Phosphorylation of the protein kinase C-theta activation loop and hydrophobic motif regulates its kinase activity, but only activation loop phosphorylation is critical to *in vivo* nuclear-factor-κB induction

Yin LIU*, Caroline GRAHAM*, Aiqun LI†, Robert J. FISHER† and Stephen SHAW*[1]

*Experimental Immunology Branch, National Cancer Institute, Bethesda, MD 20892, U.S.A., and †Science Applications International Corporation (SAIC) Frederick, National Cancer Institute Frederick Cancer Research and Development Center (NCI-FCRDC), Frederick, MD 21702, U.S.A.

Protein kinase C (PKC)-theta, a member of the 'novel' subfamily of PKC isoforms, is of singular importance in transducing signals in T-lymphocytes. Since understanding of regulatory phosphorylation of novel PKCs is fragmentary and inconsistent with findings for 'classical' PKC isoforms, we investigated three potential phosphorylation sites on PKC-theta; in the activation loop (Thr^{538}), turn motif (Ser^{676}) and hydrophobic motif (Ser^{695}). Combined evidence from phospho-specific antisera and MS demonstrates phosphorylation at all three sites. Unlike its closest paralogue, PKC-delta, lack of negative charge in the activation loop of PKC-theta results in a profound catalytic defect (> 100-fold reduction in the T538A mutant); the high sequence similarity between PKC-theta and -delta assists in the formulation of structural hypotheses to account for this major difference. In contrast with mechanisms proposed for other PKC isoforms, phosphorylation at the other two sites does not reconstitute catalytic activity. Activation loop phosphorylation is critical *in vivo*, since the T538A mutant completely lost its capacity to mediate T-cell receptor-stimulation of nuclear factor κB (NF-κB) activation in Jurkat T-cells. Hydrophobic motif phosphorylation also substantially influences PKC-theta catalytic activity (5-fold reduction in the S695A mutant), but does not impair NF-κB activation in Jurkat T-cells. Its mechanism is independent of secondary effects on activation loop phosphorylation and cannot be explained by thermal instability. Turn motif phosphorylation has a limited effect on kinase activity, but negatively regulates other aspects of PKC-theta function, since the S676A mutant is more efficient than wild-type in inducing NF-κB activation in Jurkat T-cells. These findings expand our understanding of the roles of phosphorylation in novel PKCs, and indicate that PKC-theta is a constitutively competent kinase as a consequence of constitutive phosphorylation of its activation loop.

Key words: novel isoform, PKC, T-lymphocytes, turn motif.

INTRODUCTION

Protein kinase C (PKC)-theta is the most recently identified isoform of the PKC family; it is now the subject of concentrated investigation, since it has a special role in T-cell receptor-mediated activation of T-lymphocytes [1]. Among the PKC isoforms, PKC-theta is the only one to localize to the signalling complex that forms when the T-cell contacts a stimulator cell [2]. During T-cell activation, PKC-theta is critical for the induction of transcription of the T-cell growth factor interleukin-2 (IL-2) [3]; PKC-theta is unique among PKC isoforms in its ability to induce IL-2 in Jurkat cells [3,4], an intensively studied model of T-cell activation. Despite extensive analysis of functional roles of PKC-theta in intact cells, many important aspects of PKC-theta biochemistry remain unstudied, including its sites of phosphorylation and their functional importance.

Sites of regulatory phosphorylation of PKCs have only been studied extensively in the 'classical' subfamily of PKCs (which depend on calcium and diacylglycerol for activation); there are three well-studied sites in the C-terminal halves of PKC-alpha and PKC-beta that play fundamental roles in regulating protein function, including catalytic activity, localization and degradation [5]. PKC-theta belongs to a distinct subgroup of PKC isoforms, the 'novel' PKCs (which depend on diacylglycerol, but not calcium, for activation) [1]. The possibility of systematic differences in the roles of regulatory phosphorylation between the novel and classical families has been raised by findings with PKC-delta, the only novel PKC isoform whose phosphorylation has been relatively well studied. Most striking are differences regarding the activation loop phosphorylation site on a conserved threonine residue located within the catalytic cleft. In PKC-alpha and -beta, phosphorylation of this site is critical to catalytic activity [5,6]. In contrast phosphorylation of the corresponding threonine residue in PKC-delta is irrelevant to its catalytic activity [7]. The other two conserved PKC phosphorylation sites, referred to as the turn motif and the hydrophobic motif, are in the approximately 65-amino-acid C-terminal extension (also referred to as the 'tail'). The strongest evidence of the importance of the turn motif in kinase activity comes from studies of PKC-betaII, which suggest that phosphorylation of sites in the vicinity of the turn is necessary for kinase activity (based on lack of kinase activity in a triple-alanine mutant) [8] and sufficient for kinase activity in the absence of phosphorylation at the activation loop and hydrophobic sites [6]. The importance of this site has only been studied in one novel PKC, and the results from two different groups conflict with each other. Ser^{643} of PKC-delta (rat PKC numbering, equivalent to Ser^{645} in human) has been shown by one group to have 2–4-fold reduced kinase activity in constructs with a serine to alanine mutation [9], but by another group to be normal [10]. The hydrophobic motif does not

Abbreviations used: HA, haemagglutinin; IL-2, interleukin-2; KLH, keyhole-limpet haemocyanin; mAb, monoclonal antibody; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; NF-κB, nuclear factor κB; PDK1, 3-phosphoinositide-dependent protein kinase-1; PKA, protein kinase A; PKC, protein kinase C; SEAP, secreted form of human alkaline phosphatase; TAg, large T antigen.

[1] To whom correspondence should be addressed (e-mail sshaw@nih.gov).

contribute to regulation of kinase activity in classical PKC-alpha and only does so in classical PKC-betaII by influencing thermal stability; its functional importance to novel PKCs has not been studied in isolation, but may contribute to the regulation of PKC-delta kinase activity [11].

