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Biochemical properties of rat protein kinase C-η expressed in COS cells

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Using a PKC-ε cDNA probe a cDNA for PKC-η has been cloned from a rat lung cDNA library. When expressed in COS cells, rat PKC-η appeared as an 84 kDa protein. PKC-η expressed in COS cells, was solubilized by 1% Triton X-100 and purified away from the endogenous PKC-α by ammonium sulphate fractionation. The activity of this PKC-η preparation was characterized with respect to cofactor dependence and substrate specificity. Various PKC pseudosubstrate peptides are phosphorylated by PKC-η in a phospholipid and TPA-dependent but calcium-independent manner. The polypeptide histone IIIS is a poor substrate.

Protein kinase C; Protein kinase C-η; Phosphorylation

1. INTRODUCTION

Protein kinase C is a serine/threonine protein kinase, implicated in a variety of cellular processes, e.g. proliferation, differentiation, exocytosis and modulation of ion channel and receptor function [1,2]. It was originally identified as a Ca^{2+}, phospholipid and diacylglycerol/phorbol ester-dependent protein kinase with a wide substrate specificity [3]; however recent molecular cloning has shown that PKC exists as a family of closely related isotypes (referred to as PKC-α, β, γ, δ, ε, ζ and η; reviewed in [4–6]). PKC-α, β and γ contain 4 domains of high homology (C_{1-4}) that are surrounded by 5 variable domains (V_{1-5}) [7]. PKC-δ, ε, ζ and η have a similar structure but lack C_2 and have an extended V_1 [8–13]. Also biochemically, a subdivision of the PKC family is apparent. PKC-α, β and γ are Ca^{2+}-dependent kinases that can use the polypeptide histone IIIS as a substrate [4–7]. PKC-δ and ε are poor histone IIIS kinases [8–10]. Using synthetic peptides based on their pseudosubstrate sequence, it was established that these latter PKCs are Ca^{2+}-independent [9,10]. PKC-η has been cloned and its expression and phorbol ester binding has been described in a number of studies [11–14]. However, the characteristics of PKC-η kinase activity are largely unknown. In order to investigate the biochemical properties of PKC-η, we have isolated a cDNA clone for rat PKC-η. The biochemical properties of the encoded PKC-η gene product have been assessed following expression in COS cells.

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

2. EXPERIMENTAL

2.1. *Cloning of PKC-η*

A size-fractionated rat lung cDNA library (lambda ZAP II, Stratagene) was constructed and 4×10^5 plaques were screened with a full length PKC-ε cDNA probe [10,15]. A 3 kb cDNA clone was identified, representing the rat homologue of mouse and human PKC-η [11–13]; 200 bp of 5' sequence, including the starting codon, was obtained by a slightly adapted RACE protocol [16].

2.2. *Generation of peptide antiserum and Western blotting*

A synthetic peptide based on the C-terminus of PKC-η (QDEFRNFSYVSPELQ) was used to raise an antiserum (PPA022) as described in [17]. For Western analysis, proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters [18]. Filters were incubated with antiserum (1:2000) and processed as described in [19]. Total proteins were silver stained according to Meril [20].

2.3. *Transfection and extraction of COS-1 cells and ammonium sulphate precipitation*

An *Eco*RI–*Xba*I fragment containing the full coding sequence of PKC-η was cloned into the expression vector pKS1 [pKS1 is a derivative of CDM8 [21] with a pUC backbone, constructed by P.M., Katy Smith and Mark Rolfe in the laboratory of George Stark (ICRF, London)]. pKS1 or pKS1-PKC-η plasmid DNA was introduced into COS-1 cells by calcium phosphate precipitation [15]. At day 3 after transfection, cells were homogenized at 4°C in 0.5 ml of lysis buffer [10], incubated for 10 min at 4°C and centrifuged (10 min, 12,000 rpm, 4°C). To the supernatant an equal volume of a 30% (w/v) ammonium sulphate solution was added dropwise at 4°C, mixing every 2 drops. The mixture was incubated for 10 min at 4°C and cleared by centrifugation (10 min, 12,000 rpm, 4°C). The resulting pellet was resuspended in 100 μl of enzyme dilution buffer (20 mM HEPES pH 7.5; 2 mM EGTA; 0.02% Triton X-100; 0.3% (v/v) β-mercaptoethanol). In phorbol ester down regulation experiments, cells were harvested directly in 4 × concentrated Laemmli sample buffer [22] followed by sonication (10 s) and heating (5 min, 100°C). In some experiments,

PKC-η was extracted in the absence of Triton X-100 in order to investigate the cytosol/particulate distribution (see text).

