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Studies on the primary sequence requirements for PKC-α, -β_1 and -γ peptide substrates

Richard M. Marais*, Oanh Nguyen, James R. Woodgett and Peter J. Parker

Ludwig Institute for Cancer Research, Courtauld Building, 91 Riding House Street, London W1P 8BT, UK

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The substrate specificity of purified PKC-α, -β and -γ has been investigated. A series of synthetic peptides based upon the sequence surrounding serine-7 in glycogen synthase were generated and used to determine the basic residue requirements of these PKC isotypes. While PKC-α and -β are indistinguishable in their phosphorylation of these peptides, PKC-γ shows a distinct specificity profile for these synthetic substrates.

Protein kinase C; Kinase specificity; Synthetic peptide

1. INTRODUCTION

Protein kinace C (PKC) is a ubiquitous serine- and threonine-specific protein kinase which occupies a pivotal role in signal transduction (for recent reviews see [1-3]). PKC is distinguished from other kinases in that it is dependent on phospholipids, Ca^{2+} and diacylglycerol (DG) for activation. PKC is the major cellular receptor for the tumour promoting phorbol esters [4] and has therefore been implicated in neoplastic transformation of cells. The cloning of PKC has revealed that this kinase exists as a family of at least 7 closely related proteins [1,3]. Within this family, PKC-α, -β_1, -β_2 and -γ are more closely related to each other than they are to PKC-δ, -ϵ and ζ. PKC-α, -$\beta_{(1+2)}$ and -γ mixtures have been purified from numerous sources. The purified PKC-α, -$\beta_{(1+2)}$ and -γ isotypes have been separated from each other by hydroxylapatite column chromatography and this has allowed biochemical analysis of the individual isotypes [5-9]. These isotypes all fall into the broad class of Ca^{2+}/phospholipd- and DG-dependent kinases and are all activated by phorbol esters. Studies have revealed also that there are subtle differences in their activation kinetics which may be indicative of different physiological roles for each isotype (see for example [5,7,8]).

Most comparative studies on purified PKC isotypes have employed polypeptide substrates (see for example [5-9]). However, for a more detailed analysis of substrate requirements it is desirable to employ synthetic peptides that can be manipulated readily. Studies along these lines have been carried out for undefined mixtures of PKC but not for separated isotypes [10-14]. We report here on an analysis of the substrate requirements of the PKC-α, -β_1 and -γ isotypes using synthetic peptide substrates.

Correspondence and present address: P.J. Parker, Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

2. MATERIALS AND METHODS

Chemical and biochemical reagents were as described previously [8] except where indicated below. PKC-α, -β_1 and -γ were isolated from bovine brain and separated by hydroxylapatite chromatography as previously described; the isotypes were judged pure when analysed by SDS-PAGE and immunoblot analysis using isotype-specific antisera [8].

Assays of PKC isotypes were as described previously [8] except using the various substrates indicated in the text, tables and figures. Reactions were terminated by one of two methods; either (i) to 20 μl of reaction mixture, 20 μl of Laemmli sample buffer [15] was added and the phosphopeptides were separated from free ATP on 25% polyacrylamide gels; following autoradiography, the amount of [^{32}P]phosphate incorporated into peptide was determined by Cerenkov counting; or (ii) samples (25 μl) were spotted onto P81 paper and washed in 30% (v/v) acetic acid for 2 × 10 min; incorporated [^{32}P]phosphate was determined by Cerenkov counting. One set of analyses with all synthetic peptide substrates was carried out by SDS-PAGE (method i) and the results were entirely consistent with data employing the P81 procedure (method ii). The latter was subsequently used for all further studies except for those with peptides containing only one basic residue, such as peptide R^6S^9 (see below) which did not bind to P81 efficiently. A unit of enzyme activity is defined as the amount incorporating 1 μmol of phosphate into the substrate/min at 30°C under the conditions described.