To understand the importance of phosphorylation of PKC-theta, in particular, and of novel PKCs, more generally, we have analysed the phosphorylation of the corresponding three potential sites in PKC-theta. Our studies demonstrate phosphorylation of each of the potential sites. Systematic analysis of an informative panel of mutants with regard to catalytic activity and phosphorylation at all three sites enables us to distinguish two distinct modes of regulation of catalytic activity by phosphorylation: at the activation loop and of the hydrophobic motif. Of these two, activation loop phosphorylation is of dominant importance in conferring competence for nuclear factor κB (NF-κB) activation in Jurkat cells. Turn motif phosphorylation is not important for catalytic activity, but contributes to negative regulation of NF-κB induction. These studies provide additional understanding of isoform-specific regulation of PKCs by phosphorylation, and the structural basis thereof.

EXPERIMENTAL

Reagents

Monoclonal antibodies (mAbs) and their sources were as follows: CD3 mAb 38.1 was provided by Dr Carl June (University of Pennsylvania Cancer Center, Philadelphia, PA, U.S.A.), CD28 mAb 9.3 was provided by Dr John Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.), haemagglutinin (HA) tag mAb was purchased from Babco (Denver, PA, U.S.A.), Myc tag mAb was obtained from Santa Cruz Biotechnology, and anti-(PKC-delta) T(P)505 antiserum was provided by Professor Peter Parker (Protein Phosphorylation Laboratory, Imperial Cancer Research Fund Laboratories, London, U.K.). PKC-alpha pseudosubstrate peptide was purchased from Life Technologies. PMA, ionomycin, calyculin A and okadaic acid were from Calbiochem. pEF β-galactosidase plasmid and the assay kit for β-galactosidase were obtained from ClonTech Laboratories. Alkaline phosphatase substrate *p*-nitrophenyl phosphate was from Sigma, and [γ-^{32}P]ATP was purchased from Amersham.

Expression plasmids

PKC-theta and -delta cDNAs were obtained by reverse transcriptase PCR from Jurkat-cell total RNA and cloned into T7 vectors. Each construct was tagged at its C-terminus with an influenza HA peptide. After complete sequencing, each construct was subcloned into a mammalian expression vector, pCEFL (Dr Silvio Gutkind, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, U.S.A.). The following site-directed mutants of wild-type PKC-theta were generated using the QuickChange site-directed mutagenesis kit from Stratagene: K409W, T538A, T538A/S695E, T538E, T538E/S695A, S676A, S676E, S695A, S695E, S657A/S676A, S662A/S676A, S676A/S685A and S657A/S662A/S676A/S685A. PKC-delta mutants T507A and T507E were generated in the same way. All mutants were completely sequenced. Myc-tagged 3-phosphoinositide-dependent protein kinase-1 (PDK1) cDNA in pcDNA3 was provided by Dr Leonard R. Stephens (Department of Development and Signalling, The Babraham Institute, Cambridge, U.K.). Secreted form of human alkaline phosphatase (SEAP) reporter constructs for NF-κB were provided by Dr Gerald Crabtree (Stanford University School of Medicine, Stanford, CA, U.S.A.).

Preparation of PKC-theta phospho-specific antibodies

PKC-theta phospho-peptides (MFRNFpSFMNPGC, hydrophobic motif peptide; CLNEKPRLpSFAKRAL, turn motif peptide; and CLGDAKTNpTF, activation loop peptide; where single-letter amino-acid notation has been used) were synthesized by Quality Control Biochemicals (Hopkinton, MA, U.S.A.). Each peptide (4 mg) was conjugated with Imject® maleimide-activated keyhole-limpet haemocyanin (KLH) or BSA from Pierce, using their recommended procedure. Three peptides with the same amino acid sequences as the above peptides, but without phosphate, were also synthesized and coupled to BSA by the same method. Three New Zealand white rabbits per immunogen were immunized with 100 μg of the peptide–KLH conjugate/animal at biweekly intervals. The first injection was with Freund's complete adjuvant, and the following injection was with Freund's incomplete adjuvant. Serum antibody titre was tested by ELISA in plates coated with peptide–BSA conjugate. The rabbit with the highest antibody titre was killed after 5–6 injections, and antisera were collected. When used for Western blotting, these antisera were diluted 1:1000 and their binding to non-phosphorylated protein was blocked by the presence of 1 μg of the corresponding non-phosphorylated peptide/ml.

HEK-293 T-cell culture and transfection

HEK-293 T-cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) containing 10 % (v/v) foetal calf serum. Gene transfection was accomplished using the calcium phosphate standard protocol [12]. Briefly, 1 day before the transfection, 1.5×10^6 cells were seeded into a 10 cm dish. Each dish was transfected with 10 μg of DNA. The cells were then harvested from the dishes by pipette and lysed with lysis buffer, 36 h after transfection.

Western-blot analysis

Samples in 1 × SDS sample buffer were resolved by SDS/PAGE [8 % (w/v) polyacrylamide], transferred on to nitrocellulose membranes, Western blotted with the appropriate antibody and developed with the ECL® kit from Amersham using the protocol recommended by the manufacturer. Chemiluminescence was recorded on film, and absorbance was determined and analysed using Gel-pro software (Media Cybernetics, Baltimore, MD, U.S.A.). To optimize quantification by Western-blot analysis, various volumes of sample were loaded to assure that all were within the linear range of the Western-blot quantification. Results shown are representative of at least three experiments.

