The regulatory domain of protein kinase C- ϵ restricts the catalytic-domain-specificity

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Protein kinase C (PKC) consists of a family of closely related enzymes that can be divided into two subfamilies (α , β and γ and δ , ϵ and ζ) on the basis of primary sequence. Functional differences have also been described; thus PKC- α , PKC- β and PKC- γ readily phosphorylate histone IIIS *in vitro*, whereas PKC- ϵ will not employ this substrate efficiently. We have previously demonstrated, however, that proteolytic cleavage of PKC- ϵ generates a constitutive kinase activity that is an efficient histone IIIS kinase [Schaap, Hsuan, Totty & Parker (1990) *Eur. J. Biochem.* **191**, 431–435]. In order to investigate the structural basis for this switch in specificity, we have constructed a chimaeric protein containing the regulatory domain of PKC- ϵ fused to the catalytic domain of PKC- γ . When this is expressed in COS1 cells the chimaeric kinase shows a substrate-specificity similar to that of PKC- ϵ rather than to that of PKC- γ . This demonstrates a role for the regulatory domain in substrate selection of PKC- ϵ .

INTRODUCTION

Protein kinase C (PKC), defined as a serine/threonine protein kinase dependent on Ca^{2+} , phospholipid and diacylglycerol or the tumour-promoting phorbol esters [such as phorbol 12-myristate 13-acetate (PMA)] for activation, consists of a family of at least seven members [reviewed by Nishizuka (1988) and Parker *et al.* (1989)]. The first four complete sequences (PKC- α , PKC- β_1 , PKC- β_2 and PKC- γ) were derived from cDNA clones isolated by library screening after obtaining peptide sequences from enzymes purified by monitoring the Ca^{2+} /phospholipid-dependent phosphorylation of histone (Parker *et al.*, 1986; Coussens *et al.*, 1986; Ono *et al.*, 1986; Knopf *et al.*, 1986). Comparison of the primary sequences of these clones revealed the presence of four highly conserved regions (C1–C4) interspersed by five more variable regions (V1–V5). The two most C-terminal conserved sequences (C3 and C4) show similarity to those of known protein kinases, and therefore the catalytic activity is thought to reside in this portion of the molecule. The C1 region contains two cysteine-rich repeats, deletion of which leads to a loss of phospholipid/PMA binding (Ono *et al.*, 1989; Kaibuchi *et al.*, 1989; Cazaubon *et al.*, 1990). The C2 region has no proven function as yet, but has been suggested to contain the Ca^{2+} -binding site (Parker *et al.*, 1986; Ono *et al.*, 1989). The N-terminal regulatory sequences form a distinct domain in the intact enzyme that can be separated from the catalytic domain by limited proteolysis, with cleavage occurring in the V3 hinge region (Young *et al.*, 1988; Kishimoto *et al.*, 1989). This proteolysis generates a 30 kDa fragment, which is capable of binding phospholipid/PMA, and a 50 kDa fragment (PKM), which has constitutive kinase activity (Lee & Bell, 1986; Hoshijima *et al.*, 1986; Huang *et al.*, 1989). The regulatory domain therefore normally acts to inhibit the catalytic activity of the kinase domain, and this inhibition is relieved by effector binding or proteolytic cleavage. Short sequences (pseudo-substrate sequences) found at the N-termini that have a high affinity for the active site have been proposed to bring about this inhibition by preventing substrate access (House & Kemp, 1987).

Such a role for the pseudo-substrate site in PKC- α has recently been demonstrated through mutagenesis (Pears *et al.*, 1990).

cDNA clones for three further isotypes (PKC- δ , PKC- ϵ and PKC- ζ) have been isolated by low-stringency screening (Ono *et al.*, 1987, 1988; Ohno *et al.*, 1988; Schaap *et al.*, 1989) and the enzymic properties of the encoded proteins deduced from expression of these DNA sequences. Use of the baculovirus (Schaap & Parker, 1990) and COS-cell (Schaap *et al.*, 1989; Ono *et al.*, 1988; Ohno *et al.*, 1988) expression systems has shown that histone IIIS, the most commonly used substrate for assaying PKC- α , PKC- β and PKC- γ activity *in vitro*, is a poor substrate for PKC- ϵ . The substrates that can be efficiently phosphorylated by this enzyme are small peptides based on the pseudo-substrate sequences of the PKCs, but containing a serine residue that can be phosphorylated (Schaap *et al.*, 1989). However, we have recently shown that partial proteolytic cleavage of PKC- ϵ generates an effector-independent kinase that can efficiently phosphorylate histone, suggesting that the regulatory domain of PKC- ϵ influences the substrate-specificity of the kinase domain, even after effector binding (Schaap *et al.*, 1990). We have tested the influence of the regulatory domain by creating a chimaeric PKC containing the regulatory domain of PKC- ϵ fused to the catalytic domain of PKC- γ . The activity of this protein, determined after transient expression in COS cells, confirms the role of the regulatory domain in the substrate-specificity of the intact enzyme.