Peptides GS1-10 EGFR-1, MBP-1 and $pp60^{src-1}$ were as described previously [10]. GS1-10-related peptides were synthesised on an automated Applied Biosystems 430A peptide synthesiser according to the manufacturer's instructions. The peptides were purified by reverse phase HPLC, using a C_{18} column equilibrated in 0.1% TFA and attached to a Beckman Gold System; peptides were eluted using a linear gradient from 0% to 60% acetonitrile (Rathburn). Purified peptides were lyophilised, resuspended in 0.2 M Hepes, pH 7.5, 2 mM EGTA and used directly. In order to reduce cost during synthesis, the peptides with identical C-termini were synthesised in a single batch, from residues 13-8. The beads were then split 4 ways and each peptide further extended individually. Peptide nomenclature is as follows: in

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each case the position of the basic arginine residue and the phosphorylated serine residue is given, e.g. NH_2-GGRLARALS-VAAG-COOH is abbreviated to $R^3R^6S^9$.

Kinetic analysis of data was carried out using the Enzfitter program (Elsevier Biosoft). In the case of peptides with arginine residues at positions 11 and 12 substrate inhibition was observed for PKC-α; therefore for these peptides optimal activity and the concentrations giving half-maximal activity ($S_{0.5}$) are described in place of V_{max} and K_m values, respectively (these values were determined graphically).

3. RESULTS AND DISCUSSION

3.1. *Synthetic peptides corresponding to known PKC substrates*

Peptides corresponding to the major PKC phosphorylation sites in the epidermal growth factor receptor, $pp60^{c-src}$, myelin basic protein and glycogen synthetase have all been shown to be substrates for undefined mixtures of PKC [10]. These peptides were therefore used to analyse the substrate specificity of the PKC isotypes and were all found to be phosphorylated by PKC-α, -β_1 and -γ (Table I). For PKC-α, the range of apparent K_m values with these peptide substrates varied from 15 μM to 220 μM. Although these values differ from published data (Table I), the discrepancies probably reflect different assay conditions and variations in PKC isotype content. The V_{max} values with these peptides varied from 0.56 to 2.2 μmol/min/mg for PKC-α. PKC-β_1 displayed similar kinetics to PKC-α for these peptides, the apparent K_m values being within two-fold of the values for PKC-α; however, the V_{max} values for PKC-β_1 were consistently 2–4-fold lower than the values for PKC-α. The reduced activity of PKC-β_1 compared to PKC-α has been noted previously for polypeptide substrates [5–8].

In contrast to PKC-β_1, PKC-γ displayed a significant difference in substrate specificity compared to PKC-α. The apparent K_m values of PKC-γ were within 2.5-fold of the values for PKC-α for peptides EGF-R3, p60-1 and MBP-1, but for GS1-10, the difference was greater than 10-fold. The V_{max} values of PKC-γ were approximately 2-fold lower than the values of PKC-α even for GS1-10. While the differences in V_{max} values are consistent with observations made for protein substrates, no substrate has been described previously in which there is such a large difference in K_m between the isotypes [5–8].

3.2. *Derivatives of the GS1-10 peptide*

PKC mixtures have been shown to phosphorylate serine or threonine residues flanked by basic amino acids [10–14]. Peptide GS1-10 contains only a single basic residue (R^4) in addition to the N-terminus and therefore does not conform to the PKC substrate 'consensus'. Nevertheless, this peptide is a substrate for PKC and is phosphorylated exclusively on serine residue 7 by PKC [11]; R.M.M., Ph.D. thesis). In order to investigate the basis of the differential substrate characteristics of the PKC isotypes with this peptide, the effect of the number and position of the basic residues on this peptide were altered. Peptides based on the GS1-10 sequence were synthesised, with S^3 and T^5 being replaced by alanine residues to avoid the possibility of secondary phosphorylation events which would complicate the analysis. The N- and C-termini of this family of peptides were also extended by additional residues to accommodate basic residues at these positions so that their influence on substrate specificity could be evaluated.

In initial studies, PKC-α and -β_1 were found to display indistinguishable characteristics towards these peptides (data not shown) and therefore for clarity we have detailed here the PKC-α and PKC-γ data (Table II).

