Threonine-497 is a critical site for permissive activation of protein kinase Cα

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Phosphorylation of the region containing Thr-494, Thr-495 and Thr-497, present in the catalytic domain of protein kinase Cα (PKCα), is a preliminary event necessary for subsequent PKC activation [Cazaubon and Parker (1993) J. Biol. Chem. **268**, 17559–17563]. To define the essential residues in this region, various combinations of alanine substitutions for threonine residues 494, 495 and 497 have been tested. These mutations yielded expressed polypeptides of 76 and 80 kDa in ratios that vary from 100% 80 kDa (wild-type kinase, active) to 100% 76 kDa (AAA mutant, inactive) with the hierarchy being wild-type PKCα (TTT), ATT, AAT, TTA, ATA, TAA, AAA (the nomenclature indicates the location of alanine residues substituted for Thr-494, Thr-495 and Thr-497 respectively). Only the mutants retaining Thr-497 displayed kinase activity *in vitro*. The results overall indicate that Thr-497 plays the dominant role in the regulation of PKCα activity but that in the wild-type protein, Thr-495 may also be important. Consistent with the need for phosphorylation in this region, an intrinsically active PKCα could be produced in bacteria by exchanging Thr-495 for a glutamic acid residue.

INTRODUCTION

Protein kinase C (PKC) is a serine/threonine-kinase that plays a crucial role in the regulation of a variety of physiological processes [1]. Indeed, PKC is the intracellular mediator of various external signals (hormones, growth factors, neuropeptides) that induce the production of the second messenger diacylglycerol. Initially, four isotypes of PKC; PKCα, β_1, β_2 and γ, were isolated and characterized as Ca^{2+}, phospholipid- and diacylglycerol/phorbol ester-dependent protein kinases. Subsequently, it has been shown that PKC constitutes a family of at least ten structurally related polypeptides that display subtly different enzymological properties as well as specific tissue expression and subcellular localization [2,3].

In addition to the regulation of PKC activity by allosteric effectors such as diacylglycerol and phorbol esters, the post-translational phosphorylation of PKC also appears to constitute an important aspect of control. In previous studies, it was shown that PKCα is synthesized as an unphosphorylated and catalytically inactive protein of 76 kDa that is converted into an active form of 76 kDa and then into an 80 kDa form by at least two stages of phosphorylation [4–7]. The first stage of phosphorylation, which is a preliminary event for PKC activation, seems to be initiated by an heterologous kinase ('PKC kinase'). Consistent with this, bacterially expressed PKC is a protein of 76 kDa devoid of kinase activity [8,9]. Furthermore, the observation that a PKCα mutant (AAA) mimicking the unphosphorylated form of PKCα can inhibit, in a competitive manner, the phosphorylation of newly synthesized PKCα and β [6] also argues for a putative PKC kinase. In contrast, the second stage of phosphorylation, which is responsible for the electrophoretic mobility shift of PKC (from 76 kDa to 80 kDa), can take place by autophosphorylation [5].

Recently, the phosphorylated region of PKCα that appears to be essential for the permissive activation of the enzyme was mapped [6]. The substitution of Ala residues for Thr-494, Thr-495 and Thr-497 (AAA) led to the expression, in COS-1 cells, of an unphosphorylated protein of 76 kDa, which is similar in size to that determined for the primary translation product and the bacterially expressed PKC. This PKCα mutant is a functional phorbol-ester-binding protein but retains no kinase activity, indicating that Thr-494, Thr-495 and Thr-497 contain the site(s) whose phosphorylation is necessary for PKC activity. In order to identify the position of the phosphorylated residue(s), different combinations of Ala substitution for these Thr residues have been introduced into the PKCα cDNA. These PKCα mutants have been expressed in COS-1 cells and subsequently analysed for mobility by SDS/PAGE, as well as for kinase activity *in vitro*. This study shows that Thr-497 is critical for PKC activity and that Thr-495 may also somehow be involved. In addition a further mutant was made, with a glutamic acid residue substituted for Thr-495; this mutant displays kinase activity *in vitro* following expression in *Escherichia coli*. Thus a negative charge (phosphate or glutamate) in this region of PKC is essential for catalytic activity.