MATERIALS AND METHODS

DNA constructs

pMT-PKC- ϵ contains the cDNA sequences of PKC- ϵ , isolated from mouse brain, inserted into the mammalian expression vector, pMT2 (Schaap *et al.*, 1989); similarly, pMT-PKC- γ contains the coding sequences for bovine PKC- γ . In pMT-PKC- ϵ/γ a 911 bp *NcoI*–*StuI* fragment, extending from the initiating methionine residue to the V3 hinge region of PKC- γ , has been replaced by the corresponding 1095 bp *NcoI*–*StuI* fragment from

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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Fig. 1. $(\text{NH}_4)_2\text{SO}_4$ precipitation of PKC isotypes

COS cells were harvested 48 h after transfection with pMT2, pMT-PKC-ε, pMT-PKC-γ or pMT-PKC-ε/γ in buffer containing 1% Triton X-100, and the material insoluble in 0–18% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (■) and that insoluble in 18–45% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (▨) were assayed for the phospholipid/PMA-stimulatable kinase activity with a peptide based on the pseudo-substrate site of PKC-ε (0.5 mg/ml) as substrate. For the vector control the activity is assigned to the endogenous PKC-α. As the same number of cells were transfected with each construct, any increase in stimulatable kinase activity can be assumed to be due to expression of alternative subtypes of PKC by using the exogenous DNA sequences. The results shown are from a single experiment, although comparable results were obtained on three separate occasions.

PKC-ε. This in-frame fusion allows expression of a chimaeric PKC-ε/γ containing the regulatory domain of PKC-ε and the catalytic domain of PKC-γ.

Transient expression of PKC in COS1 cells

COS1 (COS) cells were plated at 5×10^5 cells/9 cm plate and allowed to adhere for 16 h before transfection with 20 μg of plasmid DNA with the use of the calcium phosphate co-precipitation procedure. Cells were harvested 48 h after transfection in 1 ml of lysis buffer [20 mM-Tris/HCl buffer, pH 7.5, containing 1% (v/v) Triton X-100, 50 mM-NaF, 5 mM-EDTA, 25 μg of leupeptin/ml, 50 μg of phenylmethanesulphonyl fluoride/ml, 10 mM-benzamidine and 0.3% (v/v) 2-mercapto-

ethanol]. Insoluble material was removed by centrifugation at 12000 g in a refrigerated Microfuge. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to 18% (w/v), and precipitated material was removed by repeating the centrifugation procedure (fraction I); a further $(\text{NH}_4)_2\text{SO}_4$ precipitate of material insoluble up to 45% (w/v) was also generated (fraction II). These precipitates were re-dissolved in 0.5 ml of 20 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-EDTA, 10 mM-benzamidine and 0.3% (v/v) 2-mercapto-ethanol and assayed for phospholipid/PMA-stimulatable kinase activity as described in Marais & Parker (1989) with either histone IIIS (1.25 mg/ml) or a peptide based on the pseudo-substrate site of PKC-ε (0.5 mg/ml) (Schaap *et al.*, 1989) as substrate. A unit of PKC activity is defined as the amount of PKC required to transfer 1 nmol of $[^{32}\text{P}]$ phosphate into substrate/min under standard assay conditions (Marais & Parker, 1989). Western-blot analysis was carried out as in Marais & Parker (1989), with antisera specific for the C-terminus of PKC-ε (Schaap *et al.*, 1989) or PKC-γ (Marais & Parker, 1989).