2.4. *Protein kinase C-η assay*

Routinely, 5 ml of enzyme was assayed as described in [23]. In each experiment an enzyme dilution curve was made to establish linearity. In general the ammonium sulphate fraction was used in a 5- to 10-fold dilution. The PKC-η pseudosubstrate peptide (pep-η) is based on the predicted pseudosubstrate site of PKC-η with a serine for alanine substitution: RKRQRSMRRRVH. Other pseudosubstrate peptides have been described elsewhere [9,10,24]. One unit of PKC-η enzyme activity is defined as the amount of enzyme that transfers 1 nmol of phosphate into substrate per min at 30°C under standard assay conditions.

3. RESULTS AND DISCUSSION

3.1. *Structure and expression of rat PKC-η*

The amino acid sequence of rat PKC-η is 99% identical to the sequence described for mouse PKC-η [11] and 97% identical to the corrected human PKC-η sequence [12,13] (the sequence of rat PKC-η will appear in the EMBL Nucleotide Sequence Database under accession number X68400). The identity of the cDNA clone was confirmed by Northern blotting; rat PKC-η (4.6 kb) showed the tissue distribution described for mouse and human PKC-η [11,12]. Moreover, PKC-η was identified as the 4.6 kb rat lung transcript that hybridized with a PKC-ε cDNA probe [24], as a unique PKC-ε oligonucleotide probe failed to hybridize with a transcript of this size (not shown).

3.2. *Expression in COS cells*

Expression of PKC-η in COS cells was monitored by Western blotting using an antiserum against a synthetic peptide based on the C-terminal sequence of PKC-η. Transfection of COS-1 cells with the pKSI-PKC-η expression construct results in the expression of an 84 kDa protein that is immunoreactive with the antiserum (Fig. 1). The signal from the immunoreactive protein is competed out when the C-terminal synthetic peptide is included in the immunoblot. In contrast to a previous report [13], no immunoreactive protein was present in COS cells transfected with the vector alone (Fig. 1), even after long exposure of the western blots. Furthermore, in contrast to these same studies, it is found here that a substantial part of expressed PKC-η is present in the cytosolic fraction and is readily extractable (see below). The reason for these contrasting observations is, at present, unclear. The molecular weight of PKC-η is higher than the predicted molecular weight of 78 kD. This has been observed before for other members of the PKC family [7,9,10] and is thought to be caused by post-translational modification, most likely phosphorylation [25]. Evidence that PKC-η could be a phosphoprotein has been provided by Osada et al. [11] who showed phosphorylation of mouse PKC-η in immunoprecipitates from COS cells expressing mouse PKC-η. We confirmed that recombinant rat PKC-η expressed in COS cells behaves in a characteristic fashion on treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as concentrations of 1 nM and higher, applied for 48 h, effectively down regulate PKC-η in COS cells (not shown). These concentrations are comparable to those reported to down regulate the endogenous PKC-α in COS cells [26] or PKC-α, δ and ε in Swiss 3T3 cells [19].