MATERIALS AND METHODS

Materials

Peroxidase-linked donkey anti-(rabbit IgG), rabbit anti-(mouse IgG) and the ECL Western-blotting detection system were obtained from Amersham (Amersham, Bucks., U.K.). Other chemicals and biochemicals were from Sigma (Poole, Dorset, U.K.). The sequenase version 2.0 DNA sequencing kit was from United States Biochemical (Cleveland, OH, U.S.A.) and the Mono Q column was from Pharmacia (Uppsala, Sweden).

Abbreviations used: PKC, protein kinase C; TTT, wild-type PKC containing Thr-494, Thr-495 and Thr-497; TAT, mutant PKC containing Ala at residue 495 (similarly ATT, TTA, etc.); bTTT, bacterial expressed wild-type PKC; bTET, bacterial expressed mutant PKC containing Glu at residue 495; PMA, phorbol 12-myristate 13-acetate; PtdSer, phosphatidylserine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride.

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Construction of the different PKCα-mutant-cDNA expression vectors

PKCα mutant cDNAs were constructed using bovine PKCα cDNA [10]. Exchange of the Thr residue(s) with an Ala residue(s) was made using the Altered sites *in vitro* mutagenesis system (Promega). Briefly, an *Eco*RI/*Xba*I fragment containing the full coding sequence of PKCα was cloned into the pAlter-1 vector. In addition to the ampicillin-repair oligonucleotide the following mutagenic oligonucleotides that are sense with respect to the PKCα cDNA were used: (T/A)494, ATGATGGACGGCGTCGCGACCAGGACCTTCT; (T/A)497, GACGGCGTCACGACGCGCGCCTTCTGCGGGACC; $(T/A)_2$494/495, ATGATGATGGACGGCGTCGCGGCCAGGACCTTCTGC; $(T/A)_2$495/497, ATGGACGGCGTCACGGCAAGGGCCTTCCTGCGGGA; $(T/A)_2$494/497, ATGATGGACGGCGTCGCGACCAGGGCCTTCTGCGGGA. Changed bases are underlined. The introduced mutations destroy an *Mae*III or *Bst*NI site, the loss of which was utilized during the screening. The mutated oligonucleotide portion was then verified by sequence analysis using the Sequenase kit. The *Eco*RI–*Xba*I digestion of the pAlter-1 plasmid containing the different PKCα mutants, yielded a 2.3 kb fragment which was subcloned into the pKS1 vector [11] for expression in COS-1 cells.

In order to produce the TET mutant, Thr-495 was substituted by a Glu residue using PCR as described previously [6] with the following 'inside primers' containing the mutation: sense primer, GGCGTCACGGAGAGGACCTTCTGC; antisense primer, GAAGGTCCTCTCCGTGACGCC. The wild-type PKCα (TTT) and TET cDNAs cloned into the Bluescript vector as described previously [6] were subcloned into the multifunctional phagemid pTZ18R (Pharmacia) for expression in *E. coli* cells, strain HB101. For that purpose, Bluescript-TTT and Bluescript-TET were digested with *Sac*II, blunt-ended, and digested with *Sal*I, whereas the pTZ18R vector itself was digested with *Eco*RI, blunt-ended, and cut with *Sal*I. The TTT or TET cDNAs were then ligated into the blunt-end and *Sal*I site of pTZ18R. Transformation of *E. coli* with either pTZ18R-TTT or pTZ18R-TET plasmid led to the expression of the bacterial recombinant protein hereafter called bTTT and bTET respectively.