PKC-theta *in vitro* immune complex kinase assay

HA-tagged PKC-theta constructs were transfected into HEK-293 T-cells. The cells were lysed with lysis buffer [50 mM Tris/HCl (pH 7.4), 1 % (v/v) Triton X-100, 150 mM NaCl, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, protease-inhibitor cocktail tablet from Boehringer Mannheim and 50 nM calyculin A], 36 h after transfection. The cell lysate was incubated with HA tag mAb and Protein G–Sepharose 4B beads for 2 h. The beads were washed three times with lysis buffer, and resuspended in kinase buffer [100 mM Hepes (pH 7.4), 20 mM $MgCl_2$, 1 mM $CaCl_2$, 200 μg/ml phosphatidylserine and 0.05 % Triton X-100]. For kinase assays, aliquots were transferred into microcentrifuge tubes, centrifuged, the supernatant aspirated and the volume reconstituted to 40 μl with kinase buffer

to which had been added 100 μM substrate peptide, 2 μCi of [γ-^{32}P]ATP, 20 μM ATP and 100 nM PMA. The assay was carried out at 30 °C for 5 min and was stopped by adding 10 μl of 25 % (w/v) trichloroacetic acid. After brief centrifugation, the supernatant was spotted on to P81 nitrocellulose paper. The paper was washed four times with 75 mM phosphoric acid, dried and radioactivity measured in a scintillation counter. In experiments testing thermal stability, 50 nM calyculin A was added to the kinase buffer to prevent potential dephosphorylation during preincubation at 25 °C or the kinase assay. For analysis of the kinase activity at lower temperatures the assay was carried out for 7 min at 15 °C or for 15 min on ice. Parallel aliquots of beads were analysed by Western blotting and the kinase activity of each sample was normalized to the amount of PKC-theta in the same sample by dividing kinase activity by relative PKC concentration determined by Western-blot analysis. The normalized kinase activity of the wild-type was taken as 100 % and the kinase activity of the mutants was expressed as a percentage of the wild-type activity. Results shown are representative of at least three experiments; error bars indicate standard errors of the mean of replicate samples.

NF-κB reporter assay

Simian virus 40 large T antigen (TAg)-transfected Jurkat-TAg cells were grown in 10 % (v/v) foetal calf serum/RPMI 1640 (Life Technologies). For transfection, 1×10^7 Jurkat-TAg cells in 0.4 ml of RPMI 1640 with 20 mM Hepes were mixed with appropriate plasmids (5 μg of NF-κB reporter construct, 5 μg of β-galactosidase construct in pEF, together with 10 μg of PKC-theta construct in pCEFL or empty pCEFL vector) and electroporated at 310 V and 950 μF. The cells were stimulated or not with CD3 mAb (clone 38.1 ascites; 1:1000 dilution) plus CD28 mAb (clone 9.3 ascites; 1:1000 dilution) and cultured for an additional 16 h, 24 h after transfection. After transfection and stimulation of Jurkat-TAg cells, the supernatant was harvested and heat-inactivated at 65 °C for 1 h to destroy endogenous alkaline phosphatase. After incubation with substrate *p*-nitrophenyl phosphate in Tris buffer at 24 °C for 12 h, absorbance was read at 405 nM. The remaining cells were lysed with lysis buffer [100 mM potassium phosphate (pH 7.8), 0.2 % Triton X-100 and 1 mM dithiothreitol], and β-galactosidase in the cells was quantified using the Chemiluminescence kit from ClonTech, following the manufacturer's procedures. Part of the cell lysate was used for Western blotting for analysis of expression of the constructs. Results of the reporter assay for the SEAP NF-κB construct were normalized by dividing by the expression level of β-galactosidase co-transfected with the reporter construct. For experiments analysing effects over a range of expression protein concentrations, cells were transfected with a range of DNA amounts (0.33, 1.0, 3.0 and 9.0 μg).

In-gel digestion of gel-separated PKC-theta

PKC-theta immunoprecipitated from transfected HEK-293 T-cells was resolved by SDS/PAGE. The Coomassie Brilliant Blue-stained gel was cleaned, dehydrated, dried and digested with trypsin (modified, sequence grade; Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) following standard procedures [13], with slight modification. The digestion was carried out at 37 °C for 16–20 h. The extracted peptides were pooled and dried with a SpeedVac, and 6 μl of 50 % (v/v) acetonitrile in water was added to dissolve the peptides for MS analysis.

Phosphatase treatment of peptides

Trypsin-digest supernatant (5 μl) was mixed with 1.5 μl of calf intestine phosphatase (1 unit/μl; New England Biolabs, Beverly, MA, U.S.A.) in 50 mM NH_4HCO_3 and incubated at 37 °C for 2.5 h. The reaction mixture was SpeedVac dried and redissolved in 3 μl of 50 % (v/v) acetonitrile in water for MS analysis.

MS

Analysis was performed using matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI–TOF-MS) with delayed extraction (Voyager-DE PRO; Perseptive Biosystems, Framingham, MA, U.S.A.). The matrix solution was a 2-fold dilution of a saturated solution of 2,5-dihydroxybenzoic acid (Sigma, St Louis, MO, U.S.A.) in 50 % (v/v) acetonitrile in water. Aliquots of 0.5 μl of the peptide mixture and 0.5 μl of the matrix solution were mixed on the sample plate and air-dried prior to analysis. All spectra were taken in reflectron mode. Masses were calibrated internally with peptides from trypsin autolysis.

Molecular modelling

Structural models of PKC-theta and -delta were generated by SwissModel using sequences of PKC-theta (GenBank® sequence identifier = 5453976) and PKC-delta (GenBank® sequence identifier = 5453970); SwissModel used regions of 110 solved kinase domain structures having sequence similarity of at least 30 % with the submitted sequence as templates. Graphical images were generated using Rasmol.