3.3. *PKC-α requirements for substrate specificity*

PKC-α showed an apparent K_m value for peptide GS1-10 of 53 μM and a V_{max} of 2.2 units/mg. The peptide which most closely resembles GS1-10 with respect to the positioning of basic residues is peptide $R^3R^6S^9$. In this peptide, R^6 occupies the position corresponding to R^4 in GS1-10 and R^3 the position corresponding to the GS1-10 N-terminus (which is predicted to be positively charged). However, $R^3R^6S^9$ is a less efficient PKC-α substrate than GS1-10, with a lower V_{max} and a higher

Table I

Phosphorylation of physiological substrate peptides by PKC-α, -β_1 and -γ

	EGF-R3		p60-1		MBP-1		GS1-10	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
PKC-α	15 ±2.9	0.56±0.06	220±21	1.5 ±9.1	82± 7.4	0.89±0.04	53± 10	2.2 ±0.09
PKC-β_1	7.6±1.7	0.22±0.05	280±56	0.87±0.10	69± 7.8	0.21±0.01	44± 5.3	0.98±0.02
PKC-γ	15 ±3.3	0.23±0.03	260±16	0.75±0.02	146±14	0.37±0.06	550±110	1.2 ±0.10
Previous*	48[1]	0.990	48[1]	0.560	22[2]	0.213	40.3[3]	1.60

The apparent K_m and V_{max} values for the three PKC isotypes are indicated. The K_m units are μM and the V_{max} values expressed as μmol/min/mg. The assays with these peptides were performed in standard assay buffer (lacking histone; total volume = 20 μl) and the reaction were terminated (after 3 min, 30°C) by the addition of Laemmli sample buffer as described in section 2. EGF-R3: VRKTLRRL$_{NH_2}$; p60-1 GSSKSKPKDPSQRRSLE$_{NH_2}$; MBP-1 GAGRGLSLSRFSWGA; GS1-10 PLSRTLSVAA (underlined residues are the major target sites).

*The previously published values for PKC mixtures come from: [1][10], [2][13], [3][11]

apparent K_m (Table II). Thus the changes which were made in converting GS1-10 to $R^3R^6S^9$ affect both the apparent affinity of this peptide for PKC-α and the turnover rate. This suggests that it is not only the basic residues which are involved in substrate recognition by PKC-α but that other residues can also contribute. Nevertheless, $R^3R^6S^9$ is a substrate for PKC-α and is therefore a useful model peptide on which to base a study which examines how the positioning of basic residues with respect to the target residue affects PKC isotype substrate recognition. Removal of the R residue at position 3 results in peptide R^6S^9 which has almost identical kinetics as a PKC-α substrate as $R^3R^6S^9$. This suggests that R^3 does not have a major role in the ability of PKC-α to recognise peptide $R^3R^6S^9$. However, R^6 is important for PKC-α substrate interaction, since for example peptide R^5S^9 is not a PKC-α substrate (the synthetic peptide lacking any R residues was insoluble in aqueous buffers and therefore could not be tested).

Inclusion of a second basic amino acid proximal to R^6 does not significantly affect the kinetic parameters of the peptide for PKC-α as seen in peptides $R^5R^6S^9$ and $R^6R^7S^9$. Thus the presence of an R residue at position 6 appears to be the most important N-terminal residue for PKC-α substrate recognition; the presence of a second basic residue at only one of the positions 3, 5 or 7 having a minimal effect on substrate recognition. However, positions 3 and 5 together have a synergistic effect on substrate recognition, since peptide $R^3R^5R^6S^9$ has a V_{max} 3.3-fold higher and an apparent K_m value 4-fold lower than R^6S^9. From this we conclude that in the presence of an R residue at position 6, basic residues at either position 3 or 5 alone do not have a significant effect on substrate recognition by PKC-α; however, when both positions 3 and 5 are occupied by R residues, there is a significant co-operative contribution to substrate recognition. Similarly the combination of R^3 and R^7 with R^6 produces a substrate with lower apparent K_m and higher V_{max} (not shown).