Culture and transfection of COS-1 cells

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal-calf serum (FCS), penicillin (1000 units/ml) and streptomycin (100 μg/ml). pKS1, pKS1-PKCα or pKS1-PKCα mutant plasmid DNA (30 μg) was introduced into 60% confluent COS-1 cells (2×10^6) by electroporation (0.45 kV for 5 ms). After transfection (48 h), cells were lysed in 500 μl of an ice-cold buffer containing 20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 5 mM dithiothreitol (DTT), 1% (v/v) Triton X-100, 20 mM NaF, 10 mM benzamidine, 25 μg/ml leupeptin, 25 μg/ml aprotinin and 0.1 mM phenylmethanesulphonyl fluoride (PMSF). Cells were broken with 20 strokes of a Dounce homogenizer and then the Triton-insoluble fraction was removed by centrifugation (12000 *g* for 20 min at 4 °C). An aliquot of the supernatant was subjected to immunoblotting with antibodies directed against a synthetic peptide based on the C-terminal sequence of bovine PKCα as already described [12]. PKCα and the different PKCα mutants were partially purified from the supernatant by anion-exchange chromatography as described below.

Partial purification of PKC from transfected COS-1 cells

The Triton-soluble fraction from transfected COS-1 cells was loaded on a 1-ml Mono Q column, equilibrated in buffer A [20 mM Tris/HCl, pH 7.5, 2 mM EDTA, 5 mM DTT, 10 mM benzamidine and 0.02% (v/v) Triton X-100]. The column was washed in 5 ml of buffer A and a 10 ml linear gradient (200–700 mM NaCl) was used to elute proteins. Fractions (500 μl) were collected, subjected to immunoblot analysis and assayed for PKC activity as described previously [8].

Expression and partial purification of bacterial recombinant PKC

In order to induce the expression of bTTT and bTET in *E. coli* HB101 cells, the bacteria that were transformed with either pTZ18R-TTT or pTZ18R-TET plasmid were grown in 0.5 l of Luria–Bertani medium containing 100 μg/ml ampicillin at 30 °C. On reaching an A_{600} of 0.7, cultures were induced with 0.5 mM isopropyl β-D-thiogalactoside. Bacteria were harvested when the A_{600} was 1.5. Bacteria were centrifuged for 10 min at 3000 *g* and washed with an ice-cold buffer containing 20 mM Tris (pH 7.5) and 50 mM NaCl. Bacteria were resuspended in 10 ml of ice-cold STE buffer [10% (w/v) sucrose, 100 mM Tris, pH 7.5, 1.5 mM EDTA] and lysozyme was added to a final concentration of 100 μg/ml. The mixture was incubated for 20 min at 4 °C, then Triton X-100 (0.2% final concentration) and PMSF (0.5 mM) were added. To reduce the viscosity, the lysate was mildly sonicated for seven 10 s bursts at 90 W and then centrifuged for 30 min at 50000 *g*. Recombinant PKCα proteins were purified from the supernatant (Triton-soluble fraction) as described above except that the Mono Q column was washed in 10 ml of buffer A and that a 20 ml linear gradient (200–700 mM NaCl) was used to elute proteins. An aliquot of the supernatant and the fractions collected from the Mono Q column were subjected to immunoblot analysis with a monoclonal antibody specific to PKCα (MC5) as already described [13].

Other methods

Protein concentration was determined by the method of Bradford [14] using bovine γ-globulin as the standard.

RESULTS AND DISCUSSION

Expression of PKCα mutants in COS-1 cells

In a previous study, it was shown that Ala replacement of Thr-494, Thr-495 and Thr-497 leads to the expression of an unphosphorylated and inactive kinase (AAA), suggesting that this region contains the phosphorylated residue(s) necessary for PKC activity [6]. Several combinations of Ala substitution for these three Thr residues have been introduced into the PKCα cDNA in an attempt to define the important residue(s). The mutants ATT and TTA have been produced by exchanging individually Thr-494 and Thr-497 by an Ala residue. In addition, Thr-494/Thr-495, Thr-494/Thr-497 or Thr-495/Thr-497 were replaced by two Ala residues to obtain the AAT, ATA or TAA mutants respectively.

Expression of the different PKCα mutants in COS-1 cells was monitored by immunoblotting using antibodies specific to the C-terminal sequence of PKCα (Figure 1). As determined previously, the wild-type PKCα (TTT), expressed in COS-1 cells, migrates as an 80 kDa polypeptide similar to the endogenous PKCα. Under the same loading conditions (50 μg of protein), the endogenous PKCα was not detectable in COS-1 cells transfected with the pKS1 vector alone (Figure 1; C). In contrast, AAA, which does not contain stoichiometric levels of phosphate normally responsible for the shift of about 3–4 kDa, clearly displayed a faster mobility on SDS/PAGE (molecular mass 76 kDa).