RESULTS

PKC-theta is constitutively phosphorylated at Thr^{538}, Ser^{676} and Ser^{695}

Multiple sequence alignment of PKC-theta and other PKC isoforms indicates that PKC-theta has strong sequence similarity to other PKC isoforms in the vicinity of the three phosphorylation sites found in other PKCs, including conservation of the pertinent serine and threonine residues: Thr^{538}, Ser^{676} and Ser^{695} (Figure 1). We generated phospho-specific antisera against the corresponding phosphopeptides to confirm that these residues are phosphorylated and to facilitate systematic analysis of the state of phosphorylation of PKC-theta at these three sites. The specificity of these antisera was confirmed using ELISA to determine binding of the antisera to phosphorylated peptides but not non-phosphorylated peptides (results not shown). Concurrently, we made PKC-theta constructs having mutations at these residues, replacing the threonine or serine with either an alanine residue, to prevent phosphorylation, or a glutamic acid residue, to mimic phosphorylation. The specificity of the antibodies was confirmed in Western-blot analysis by comparisons of reactivity with PKC proteins produced by wild-type and mutant constructs expressed in HEK-293 T-cells (Figure 2). Each recognized the region of PKC corresponding to the immunizing peptide, since they bound wild-type PKC-theta but failed to bind a PKC-theta construct having an alanine substitution at the corresponding site. Each failed to bind to kinase-dead PKC-theta (K409W), consistent with loss of phosphorylation of the corresponding site in the kinase-dead mutant (also see the Discussion section). Similarly, mutation to glutamic acid abolished antisera reactivity for two of the three antisera, but the anti-pS695 antisera did not distinguish between glutamic acid and phosphoserine. Mutation of the corresponding sites in classical PKCs increases the electrophoretic mobility of these

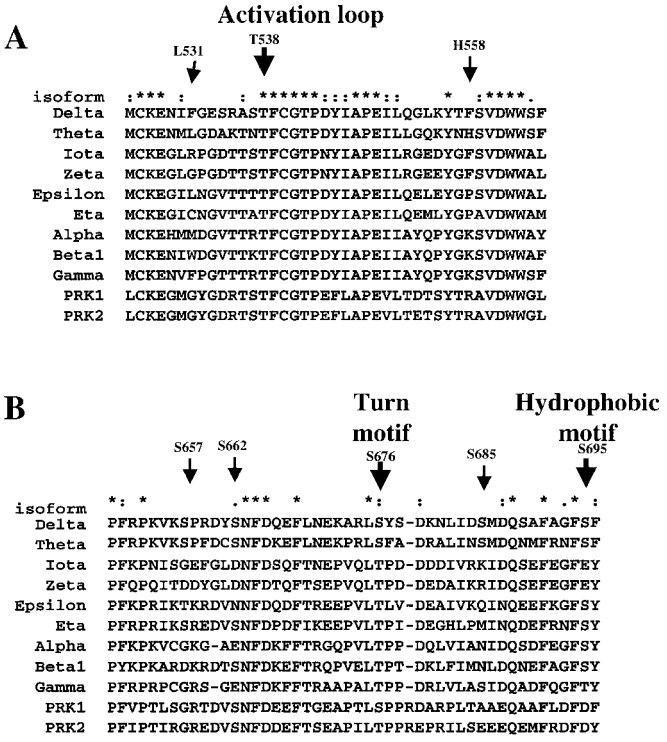

Figure 1 Multiple sequence alignment of human PKC family members at the activation loop and C-terminal extension

(**A**) Multiple sequence alignment of the activation loop. (**B**) Sequence alignment of the C-terminal extension. Numbers and arrows indicate the residues in PKC-theta that are referred to in the text. Degree of conservation within the family is indicated by symbols above the alignment: *, absolute conservation; :, strong conservation; and ·, weak conservation. PRK, protein kinase C-related kinase.

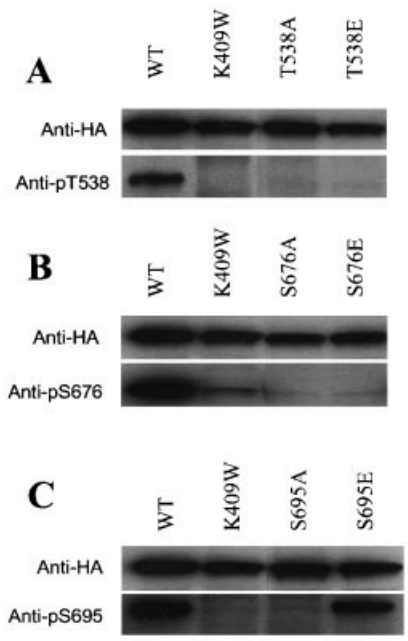

Figure 2 Specificity of anti-phosphopeptide antibodies

Antisera raised against phosphopeptides were tested for specificity by binding in Western blots to four PKC-theta constructs: wild-type (WT), kinase-dead (K409W), and constructs having mutations of the corresponding serine/threonine residue to alanine or glutamic acid. (**A**) Anti-pT538 antibody. (**B**) Anti-pS676 antibody. (**C**) Anti-pS695 antibody. Loading of gels was evaluated by parallel blots with anti-HA antibody.

PKC isoforms resolved by SDS/PAGE; no such alteration in electrophoretic mobility was observed in PKC-theta mutants (Figure 2).

MS analysis of tryptic digests of PKC-theta by MALDI–TOF-MS confirmed phosphorylation of the turn motif and the hydrophobic motif (Figure 3). Analysis revealed 58 peaks not attributable to trypsin, of which 35 matched PKC-theta within a delta p.p.m. of 50, giving a MOWSE score of 3×10^{15} (MOWSE refers to MOlecular Weight SEarch, see http://www.hgmp.mrc.ac.uk/Bioinformatics/Webapp/mowse/). Purity of the protein is evident from findings that the next closest matching human protein has only five matching peptides and a MOWSE score which is only 6×10^3. Peaks were found at the predicted masses for phosphorylated peptides corresponding to the turn and hydrophobic motifs, and at the predicted masses for unphosphorylated peptides following phosphatase treatment. The sequence assignment for the tryptic phosphopeptide in the hydrophobic motif was further confirmed by observation of its methionine-oxidized forms. No peaks were found corresponding to either phosphorylated or unphosphorylated activation loop, which is unremarkable given the approximately 62 % coverage of peptides we achieve and that the predicted masses for activation loop tryptic peptides are above the optimal size sensitivity of MALDI–TOF-MS and greater than any of the peptides that we have detected in this digest. Confirmation of activation loop phosphorylation was provided by Western-blot analysis with a commercial antibody specific for the predicted PKC-theta activation loop phosphorylation site (results not shown).