C-terminal residues also influence the recognition of substrates by PKC-α. Thus, peptide $R^6S^9R^{12}$ has a 2.9-fold higher V_{max} and a 2.3-fold lower apparent K_m than R^6S^9. This effect of R^{12} on peptide recognition is further enhanced for those peptides with more than one basic residue N-terminal to the target site. Thus $R^6R^7S^9R^{12}$, $R^5R^6S^9R^{12}$ and $R^3R^6S^9R^{12}$ have significantly higher V_{max} values and very much lower apparent K_m values than the corresponding peptides lacking R^{12}. As mentioned above, peptides containing only two N-terminal basic amino acids were marginally better substrates than peptides with one N-terminal basic residue (see above). By contrast, peptides with two N-terminal basic residues in the presence of R^{12} have apparent K_m values of the order of 10–50-fold lower than peptides with only a single N-terminal basic residue. It therefore appears that the C-terminal basic residue is able to synergise with multiple N-terminal basic residues more efficiently than with single N-terminal basic residues. There is nonetheless a requirement for N-terminal basic residues since the peptide S^9R^{12} is not phosphorylated by PKC-α indicating that a basic residue at position 12 is not sufficient on its own to direct PKC-α substrate recognition.

Increasing the number of basic residues C-terminal to the target had unusual effects on PKC-α substrate kinetics for this series of peptides. Peptides containing

Table II

PKC-α and -γ activities towards synthetic peptide substrates

	PKC-α		PKC-γ	
Peptide	K_m	V_{max}	K_m	V_{max}
R^6S^9	180 ± 43	0.13 ± 0.01	N/S	
$R^6R^7S^9$	350 ± 250	0.32 ± 0.01	N/S	
$R^5R^6S^9$	330 ± 69	0.23 ± 0.03	N/S	
$R^3R^6S^9$	190 ± 2.0	0.13 ± 0.00	N/S	
$R^3R^5R^6S^9$	46 ± 2.6	0.44 ± 0.01	170 ± 24	0.19 ± 0.02
$R^6S^9R^{12}$	80 ± 19	0.40 ± 0.02	170 ± 12	0.26 ± 0.01
$R^6R^7S^9R^{12}$	7.9 ± 0.9	0.69 ± 0.02	16 ± 5.0	0.50 ± 0.05
$R^5R^6S^9R^{12}$	12 ± 1.6	0.55 ± 0.02	38 ± 8.0	0.51 ± 0.04
$R^3R^6S^9R^{12}$	19 ± 4.0	0.52 ± 0.03	110 ± 11	0.42 ± 0.02
$R^6S^9R^{11}R^{12}$	7.6 ± 2.2*	0.57 ± 0.11*	20 ± 17	0.34 ± 0.01
$R^6R^7S^9R^{11}R^{12}$	3.1 ± 1.6*	0.59 ± 0.02*	3.6 ± 0.5	0.31 ± 0.01
$R^5R^6S^9R^{11}R^{12}$	2.5 ± 0.8*	0.43 ± 0.05*	5.4 ± 0.9	0.32 ± 0.02

Peptides were employed as substrates for PKC-α and PKC-γ as described in section 2. Values shown are means and SD for one of three similar determinants. Apparent K_m values are in μM units and V_{max} values are expressed as μmol/min/mg. N/S, not saturating; low activity is observed with PKC-γ as shown in Fig. 1 for R^6S^9.

*Due to substrate inhibition (see Fig. 2) these values are not K_m and V_{max} values but substrate concentrations giving half-optimum activity (μM) and optimum activity values (μmol/min/mg)

basic residues at positions 11 and 12 were all phosphorylated by PKC-α with $S_{0.5}$ values between 2 and 8 μM, but in all cases the PKC-α activity was inhibited at high peptide concentrations (see for example Fig. 2). It was therefore not possible to obtain V_{max} values for these peptide substrates, and optimum activity values are shown in Table II. The inhibition observed would not appear to be non-specific since: (i) following synthesis, deprotection and precipitation, the peptides were all repurified by HPLC (as described in section 2); and (ii) no substrate inhibition is observed with PKC-γ (see below). The reason for this inhibition of PKC-α (and PKC-β; data not shown) is not clear.