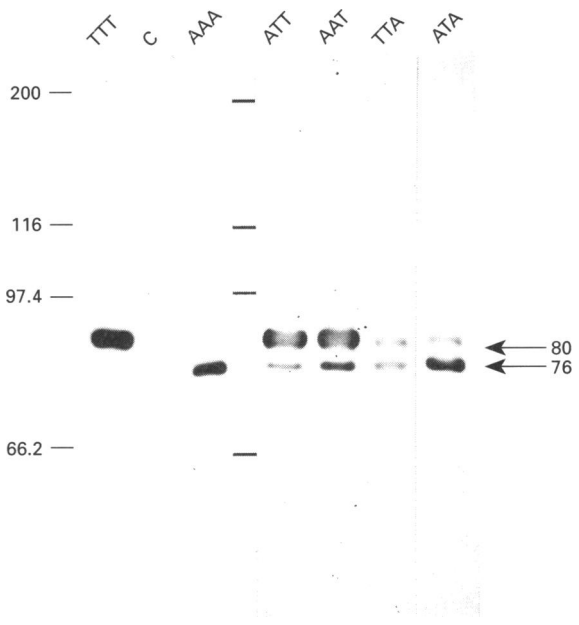

Figure 1 Expression of PKCα mutants in COS-1 cells

COS-1 cells transfected with pKS1-PKCα (TTT), pKS1 (C), pKS1-AAA, pKS1-ATT, pKS1-AAT, pKS1-TTA or pKS1-ATA were lysed in SDS/PAGE sample buffer 2 days after transfection. Fractions (50 μg of protein) were electrophoresed on SDS/8% (v/v) PAGE and transferred to nitrocellulose membrane followed by incubation with antibodies specific to the PKCα C-terminus (1/5000). Molecular-mass markers (kDa) are shown on the left-hand side and arrows indicate migration of the 80 kDa and 76 kDa forms of PKCα.

Table 1 Comparative analysis of the biochemical properties of the different PKCα mutants

The PKCα mutants expressed in COS-1 cells were compared with the wild-type PKCα (TTT) and the unphosphorylated and inactive mutant (AAA). Densitometric scanner analysis of autoradiographs has allowed evaluation of the percentage of conversion of the 76 kDa form into the 80 kDa form and the relative kinase activities of TTT, ATT and AAT. A range of values is shown for the percentage of the 80 kDa form (from two or three separate determinations). Specific activities are values from a representative experiment. Activities for TTA, ATA and AAA were not significantly different from vector transfected controls.

	80 kDa species (% of 80 + 76 kDa)	Specific activity (arbitrary units)
TTT	100	1.00
ATT	85–90	0.83
AAT	76–80	0.44
TTA	20–45	< 0.04
ATA	11–24	< 0.04
AAA	0	< 0.04

Transfection of COS-1 cells with the different mutated constructs resulted in the expression of two immunoreactive proteins migrating at 76 kDa and 80 kDa. The ratio of these two forms of mutated PKCα was dependent upon the position of the substituted Thr residue. While ATT and AAT mainly migrated as 80 kDa proteins, the Ala replacement of Thr-497 led to a significant relative decrease of the 80 kDa form and increase in the 76 kDa form (see Figure 1 and Table 1). The prominence of the 76 kDa form of TTA, ATA and TAA (not shown) suggests that these PKCα mutants do not completely autophosphorylate