RESULTS

The plasmids pMT-PKC-ε and pMT-PKC-γ contain the coding sequences for PKC-ε and PKC-γ under the control of the adenovirus major late promoter, allowing expression of these sequences after transfection into mammalian cells. In pMT-PKC-ε/γ sequences coding for the regulatory domain of PKC-ε replace the corresponding region of PKC-γ, leaving the PKC-γ catalytic sequences intact. After transfection of pMT-2, pMT-PKC-ε, pMT-PKC-γ or pMT-PKC-ε/γ, COS cells were lysed in 1% Triton X-100 and the insoluble material was removed by centrifugation. Two $(\text{NH}_4)_2\text{SO}_4$ fractions [I, 0–18% (w/v); II, 18–45% (w/v)] were prepared and assayed for their phospholipid/PMA-stimulatable kinase activity (Fig. 1) with as substrate a peptide based on the PKC-ε pseudo-substrate sequence, which is known to be phosphorylated efficiently by both PKC-γ and PKC-ε (Schaap *et al.*, 1989; Marais & Parker, 1989). The endogenous PKC-α in COS cells is present in fraction II, as is the expressed PKC-γ. A large proportion of the activity associated with PKC-ε is present in fraction I, and the large increase in effector-stimulated activity in cells transfected with pMT-PKC-ε/γ suggests that most of the chimaeric PKC-ε/γ is also present in fraction I.

This distribution was confirmed by Western-blot analysis with antibodies specific for the C-terminal V5 regions of PKC-γ (Marais & Parker, 1989) and PKC-ε (Schaap *et al.*, 1989) (Fig.

Fig. 2. Western-blot analysis of transient PKC expression in COS cells after $(\text{NH}_4)_2\text{SO}_4$ fractionation

COS cells were harvested 48 h after transfection with pMT-2, pMT-PKC-ε, pMT-PKC-γ or pMT-PKC-ε/γ and fractionated to generate fraction I [0–18% (w/v) $(\text{NH}_4)_2\text{SO}_4$] and fraction II [18–45% (w/v) $(\text{NH}_4)_2\text{SO}_4$] as described in the text. Portions of each fraction were subjected to Western-blot analysis with antisera raised against the C-terminal peptides of PKC-ε (Schaap *et al.*, 1989) (panel a), PKC-γ and PKC-α (Marais & Parker, 1989) (panels b and c respectively).

Fig. 3. Histone kinase activity of PKC isotypes

The 18–45% (w/v) $(\text{NH}_4)_2\text{SO}_4$ fraction from cells transfected with pMT2 and pMT-PKC- γ and the 0–18% (w/v) $(\text{NH}_4)_2\text{SO}_4$ fraction from cells transfected with pMT-PKC- ϵ and pMT-PKC- ϵ/γ were assayed for their ability to phosphorylate histone IIIS (1.25 mg/ml) in the presence of the activators phosphatidylserine, PMA and Ca^{2+} . For the vector control the activity is assigned to the endogenous PKC- α . The activities are expressed as percentages of the activity seen towards the peptide substrate with the same dilution of enzyme. The percentages are the averages of three separate series of transfection experiments.

2). As expected, transfection of pMT-PKC- ϵ led to the expression of a 90 kDa protein recognized only by the PKC- ϵ -specific antiserum; pMT-PKC- γ generated an 80 kDa protein recognized only by the PKC- γ -specific antiserum. The chimaeric PKC- ϵ/γ protein was, as predicted, of 90 kDa and recognized only by the PKC- γ specific antiserum, as the C-terminal portion of the protein is derived from PKC- γ . This is consistent with expression of pMT-PKC- ϵ/γ giving rise to a protein containing the regulatory domain of PKC- ϵ and the catalytic domain of PKC- γ . The PKC- ϵ and PKC- ϵ/γ were therefore separated from most of the endogenous PKC activity in COS cells whereas most of the PKC- γ was co-precipitated with the bulk of the endogenous PKC- α activity. The relative kinase activities in fraction II from cells transfected with pMT2 and pMT-PKC- γ indicate that the ratio of PKC- γ to endogenous PKC- α is approx. 10:1 in this particular experiment. Serial dilution of samples before Western-blot analysis revealed that less than 10% of the endogenous PKC- α is present in fraction I and its distribution is unaltered by expression of any of the exogenous PKCs (results not shown).

After $(\text{NH}_4)_2\text{SO}_4$ precipitation of COS-cell extracts, the relevant fractions were diluted to give similar activities towards the peptide substrate and assayed for their ability to phosphorylate histone IIIS in the presence of phospholipid/PMA and Ca^{2+} (Fig. 3). PKC- α and PKC- γ were both able to phosphorylate the histone efficiently, whereas PKC- ϵ and PKC- ϵ/γ showed a very low level of activity towards this protein substrate. This was found to be true over a range of histone concentrations from 6.25 to 0.125 mg/ml (results not shown). Western-blot analysis confirms (Fig. 2) that the low level of histone phosphorylation seen in the PKC- ϵ and PKC- ϵ/γ fractions is due to contamination of fraction I with a small amount of endogenous PKC- α , though some activity towards histone has been reported for PKC- ϵ (Ohno *et al.*, 1988; Schaap *et al.*, 1989).