Activation loop Thr^{538} is critical to PKC-theta kinase activity, unlike PKC-delta

The *in vitro* kinase activity of mutant proteins was compared with that of wild-type PKC-theta (Figure 4). The most critical site was the activation loop. Mutation of the activation-loop threonine residue to alanine (T538A) resulted in more than a 100-fold decrease in activity. Mutation to glutamic acid (T538E) preserved much of the kinase activity. T538E was 30-fold more active than T538A, but still 3-fold less active than wild-type; this behaviour is consistent with glutamic acid providing a partial mimic of phospho-threonine. These findings are diametrically opposed to the published findings with PKC-delta, in which mutation of the corresponding activation-loop threonine residue to alanine does not impair catalytic activity [7]. To address the possibility that this difference reflects the anomalous behaviour of PKC-delta produced in bacteria, we made constructs of PKC-delta for expression in mammalian cells and analysed their catalytic activity. Our results confirm that PKC-delta produced in mammalian cells, unlike PKC-theta, does not require activation loop phosphorylation for catalytic activity (Figure 4). By Western-blot analysis we have confirmed that wild-type PKC-delta expressed in mammalian cells is phosphorylated at its activation loop (results not shown) as previously described [11], but findings from the mutant constructs indicate that this phosphorylation is not required for normal kinase activity [10].

Regulation of turn motif and hydrophobic motif phosphorylation

Phosphorylation of one site on PKC can influence phosphorylation of another site. We anticipated that phosphorylation of the

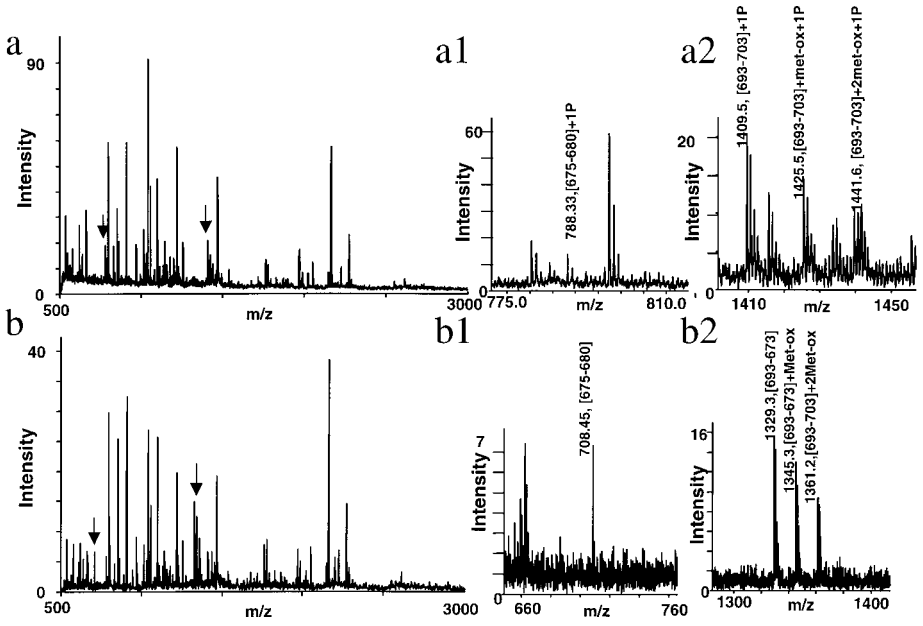

Figure 3 MS analysis demonstrates phosphorylation of Ser^{676} and Ser^{695} in PKC-theta

PKC-theta purified from transfected HEK-293 T-cells was digested with trypsin and analysed by MS. (**a**) Mass spectrum of trypsin digest and subregions containing peaks corresponding to peptide 675–680 (**a1**) and to peptide 693–703 (**a2**). (**b**) Mass spectrum following phosphatase treatment and subregions corresponding to peptide 675–680 (**b1**) and to peptide 693–703 (**b2**). Met-ox, methionine-oxidized.