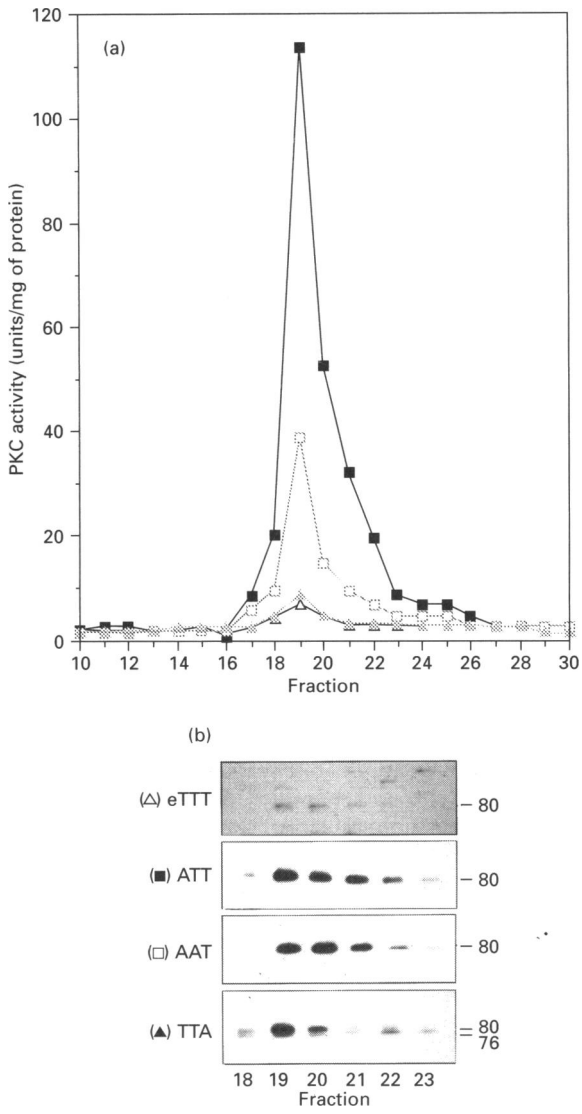

Figure 2 Partial purification and biochemical characterization of PKCα mutants expressed in COS-1 cells

The Triton-soluble fractions of COS-1 cells transfected with pKS1-ATT (■), pKS1-AAT (□), pKS1-TTA (▲) or pKS1 (△) were subjected to Mono Q chromatography as described in the Materials and methods section. Fractions were assayed for PKC activity using histone III-S as substrate (**a**). Units are expressed as a function of protein concentration. Fractions were subjected to 10% SDS/PAGE and immunoblotting (**b**) as described in Figure 1.

to produce the 80 kDa form and probably retain low kinase activity.

Kinase activity of PKCα mutants partially purified from COS-1 cells

In order to verify the above activity predictions, the kinase activities of the partially purified mutants were determined *in vitro*. The Triton-soluble fractions of COS-1 cells transfected with the various PKC mutants were chromatographed on a Mono Q column. Collected fractions were tested for their ability to phosphorylate histone III-S in the presence or absence of the effectors: PtdSer, Ca^{2+} and phorbol 12-myristate 13-acetate (PMA). Figure 2(a) shows the effector-dependent PKC activity; no histone III-S phosphorylation was detectable in the absence

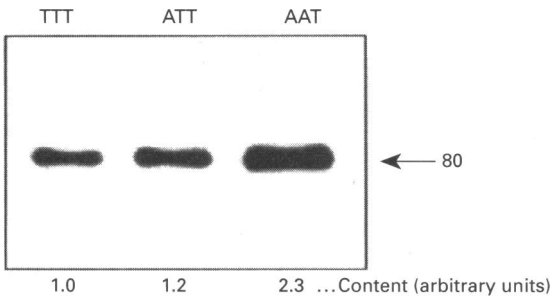

Figure 3 Determination of the relative activity of TTT, ATT and AAT

TTT, ATT and AAT were expressed in COS-1 cells and partially purified as described in Figures 1 and 2(a). Proteins (TTT, ATT, AAT) corresponding to 0.5 unit of PKC activity eluted in fraction 19 from each chromatogram were analysed by immunoblot as described in Figure 1. Densitometric scanning data of the autoradiograph are shown underneath, in arbitrary units.

of effectors, suggesting that no significant amounts of catalytic domain fragments were generated *in vivo* or during the purification (data not shown).