DISCUSSION

The influence of the regulatory domain over the catalytic activity of PKC- ϵ has been investigated here by creating a chimaeric protein with the regulatory sequences of PKC- ϵ fused

to the catalytic domain of PKC- γ . This protein was expressed in COS cells with the use of the mammalian expression vector pMT2 and reached a steady-state concentration comparable with that of PKC- ϵ or PKC- γ expressed with the use of the same vector system. Antiserum specific for the catalytic domain of PKC- γ confirmed the identity of the protein. The PKC- ϵ/γ generated behaves as a functional protein kinase C as judged by its effector-dependent phosphorylation of a peptide substrate *in vitro*. In NIH3T3 cells co-transfection of these sequences with a plasmid containing the chloramphenicol acetyltransferase (CAT) gene under the control of the PMA-inducible collagenase promoter (Angel *et al.*, 1987) confirms that PKC- ϵ/γ can function in a regulatable manner in the intact cell, as its expression causes an increased induction of chloramphenicol acetyltransferase activity in response to PMA treatment; induction to a similar level occurs after co-expression with PKC- ϵ or PKC- γ (C. Pears & P. J. Parker, unpublished work). Transfection of the corresponding construct containing the regulatory domain of PKC- γ fused to the catalytic domain of PKC- ϵ did not give rise to any stable chimaeric PKC- γ/ϵ in COS cells, as judged by Western-blot analysis or increased kinase activity.

Catalytically, the chimaeric PKC- ϵ/γ behaves like PKC- ϵ in its inability to phosphorylate histone efficiently, despite containing the catalytic sequences of PKC- γ , which uses this substrate efficiently. It was originally considered that effector binding, by releasing the interaction of the regulatory domain with the catalytic domain, permits the latter to function independently. However, in the case of PKC- ϵ at least, it now seems that the regulatory domain can play a more complex role, influencing substrate selection by regulating access to the active site following activation. Presumably small peptides such as those based on the PKC pseudo-substrate sequences can gain access to the active site, whereas the entry of certain polypeptide substrates is blocked. The physiological substrate(s) for PKC- ϵ may be very small peptides or may be of a specific conformation that can interact with the 'gating' regulatory domain sequences and so gain access to the catalytic function. Alternatively a further activating signal may be required for complete PKC- ϵ function, such as phosphorylation or phosphatase action or binding of an as yet unidentified effector.

The V1 region is a likely candidate for the sequence regulating substrate selection, as this is greatly extended in PKC- ϵ (Schaap & Parker, 1990), compared with PKC- α , PKC- β and PKC- γ . The initial cleavage of PKC- ϵ during trypsin digestion, which is known to generate a PKM form able to phosphorylate histone, has been mapped to the region immediately C-terminal to the C1 region, and so removal of the V1 and C1 regions is sufficient to alter the substrate range (Schaap *et al.*, 1990). The lack of conservation of the V1 region between the isotypes of PKC suggests that each may have specific substrate targets that cannot be phosphorylated by all members of the family. This specificity may be important in generating the diversity of signal-transduction pathways present in the cell. There is evidence that, at least in some cell types such as neutrophils and platelets (Tapley & Murray, 1985; Melloni *et al.*, 1986), PKM forms may be generated on activation of PKC and may play a role in signal transduction. If this were found to be true for PKC- ϵ , one could imagine that the initial activation event could generate a kinase capable of phosphorylating only a limited range of substrates but that subsequent proteolytic cleavage may increase this range at a later time or elsewhere within the cell. Thus PKC- ϵ may have a different substrate range within the same cell depending on the mechanism of activation.

It may be noted that there is evidence for alternative splicing of the PKC- ϵ gene, since a cDNA clone encoding a truncated version of the enzyme (PKC- ϵ') has been isolated from rat brain

(Ono *et al.*, 1988) and shorter transcripts that differ from PKC- ϵ at the 5'-end have been reported in rat lung and brain (Schaap *et al.*, 1990). It will be of interest to see if these truncated versions have an increased substrate range relative to PKC- ϵ , suggesting yet another mechanism for the generation of a diversity of signal-transduction pathways within the cell. As more functional differences between the isotypes of PKC emerge the use of chimaeric proteins such as that used here will be of increasing importance to try and unravel both the structural basis for these differences and importantly the role of these gene products in the complex mechanisms of signal transduction.

We thank Nigel Goode, Julian Downward and Doreen Cantrell for helpful comments on the manuscript and Amanda Wilkinson for her help in preparation of the manuscript.

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Received 11 October 1990/12 March 1991; accepted 19 March 1991