3.4. *PKC-γ substrate specificity*

PKC-γ displayed very low rates of phosphorylation with peptides R^6S^9, $R^6R^7S^9$, $R^5R^6S^9$ or $R^3R^6S^9$ and it was not possible to saturate the activity (Table II and see Fig. 1). However, PKC-γ was able to phosphorylate the peptide $R^3R^5R^6S^9$, indicating that in this model system PKC-γ can recognise substrates with three basic residues N-terminal to the target site in the absence of C-terminal basic residues (see below).

PKC-γ was able to phosphorylate peptides with both C- and N-terminal basic residues. Peptide $R^6S^9R^{12}$ was phosphorylated by PKC-γ with similar kinetics (the V_{max} is slightly higher) to $R^3R^5R^6S^9$, indicating that in the presence of a C-terminal basic residue, the requirement of PKC-γ for multiple N-terminal basic residues is in part relieved. However, N-terminal basic residues are still required (in the presence of a single C-terminal basic residue) since peptide S^9R^{12} is not a PKC-γ substrate; in contrast, the peptide $S^9R^{11}R^{12}$ is a substrate for PKC-γ albeit with a high apparent K_m and a low V_{max} (data not shown).

Peptide $R^6R^7S^9R^{12}$ is an excellent PKC-γ substrate, with an apparent K_m 10.7-fold lower and a V_{max} 2-fold higher than $R^6S^9R^{12}$. Evidently the inclusion of an additional basic residue to peptide $R^6S^9R^{12}$ creates a good PKC-γ substrate. The precise location of the pair of N-terminal basic residues has a significant influence on K_m. This can be seen by comparing peptides $R^6R^7S^9R^{12}$, $R^5R^6S^9R^{12}$, and $R^3R^6S^9R^{12}$; the apparent K_m values for PKC-γ of $R^5R^6S^9R^{12}$ and $R^3R^6S^9R^{12}$ are 2.4- and 7-fold higher than $R^6R^7S^9R^{12}$, respectively, although the V_{max} values of these peptides are similar (Table I). Interestingly the peptide $R^5S^9R^{12}$ was not a good PKC-γ substrate and behaved as the R^6S^9 (see Fig. 1). Thus PKC-γ is sensitive to the precise positioning of basic residues surrounding the target site in substrate recognition.

Inclusion of a second C-terminal basic residue further improves the efficiency with which PKC-γ is able to phosphorylate these peptides (Table II). Unlike PKC-α, substrate inhibition is not observed for PKC-γ with these peptides (see Fig. 2). Thus peptide $R^6S^9R^{11}R^{12}$ has an 8.6-fold lower apparent K_m and similar V_{max} to $R^6S^9R^{12}$ (Table II). Inclusion of more than one N-terminal basic residue further increases the apparent affinity o this class of peptides for PKC-γ, with peptides $R^6R^7S^9R^{11}R^{12}$ and $R^5R^6S^9R^{11}R^{12}$ having 5.6- and 3.7-fold lower K_m values, respectively, than

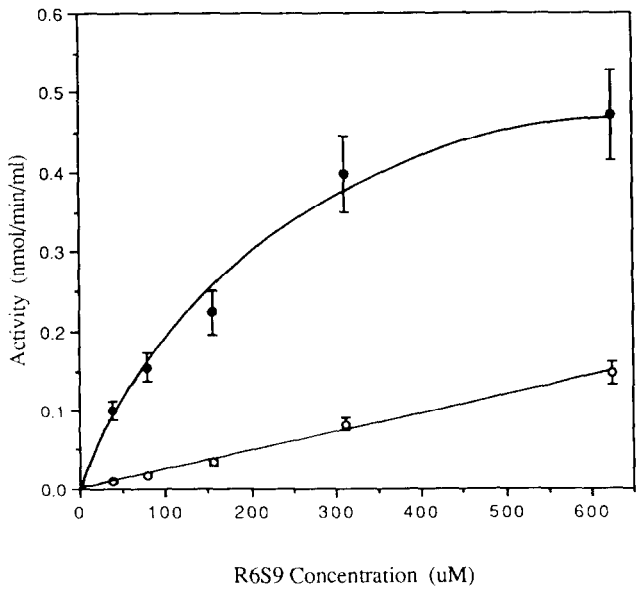

Fig. 1. A comparison of PKC-α and PKC-γ activity with the peptide substrate R^6S^9. Peptide R^6S^9 was assayed with purified PKC-α (•—•) and PKC-γ (○—○) as described in section 2. Data points are means of duplicate observations from a single set of analyses and are representative of 3 separate analyses.