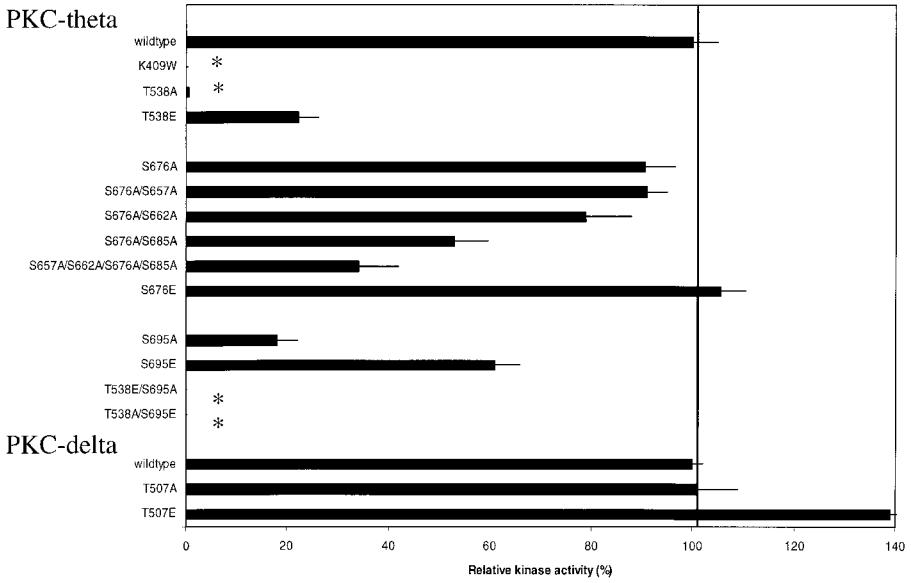

Figure 4 *In vitro* kinase activity of PKC-theta and -delta mutant constructs

Wild-type and mutant proteins were purified by immunoprecipitation from HEK-293 T-cells transfected with PKC-theta or -delta constructs. Catalytic activity was evaluated by an *in vitro* kinase assay and results are expressed as a percentage of the activity of wild-type. Four constructs highlighted with an asterisk had activity that was not distinguishable from the negative control (0.1–0.2% of wild-type).

turn motif would be markedly decreased in the T538A mutant, since the turn motif is an autophosphorylation site in all PKCs studied. Remarkably the T538A mutant, which has less than 1% of wild-type catalytic activity, maintains 20–50% of normal phosphorylation at the turn motif (Figure 5) (quantified by Western-blot analysis in multiple experiments). Predictions regarding phosphorylation of the hydrophobic motif were less certain, since the hydrophobic motif can be either a site of autophosphorylation or of phosphorylation by another kinase. We find that the T538A protein has completely lost phosphorylation of its hydrophobic motif. The simplest interpretation of these observations is that both the turn motif and the hydro-

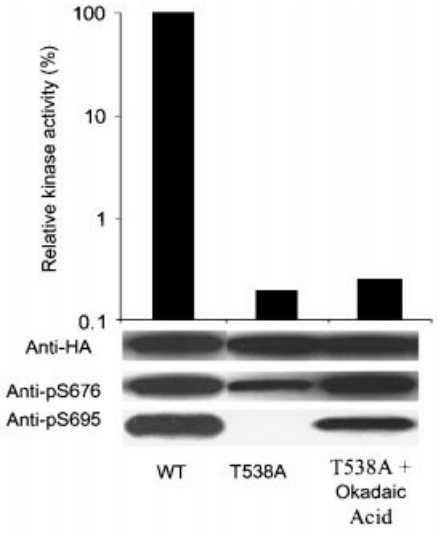

Figure 5 Okadaic acid treatment increases tail phosphorylation of T538A, but fails to increase its kinase activity

Lysates were prepared from HEK-293 T-cells transfected with wild-type (WT) and the T538A mutant PKC construct. Half the T538A-transfected cell culture was treated with 1 μM okadaic acid for 1 h. Part of the lysates was immunoprecipitated and evaluated for *in vitro* kinase activity. Kinase activity is represented as a percentage of wild-type. The remainder of the lysates was separated by SDS/PAGE. Phosphorylation of turn and hydrophobic motifs was evaluated by Western-blot analysis with anti-pS676 and anti-pS695 antibodies respectively. Loading of gels was evaluated by parallel blots with anti-HA antibody.

phobic motif are sites of autophosphorylation, but that the turn motif is an unusually favourable site of autophosphorylation (see the Discussion section).

Since the turn and hydrophobic motifs are hypophosphorylated in the T538A mutant, those secondary changes in phosphorylation may contribute to its catalytic defect. Indeed, Keranen et al. have demonstrated that phosphorylation of the turn motif of PKC-beta can overcome the catalytic defect caused by lack of activation loop phosphorylation [6]. Therefore we investigated whether phosphorylation of either turn or hydrophobic motifs in PKC-theta would reconstitute normal kinase activity in the T538A mutant. We observed that treatment of cells expressing the T538A mutant with the phosphatase inhibitor okadaic acid (Figure 5) increased phosphorylation of both the turn motif and the hydrophobic motif. However, there was no associated increase in catalytic activity. Since the extent of turn motif phosphorylation in the T538A mutant under these conditions resembles wild-type, these findings indicate that turn motif phosphorylation cannot substitute for activation loop phosphorylation to normalize kinase activity. However, the hydrophobic motif is still incompletely phosphorylated under these conditions, and thus its potential to substitute cannot be assessed with confidence. Therefore we constructed a double mutant, T538A/S695E, to test this possibility. Analysis of *in vitro* kinase activity (Figure 4) demonstrates that: (1) glutamic acid is a satisfactory mimic of hydrophobic motif phosphorylation (S695E); and (2) the defect of T538A/S695E in kinase activity is as profound as that of T538A, and therefore hydrophobic-site phosphorylation of PKC-theta is unlikely to be able to substitute for activation loop phosphorylation. Thus the greater than 100-fold reduction in kinase activity of the T538A mutant appears to be largely due to that mutation, and not an overestimate reflecting contributions from turn- and hydrophobic-motif hypophosphorylation.

Catalytic activity of turn mutants is close to normal

Replacement of the turn-motif serine residue with alanine (S676A) does not cause a measurable change in kinase activity (Figure 4). Studies of PKC-betaII [8] indicate that a single amino acid mutation at the turn motif does not reveal an abnormal phenotype because compensatory phosphorylation occurs at nearby residues. One additional serine/threonine residue (S685A) in PKC-theta is located within the region corresponding to the region of PKC-betaII in which compensatory phosphorylation has been demonstrated, and there are two other serine residues located 14 and 19 residues N-terminal to the turn motif (Ser^{662} and Ser^{657}). To analyse the roles of the additional serine residues, we generated three double-alanine mutant constructs (S676A mutation together with a second alanine mutation) and a quadruple-alanine mutant construct. None of these mutations, individually or in combination, caused a profound defect in kinase activity; rather there was a modest progressive reduction of kinase activity with each successive mutation (Figure 4).