The PKC activity from COS-1 cells transfected with the pKS1 vector as a control corresponds to the endogenous activity, and was just detectable, mostly eluting in fraction 19 (approx. 300 mM NaCl). Transfection of COS-1 cells with either pKS1-ATT or pKS1-AAT led to a 16-fold and 5.5-fold increase in PKC activity respectively, both of which peaked in fraction 19. In contrast, there was no detectable change in PKC activity from pKS1-TTA-transfected COS-1 cells, suggesting that TTA is not a functional histone kinase. In the same way, the PKC activity recovered from COS-1 cells transfected with pKS1-ATA or pKS1-TAA was also similar to that of the endogenous PKC (data not shown).

The presence of the different PKCα mutants in each fraction was analysed by immunoblot (Figure 2b). Under the same loading conditions, no immunoreactive protein was detectable in COS-1 cells transfected with the pKS1 vector alone. Endogenous PKCα (eTTT, 80 kDa) became detectable in fractions 19 and 20 when the period of exposure of the membrane to the autoradiography film was 10 times longer. The increase in PKC activity in cells transfected with either pKS1-ATT or pKS1-AAT correlated with the expression of the 80 kDa form of the PKCα mutant in these cells (fractions 19–22). The very low amounts of the 76 kDa forms of ATT and AAT (see Figure 1 and Table 1) were undetectable, probably because these proteins were eluted over several fractions (as described below for TTA). While ATT and AAT appear to be expressed at a similar level, the kinase activity of these two mutants was clearly different. To determine their relative specific activities and also to compare them with that of the wild-type protein, samples of ATT, AAT and TTT displaying the same amount of PKC activity (0.5 unit) were analysed by immunoblot (Figure 3). Densitometric scanning of the autoradiograph indicates that increased amounts of ATT (1.2-fold) and AAT (2.3-fold), with respect to TTT, are required to obtain the same activity. Hence, in the *in vitro* kinase assay AAT was about 2-fold less active than ATT which itself retained about 80% of the wild-type PKCα activity.

In contrast with these two PKCα mutants, TTA was expressed as an 80 kDa and a 76 kDa form (Figure 2b). While the 80 kDa form peaked in fraction 19 and became undetectable in fraction 22, the 76 kDa form was eluted in fractions 18–23 with a maximum level in fraction 22. The apparent low recovery of the 76 kDa form following extraction and chromatography reflects the elution of this form in a broad peak causing substantial dilution. Analysis of the loaded material (i.e. the Triton-soluble PKC) showed that the 80 kDa/76 kDa ratio was indistinguishable from that observed for whole cell lysates (not shown). The faster-migrating form clearly did not co-elute with kinase activity. The 80 kDa form, mostly present in fraction 19, was in large excess with respect to the endogenous PKCα. However, all the PKC activity measured in this fraction was attributable to the endogenous PKCα, indicating that both the 80 kDa form and the 76 kDa form of TTA was inactive *in vitro*. The presence of the 80 kDa form of TTA (see also Figure 1) suggests that this mutant was capable of autophosphorylation in intact cells. This reaction might have been very slow as the expression of the recombinant TTA in COS-1 cells was analysed 2 days after transfection. Consistent with this interpretation, the decrease in kinase activity measured *in vitro* for AAT, as compared with ATT or wild-type PKCα, was accompanied by a reduced extent of conversion into the 80 kDa species (see Figure 1).

Kinase activity of a bacterial recombinant PKCα mutant: bTET

In previous studies, it was shown that bacterially expressed PKC, while retaining effector-binding ability is devoid of kinase activity [8,9]. In order to determine whether the addition of a negative charge in the region containing the Thr-494, -495 and -497 residues can lead to the expression of an active PKC in bacteria, Thr-495 of PKCα was substituted by a glutamic acid residue to produce the bacterial recombinant protein bTET.

Expression of the bTET mutant and the wild-type PKCα (bTTT) in *E. coli* was analysed by immunoblotting the Triton-soluble fraction of transformed bacteria using a monoclonal antibody specific to PKCα (Figure 4a). No immunoreactive protein was detected in untransformed bacteria (Figure 4; C), indicating that no endogenous PKCα-related protein was present in *E. coli*. Transformation of *E. coli* with either pTZ18R-TTT or pTZ18R-TET resulted in the expression of proteins bTTT and bTET respectively, both migrating at 76 kDa on SDS/PAGE. These results suggested that bTET, as well as bTTT, does not contain the stoichiometric levels of phosphate responsible for the expression of the 80 kDa form seen in mammalian cells.