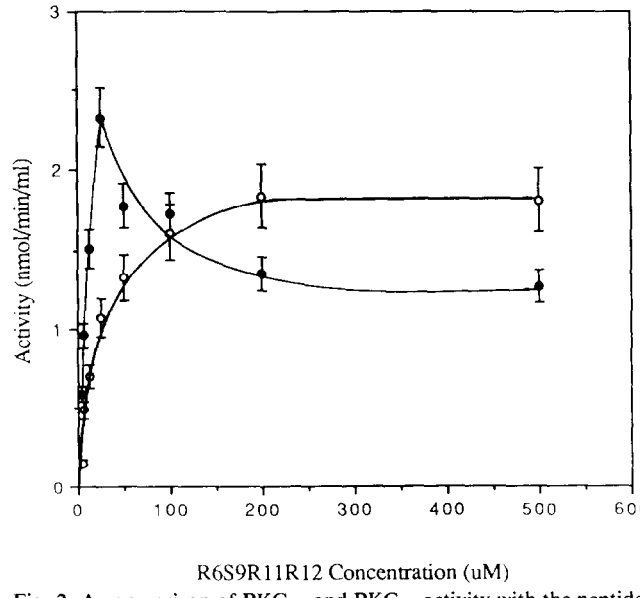

Fig. 2. A comparison of PKC-α and PKC-γ activity with the peptide substrate $R^6S^9R^{11}R^{12}$. Peptide $R^6S^9R^{11}R^{12}$ was assayed with purified PKC-α (•—•) and PKC-γ (○—○) as described in section 2. Data points are means of duplicate observations; this is one of three sets of analyses with this substrate. Similar substrate inhibition of PKC-α is seen with peptides $R^6R^7S^9R^{11}R^{12}$ and $R^5R^6S^9R^{11}R^{12}$.

$R^6S^9R^{11}R^{12}$ (Table II). The K_m of $R^6R^7S^9R^{11}R^{12}$ is the lowest for this class of peptides for PKC-γ and once again shows how the position of N-terminal basic residues affects the interaction of PKC-γ with these peptide substrates.

4. CONCLUSIONS

The different kinetics with which PKC-γ phosphorylates the GS1-10 peptide compared to PKC-α or -β has provided a model in which to investigate and compare the substrate specificity of these PKC isotypes. Based upon the GS1-10 sequence a series of substrate peptides have been synthesised that reveal differences in the primary structural determinants for these isotypes. Both PKC-α and -β isotypes display a preference for a basic residue at position 6 relative to the target serine at 9 and substantially lower apparent K_m values when a single basic residue C-terminal to the target serine is included at position 12. It can be concluded that within the context of the parent peptide, PKC-α and -β display the same basic residue requirements. By contrast, PKC-γ showed a distinct pattern of specificity, showing selectivity for peptide substrates that retain a basic residue C-terminal to the phosphorylated serine. In the absence of a C-terminal basic residue PKC-γ phosphorylation could not be saturated within the limits of peptide solubility unless multiple N-terminal basic residues were also present. However, as exemplified in Fig. 1, PKC-γ was able to phosphorylate these peptides albeit at a low rate. It is therefore possible that the V_{max} values of PKC-γ for these peptides may not differ substantially from those determined for PKC-α (or PKC-β) but that the apparent K_m values are significantly different. This is consistent with the 'parent' peptide GS1-10 which showed a 10-fold higher K_m value for PKC-γ relative to -α or -β.

It is anticipated that these differences in primary structural determinants for synthetic substrates for PKC-α, -β and -γ relate to the selective roles played by these kinases in vivo. Further studies with physiological substrates will no doubt define the consequences of this specificity.

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