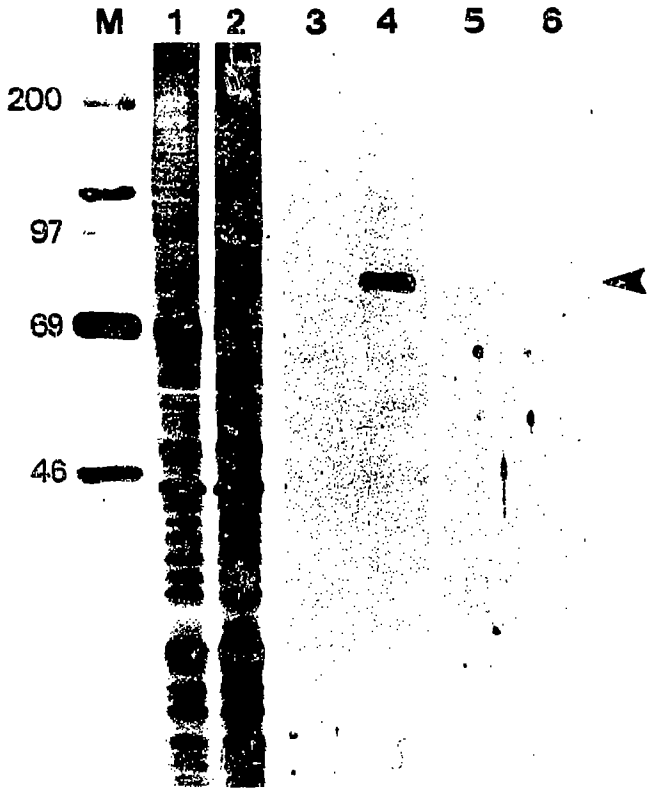

Fig. 1. Expression of PKC-η in COS cells after transfection. COS cells were transfected with pKS1 or pKS1-PKC-η and after three days homogenized in harvesting buffer (see section 2). 1% of the homogenate was electrophoresed by 10% SDS-polyacrylamide gel electrophoresis. The gel was stained for proteins by silver staining (lanes 1 and 2) or transferred to nitrocellulose followed by incubation with antiserum PPA022 (lanes 3 and 4) or antiserum PPA022 in the presence of 2 μg of synthetic peptide against which the antiserum was raised (lanes 5 and 6). 1, 3, 5: cells transfected with pKS1; 2, 4, 6: cells transfected with pKS1-PKC-η. M: molecular weight markers (kDa). Arrowhead indicates PKC-η.

3.3. *Extraction and ammonium sulphate fractionation*

Only limited information is available on the biochemical characteristics of PKC-η. Mouse PKC-η is a phosphoprotein in immunoprecipitates from COS cells [11]. Human PKC-η is also a phosphoprotein in immunocomplexes; however the antibody employed in this study had been raised against a peptide sequence which was later reported to be partially incorrect [12,13]. PKC-η has been shown to have histone kinase activity when assayed in crude extracts from sf9 cells infected with recombinant baculovirus [14].

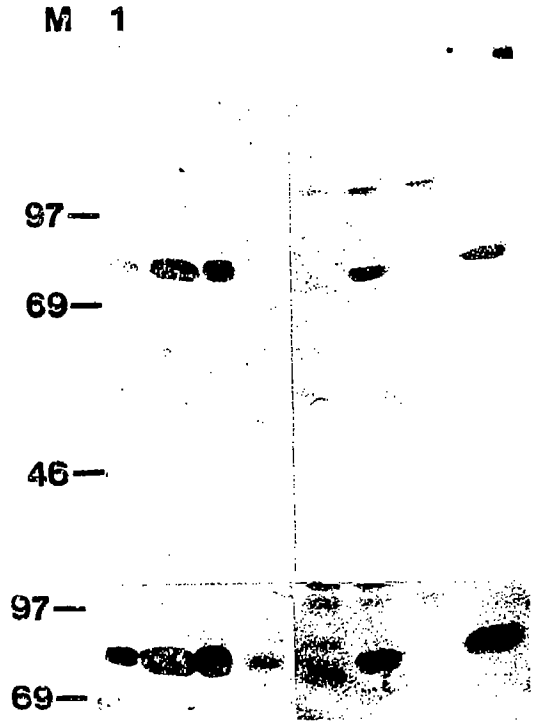

Fig. 2. Extraction of PKC-η from transfected COS cells and ammonium sulphate precipitation. COS cells were transfected with pKS1-PKC-η and after 3 days homogenized in harvesting buffer (see section 2). The Triton-insoluble fraction (lanes 1 and 5), Triton-soluble fraction (lanes 2 and 6), 0–15% ammonium sulphate precipitate of the Triton-soluble fraction (lanes 3 and 7) and the '15–45%' ammonium sulphate precipitate of the Triton-soluble fraction (lanes 4 and 8) were analyzed by 10% SDS-polyacrylamide gel electrophoresis and Western blotting. Lanes 1–4: PKC-η antiserum; lanes 5–8: PKC-α antiserum. M: positions of molecular weight markers (kDa). The lower panel shows a long exposure of the blot for proteins of MW 69–97 kDa.