To determine their respective PKC activities, bTTT and bTET were partially purified by chromatography on a Mono Q column and collected fractions were tested for their ability to phosphorylate histone III (Figure 4b). Consistent with previous work [8,9], no PKC activity was detected in fractions from *E. coli* expressing bTTT. In contrast, bacteria transformed with pTZ18R-TET displayed PKC activity which peaked in fractions 32–34. Immunoblot analysis of these fractions (Figure 4b inset) indicated that bTET co-eluted with PKC activity. Based upon Western-blot analysis the bTET activity was $\leqslant 2\%$ of mammalian-expressed wild type activity. An equivalent mutation replacing Thr-495 with an aspartic acid residue (TDT) was also prepared and expressed in *E. coli*. However, unlike bTET, bTDT did not display PKC activity (results not shown). These results indicate that the phosphorylation of bTTT necessary for its catalytic activity does not occur in *E. coli*, and show that the Glu substitution for Thr-495 is sufficient for the expression of an intrinsically active PKCα (bTET) while an Asp substitution (bTDT) is not.

Summary of PKCα mutants

The biochemical characterization of the different PKCα mutants is summarized in Table 1. These data reveal that AAA, ATA and TTA cannot phosphorylate exogenous substrate *in vitro*,

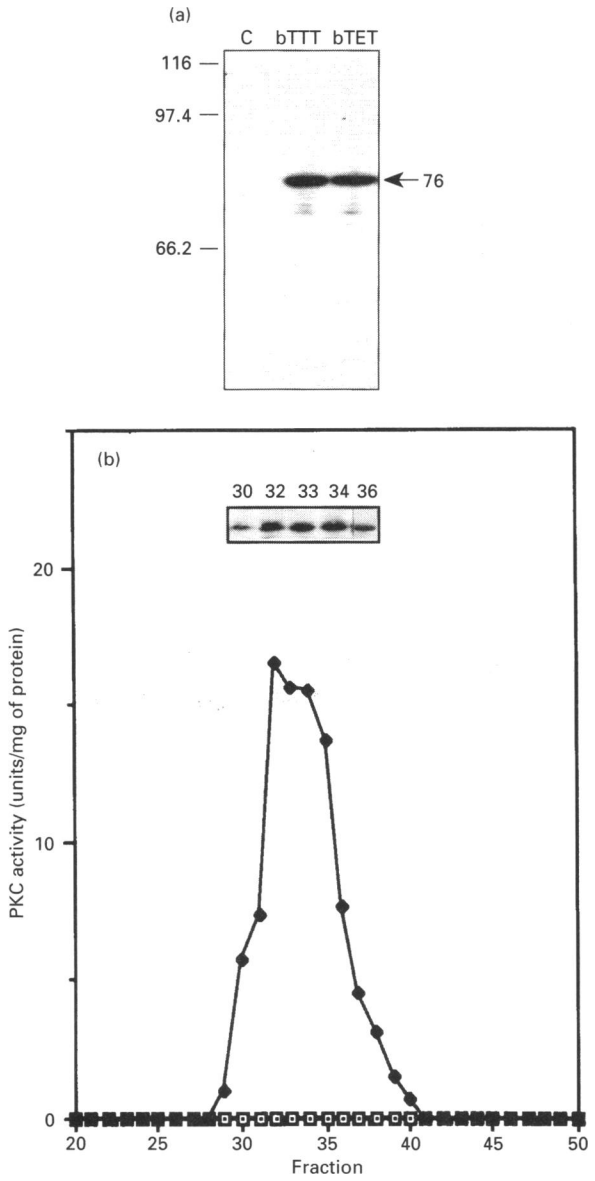

Figure 4 Characterization of a bacterially expressed PKCα mutant: bTET

Triton-soluble fractions of *E. coli* untransformed (C) or transformed with either pTZ18R-TTT or pTZ18R-TET were first analysed by immunoblotting. Molecular-mass markers (kDa) are shown on the left hand side and arrows indicate migration of the 76 kDa form of PKCα. Triton-soluble fractions containing either bTTT (□) or bTET (◆) were then subjected to Mono Q chromatography (**b**) as described in the Materials and methods section. Collected fractions were assayed for PKC activity using histone III-S as substrate. Units are expressed as a function of protein concentration. The presence of bTET in fractions 30, 32–34 and 36 was monitored by immunoblot (inset).