Negative charge at the hydrophobic motif makes an important contribution to catalytic activity

In contrast with the turn motif, a single-alanine mutation at the hydrophobic motif (S695A) results in a 5-fold reduction of catalytic activity (Figure 4). We investigated whether or not this catalytic defect could be accounted for by known mechanisms: thermal instability or effects on activation loop phosphorylation. Thermal instability is a potential contributor to defective kinase activity, and has been observed in classical PKCs having mutations in the turn motif and the hydrophobic motif [14,15]. Two lines of evidence indicate that the defect in kinase activity in the S695A mutant is not primarily a result of thermal instability. First, reducing the temperature of the kinase assay did not restore its kinase activity relative to the activity of the wild-type (Figure 6A). Secondly, although the S695A mutant showed some enhanced thermal instability, that thermal instability was no more than the double-alanine mutant S676A/S685A and therefore the 2–3-fold greater loss of kinase activity by hydrophobic-site mutant S695A than double-turn mutant S676A/S685A cannot be attributed to thermal instability (Figure 6B).

Another potential mechanism whereby a mutation at the hydrophobic site could influence kinase activity would be from a secondary effect on phosphorylation of the activation loop. This is especially plausible since the most likely candidate kinase to mediate activation loop phosphorylation, PDK1, binds to other kinases via tail regions, including the hydrophobic motif [16,17] (also see the Discussion section). Therefore we investigated possible PDK1 association with PKC-theta, and found that PDK1 associates with PKC-theta (Figure 7A). This association is reduced by approximately half in both of the hydrophobic site mutants. We therefore investigated whether activation loop phosphorylation is influenced by hydrophobic site mutations (Figure 7B). The results indicate no major change in the extent of activation loop phosphorylation in these mutants. We used a genetic approach to directly confirm this interpretation; we evaluated whether the hydrophobic site S695A mutation influences kinase activity in the T538E construct, which is not dependent on activation loop phosphorylation. The results (Figure 4) demonstrate that this T538E/S695A double mutant

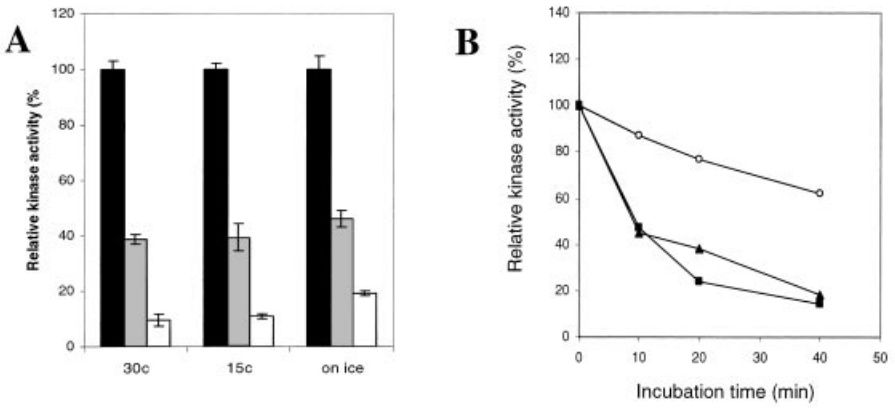

Figure 6 Decreased kinase activity of the S695A construct is not primarily due to thermal instability

(**A**) Relative kinase activity is largely independent of kinase assay incubation temperature. Constructs tested: wild type (black bars), S676A/S685A (grey bars) and S695A (white bars). c, °C. (**B**) The *in vitro* thermal stability of two PKC mutant constructs was compared with wild-type by determination of *in vitro* kinase activity following preincubation at 25 °C; 50 mM calyculin A was present to prevent dephosphorylation. The kinase activity is expressed as a percentage relative to the kinase activity of the same construct without preincubation. Constructs tested: wild-type (○), S695A (■) and S676A/S685A (▲).

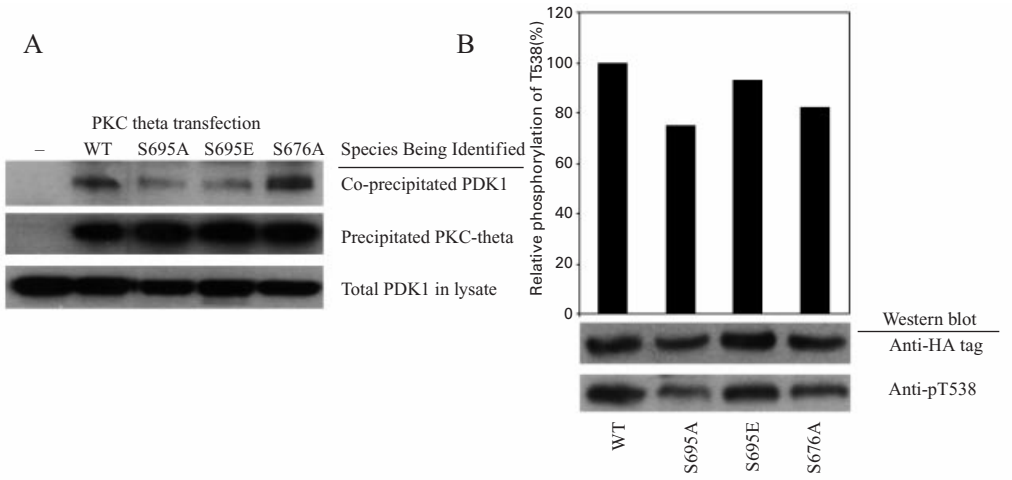

Figure 7 Limited effects of hydrophobic motif mutations on activation loop phosphorylation