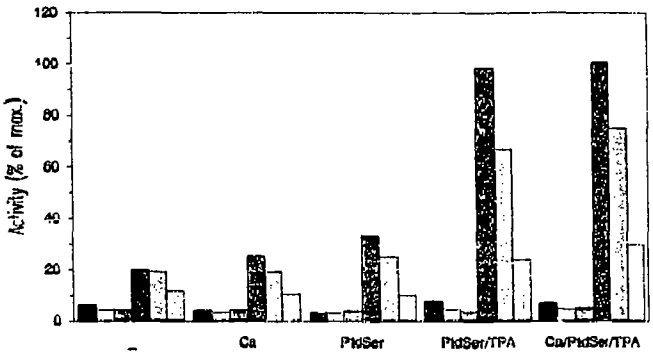

Fig. 3. Kinase activity of 0–15% ammonium sulphate fraction of control and transfected cells. 0–15% ammonium sulphate precipitates were made of COS cells transfected with pKS1 or with pKS1-PKC-η. The precipitates were diluted in enzyme dilution buffer and tested for kinase activity using 10 μg pep-η, 10 μg pep-ε or 50 μg Histone IIIS as substrate. The assay buffer [23] contained 1.5 mM EGTA (Ca^{2+}-independent) or 0.75 mM EGTA + 0.75 mM $CaCl_2$ (Ca^{2+}-dependent) and, when indicated, 50 μg PtdSer and 50 ng TPA. Kinase activity is expressed as % of maximal activity in the presence of Ca^{2+}, PtdSer and TPA with pep-η as substrate. Columns under each condition indicate from left to right (transfected plasmid/substrate): pKS1/pep-η; pKS1/pep-ε; pKS1/Histone IIIS; pKS1-PKC-η/pep-η; pKS1-PKC-η/pep-ε; pKS1-PKC-η/Histone IIIS.

The lack of a more substantial characterization of PKC-η kinase activity may be due to the reported difficulty in extracting the protein from transfected COS cells [11] and/or to the particular effector dependence/substrate specificity of this PKC isotype. In order to investigate this issue, COS cells, expressing PKC-η, were extracted and the extracts were fractionated. PKC-η can be solubilized by 1% Triton X-100 and is present in the supernatant after centrifugation (Fig. 2, lane 2). A small portion of PKC-η is Triton-insoluble and appears in the particulate fraction (Fig. 2, lane 1). In contrast to PKC-η, all of the endogenous PKC-α is present in the Triton-soluble fraction (Fig. 2, lanes 5 and 6). Extraction of the transfected COS cells in extraction buffer without Triton X-100 resulted in a poorer recovery of PKC-η in the soluble fraction (50% soluble); the presence or absence of Ca^{2+} in this extraction buffer did not affect the membrane association (not shown). This parallels the characteristics of PKC-δ and -ε and is in contrast to the Ca^{2+}-dependent membrane association of PKC-α and -β [19].

Ammonium sulphate precipitation of the 1% Triton X-100-soluble fraction allows separation of the endogenous PKC-α from PKC-η. PKC-η precipitates in the 0–15% (w/v) ammonium sulphate fraction (Fig. 2, lanes 3 and 4), whereas PKC-α is present in the 15-45% (w/v) fraction (lanes 7 and 8). The 100 kDa protein that reacts with the PKC-α antiserum and that coprecipitates with PKC-η (Fig. 2, lane 7) is non-specific as its immunoreactivity is not competed by peptide antigen (not shown).

3.4. *Characterization of rat PKC-η kinase activity*

The 0–15% ammonium sulphate precipitate was used to assess the biochemical properties of PKC-η. Precipitates from cells transfected with the control plasmid pKS1 or with pKS1-PKC-η were tested for their ability to phosphorylate the pseudosubstrate peptides pep-η and pep-ε as well as the polypeptide histone IIIS (Sigma) in the presence or absence of various cofactors. In control precipitates, very little kinase activity was observed that was neither dependent on the exogenous substrate nor on the cofactors used (Fig. 3). In the absence of any cofactors, the extracts from cells transfected with pKS1-PKC-η, showed a 3-fold increase in basal phosphorylation of pep-η (Fig. 3). In the presence of phosphatidyl serine (PtdSer) and TPA this basal pep-η kinase activity was enhanced 5-fold and Ca^{2+} did not cause any further stimulation. Pep-ε can also serve as a substrate for the kinase, its phosphorylation increasing 4-fold in the presence of PtdSer and TPA. Histone IIIS, which is used as a substrate for many members of the PKC family, is a poor substrate for the kinase; its phosphorylation increased 2.5-fold in comparison to

cofactor-independent histone IIIS phosphorylation. The presence of histone IIIS kinase activity was dependent on the enzyme preparation, being absent in some preparations. By contrast, PtdSer/TPA dependent pep-η and pep-ε kinase activity was consistently present in cells transfected with the PKC-η expression construct.

