed before that this residue is important for peptides to compete with the pseudosubstrate site in PKC-α[15,16]. Replacing the 6 residues downstream of Ala^{161} induces dramatic changes in cofactor dependence and substrate selectivity of the kinase. Thus it appears that these residues are of critical imporatance for the pseudosubstrate site inhibition in PKC-η. It is difficult to attribute the changes to a particular residue, however, converting Arg^{164} into Gln could be the modification mainly responsible for the functional changes in the kinase.

We have reported previously that pep-α (a synthetic peptide based on the pseudosubstrate sequence of PKC-α with a serine for alanine substitution) is a poor substrate of PKC-η due to very low apparent affinity [12]. The present findings provide some explanation for this observation. The characteristics of PKC-η-Rα and PKC-η-L/Rα indicate that residues in the pseudosubstrate site of PKC-α do not interact strongly with the PKC-η substrate binding domain. Since the sequence of pep-α is in fact based on these residues, it is nor surprising that the competitive capacity of pep-α is not strong enough to overcome the pseudosubstrate site inhibition retained in activated PKC-η, and thus behaves as a poor substrate in vitro.

In conclusion, our data indicate that the pseudosubstrate site of PKC-η is of critical importance to be observed substrate selectivity of this kinase and, in particular, they indicate the relevance of the amino acids downstream of Ala^{161}. The observation that loss of substrate selectivity is always parallelled by a loss in cofactor dependence indicates that both are possibly consequences of the same phenomenon, the pseudosubstrate site inhibition. This is consistent with the idea that (for PKC) substrate phosphorylation essentially consists of two components. First a weakening (but not loss) of the interaction between pseudosubstrate site and substrate binding domain is induced by PS and TPA. Then substrate competes with the pseudosubstrate site for occupation of the substrate binding domain. This provides an attractive working hypothesis to further investigate substrate selectivity at the molecular level.

Table I

K_m and V_{max} values for histone phosphorylation by PKC-η pseudosubstrate mutants

	K_m (μg/ml)		V_{max} (nmol/min/ml)	
	−PS/TPA	+PS/TPA	−PS/TPA	+PS/TPA
PKC-η-Lα	ns	178	0.6*	1.2
PKC-η-Rα	13	11	1.4	1.3
PKC-η-L/Rα	6.8	11	1.2	1.2

Values were calculated on the basis of Fig. 3. For comparison, the values previously obtained for intact PKC-η and for the PKC-η catalytic domain have been included (see [13]). ns, no saturation of the PKC-Lα mutant by histone under these conditions.
*0.6 nmol/min/ml is the activity at 520 μg/ml histone.

REFERENCES

[1] Nishizuka, Y. (1984) Nature 308, 693–698.
[2] Kikkawa, U. and Nishizuka, Y. (1986) Ann. Rev. Cell Biol. 2, 149–178.
[3] Shearman, M., Sekiguchi, K. and Nishizuka, Y. (1989) Pharmacol. Rev. 41, 211–237.
[4] Nishizuka, Y. (1988) Nature 334, 661–665.
[5] Nishizuka, Y. (1992) Science 258, 607–614.
[6] Parker, P.J., Kour, G., Marais, R., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1–11.
[7] Parker, P.J. (1992) in: Protein kinase C: Current Concepts and Future Perspectives (Lester, D.S. and Epand, R.M. eds.) Ellis Horwood, New York.
[8] Schaap, D., Parker, P.J., Bristol, A., Kriz, R. and Knopf, J. (1989) FEBS Lett. 243, 352–357.
[9] Schaap, D. and Parker, P.J. (1990) J. Biol. Chem. 265, 7301–7307.
[10] Schaap, D., Hsuan, J., Totty, N. and Parker, P.J. (1990) Eur. J. Biochem. 191, 431–435.
[11] Olivier, A.R. and Parker, P.J. (1991) Eur. J. Biochem. 200, 805–810.
[12] Dekker, L.V., Parker, P.J. and McIntyre, P. (1992) FEBS Lett. 312, 195–199.
[13] Dekker, L.V., McIntyre, P. and Parker, P.J. (1993) J. Biol. Chem. (in press).
[14] House, C. and Kemp, B.E. (1987) Science 238, 1726–1728.
[15] House, C. and Kemp, B.E. (1990) Cell. Signalling 2, 187.
[16] Kemp, B.E., Pearson, R.B. and House, C.M. (1991) Methods Enzymol. 201, 287–304.
[17] Gschwendt, M., Kittstein, W. and Marks, F. (1991) Trends Biochem. Sci. 16, 167–169.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.

[20] Laemlli, U.K. (1970) Nature 227, 680–685.
[21] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[22] Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) J. Biol. Chem. 265, 22434–22440.
[23] Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. and Ullrich, A. (1986) Science 233, 853–859.
[24] Pears, C., Kour, G., House, C., Kemp, B.E. and Parker, P.J. (1990) Eur. J. Biochem. 194, 89–94.