inferring that the substitution of Ala for Thr-497 is responsible for this lack of activity. This is also consistent with the TAA mutant which is likewise inactive (data not shown). Consistent with this conclusion, the PKCα mutants bearing a Thr at position 497 (AAT and ATT) were active kinases *in vitro*. We have shown previously that phosphorylation of the region containing Thr-494, Thr-495 and Thr-497 is a preliminary event for PKC activation. Altogether, these results strongly suggest that Thr-497 is the phosphorylated residue largely responsible for the permissive activation of PKC. The observation that AAT was about 2-fold less active than ATT could indicate that a subsequent phosphorylation of Thr-495 is also required to achieve a fully active PKCα (at least 80% of the wild-type PKCα activity), although we cannot exclude the possibility that Thr-495 might play an important role in the recognition of the Thr-497 site of phosphorylation by the 'PKC-kinase'.

The introduction of a glutamic acid residue at position 495 is sufficient to produce a catalytically active PKCα in bacteria albeit with a reduced specific activity. The inability of aspartic acid to perform the same function indicates that the longer glutamic acid side-chain makes some essential contact not made by aspartic acid. In view of this it is not possible to conclude that position 495 is a likely phosphorylation site, it is quite possible that the charge introduced at this site in some way mimics the effect of phosphorylation at a neighbouring Thr position. Such acidic mutations in regions of potentially clustered sites are clearly of limited value in defining the action of individual sites. However, the important issue here is that the introduction of a negative charge in this region of PKCα appears to be sufficient to confer activity on the bacterially expressed protein. This strongly supports the concept that this region is essential in the permissive activation of PKCα.

Comparative analysis of the amino acid sequence of the isotypes of PKC reveals that among the three Thr residues in this region of PKCα, only Thr-497 is conserved between all the members of the PKC family. This observation suggests that phosphorylation of this residue could be a common event for PKC activation. Indeed, we have shown previously that the post-translational modification of PKCα and PKCβ1 seems to be catalysed by a common 'PKC-kinase' [6]. Thr-497 is localized in the consensus sequence of subdomain VIII of the kinase superfamily [15] and phosphorylation or autophosphorylation of sites in this region have been reported to stimulate the enzymic activity of other protein serine/threonine kinases (cyclic AMP-dependent protein kinase, $p34^{cdc2}$ and $p42^{mapk}$) [16–18]. The role of a phosphorylation at this site is also evidenced by the observation that for several protein tyrosine kinases, autophosphorylation of the corresponding site (containing a Tyr residue) leads to increased catalytic activity [19]. The recent analysis of the crystal structure of the catalytic subunit of cyclic AMP-dependent protein kinase also supports the idea that phosphorylation of this site is essential for kinase activity [20].

In conclusion, the results demonstrate that replacement of Thr-497 by an alanine residue abrogates PKCα activity; implying that this is likely to be a critical site of phosphorylation. In addition, the subsequent phosphorylation or autophosphorylation of Thr-495 could prove to be important in obtaining a fully active kinase. Consistent with this conclusion, the presence of a negatively charged or phosphorylated residue in an equivalent region appears to be required for the catalytic activity of several protein kinases.

F.B. was supported by the Fondation pour la Recherche Médicale (FRM) and subsequently by a fellowship from the European Commission. P.J.P. also acknowledges concerted action support (Biomed I) from the European Commission. S.C. was supported by a fellowship from the European Community. We thank Lesley Polak for preparation of the manuscript.

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Received 18 November 1993/10 February 1994; accepted 22 February 1994