Fig. 4 shows the kinetics of pep-η, pep-ε and pep-δ phosphorylation by PKC-η. The K_m value for pep-η phosphorylation is 9.60 ± 3.51 mM (mean ± S.E.M. of 4 independent experiments). K_m values for pep-ε and pep-δ were 16.27 ± 6.35 and 27.33 ± 3.29 mM respectively. The K_m for pep-α phosphorylation was variable but in each individual experiment higher than that for pep-δ. In some experiments enzyme could not be saturated by pep-a (K_m > 1 mM).

It is clear from Fig. 2 that the 0–15% ammonium sulphate fraction made from cells transfected with pKS1-PKC-η is free of any PKC-α but contains approximately 90% of the PKC-η protein. Moreover, the same fraction made from control cells is free of Ca^{2+}, PtdSer and TPA-dependent kinase activity (Fig. 3), providing another indication that the endogenous PKC-α does not precipitate in this fraction. The activity measured in the 0–15% ammonium sulphate precipitate made from cells transfected with pKS1-PKC-η can thus be attributed to PKC-η.

The main kinase activity associated with PKC-η protein in the 0–15% ammonium sulphate fraction is dependent on phospholipid and TPA and cannot be further stimulated by Ca^{2+}. This apparent Ca^{2+} independence of PKC-η was also indicated by the fact that, in contrast to PKC-α [19] no Ca^{2+}-dependent membrane association was observed while extracting PKC-η from transfected COS cells. Various pseudosubstrate peptides are good substrates for PKC-η. By comparison, histone IIIS appeared to be a poor substrate. These data are largely in line with those observed for PKC-δ and e, PKCs that are also phospholipid and TPA-dependent but Ca^{2+}-independent and that are good peptide kinases but poor histone kinases [9,10]. Like PKC-δ and -ε, PKC-η shows highest affinity for its own pseudosubstrate peptide [9,10] (Fig. 4). In contrast to PKC-δ, ε and η, PKC-α, β and γ are dependent on Ca^{2+} for full activation; moreover these PKCs are good histone IIIS kinases (discussed in [6]).

The present findings are interesting with respect to current ideas concerning the structure-function relationship of proteins in the PKC family. Firstly, PKC-η is yet another example of a PKC isotype that lacks a C_2 domain and does not require Ca^{2+} for effector dependent activation (for reviews see [4–6]), supporting the idea that the C_2 domain is of importance for interaction of PKC with Ca^{2+}. Secondly, like PKC-δ and e, PKC-η contains an extended V_1 domain and functionally these isotypes are similar in that histone IIIS is a very poor substrate (Fig. 3; [9, 10]). This restricted specificity of PKC-ε can be conferred on PKC-γ (normally a potent histone kinase) by exchanging their amino terminal domains [24]. It seems then that the extended V_1 domain of PKC-δ and ε is related to their restricted substrate range and our present observations further extend this correlation to PKC-η.

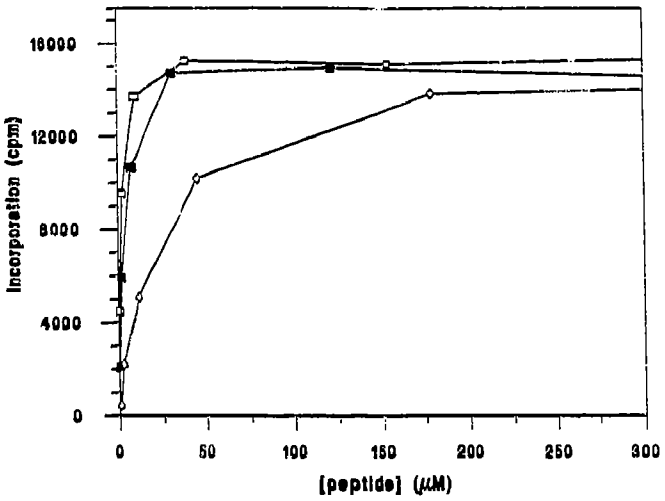

Fig. 4. Kinetics of pseudosubstrate peptide phosphorylation by PKC-η. 0–15% ammonium sulphate precipitates were made of COS cells transfected with pKS1-PKC-η. The precipitates were diluted in enzyme dilution buffer and tested for kinase activity using various concentrations of pep-η (open squares), pep-ε (filled squares) or pep-δ (diamonds) as substrate. The experiment shown is an example of 4 independent experiments.

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