(**A**) Association of PDK1 with PKC-theta was evaluated by analysis of co-precipitation from HEK-293 T-cells that were co-transfected with PDK1 and wild-type PKC-theta, mutant constructs of PKC-theta or nothing (as a control). Cell lysate was immunoprecipitated with anti-HA to precipitate PKC-theta, the product was resolved by SDS/PAGE and Western blotted with anti-HA ('precipitated PKC-theta') and anti-Myc ('co-precipitated PDK1'). Equal expression of PDK1 in each lysate was confirmed by anti-Myc Western-blot analysis of the total lysate. (**B**) Lysates from HEK-293 T-cells transfected with HA-tagged wild-type (WT) and mutant PKC constructs were separated by SDS/PAGE. Phosphorylation of the activation loop was evaluated by Western-blot analysis with anti-pT538 antibody. Loading of gels was evaluated by parallel blots with anti-HA antibody. Amounts of phosphorylation and of protein loading were determined by densitometry and relative levels of phosphorylation were calculated.

shows a severe defect in kinase activity, confirming a functional role for the hydrophobic site that is independent of regulating activation loop phosphorylation.

Only the mutation that mimics dephosphorylated activation loop has a defect in induction of NF-κB-mediated transcription in intact cells

To assess the relevance of these phosphorylation sites to the intracellular function of PKC-theta, we analysed the effects of these phosphorylation site mutations on PKC-theta-mediated induction of transcription of an NF-κB reporter. This function of PKC-theta is specifically inducible by the stimulation of the T-cell receptor in T-lymphocytes. To assay this function we employed a model system developed by Coudronniere et al. [3] in which PKC-theta is transfected into Jurkat T-cells together with an NF-κB reporter construct and the cell is stimulated with antibodies against the T-cell receptor complex (anti-CD3) and a co-stimulatory molecule (CD28) [3]. Transfection with wild-type PKC-theta causes an increase in the CD3/CD28-induced response above the baseline, attributable to the endogenous PKC-theta (Figure 8). Of the mutant constructs, only one has a defect in inducing NF-κB activation, namely the mutant that mimics activation-loop dephosphorylation (T538A). It is noteworthy that constructs with very substantial reduction in catalytic activity (e.g. 5-fold reduction in S695A and T538E) have no evident

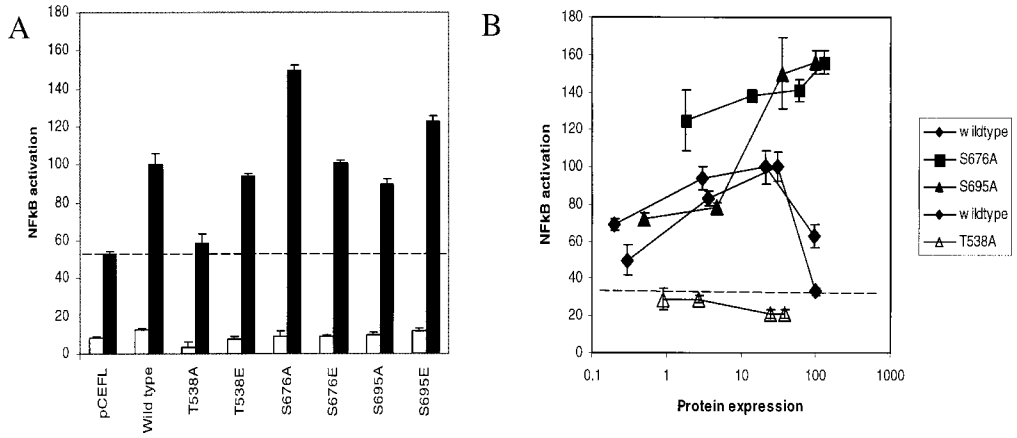

Figure 8 PKC-theta mediation of receptor-induced NF-κB activation is abrogated only in a mutant lacking negative charge in its activation loop

Jurkat T-cells were transfected with each of a panel of PKC-theta constructs in pCEFL (or empty pCEFL vector) together with two reporter constructs: an NF-κB reporter and an elongation factor-1 promoter-driven β-galactosidase reporter used for normalization. Results shown are NF-κB activation after normalization for β-galactosidase activity. A horizontal dashed line is drawn to highlight baseline for cells transfected with vector alone. (**A**) pCEFL construct (10 μg) was transfected and after 24 h of culture, the transfected Jurkat cells were either left untreated (white bars) or stimulated with a combination of CD3 and CD28 antibodies to mimic physiological T-cell activation (black bars). Results shown are representative of three experiments. (**B**) Jurkat cells were transfected with a range of DNA concentrations and the results plotted as a function of the relative amount of PKC protein expressed. The results shown are combined from two experiments; there was a consistent response to the wild-type constructs in both experiments.

defect in NF-κB induction in this *in vivo* model system; indeed, some appeared to have increased capacity to induce NF-κB.

We refined the functional analysis by titrating the amount of DNA transfected over a 27-fold range for an informative subset of the constructs, since *in vivo* function would be expected to be sensitive to levels of expression of the transfected proteins. This approach was applied to constructs having alanine mutations at each of the phosphorylation sites and the results show that this strategy results in informative titration of expressed proteins; analysis of the observed NF-κB activity confirms and extend the simpler analysis. Wild-type PKC-theta is most effective in mediating NF-κB activation at intermediate levels of expression. T538A is unable to induce NF-κB activity statistically greater than pCEFL control, demonstrating a profound defect in its *in vivo* function. It should be noted that T538A does not reduce the activity level much below that of the normal endogenous response, i.e. it does not function efficiently as a dominant negative. In keeping with the simpler analysis, the S676A mutant is strikingly more efficient in inducing NF-κB, since it requires a tenth as much expressed S676A to induce a better NF-κB response than wild-type. Finally, despite the substantial decrease in *in vitro* kinase activity of S695A, it is at least as efficient as wild-type in inducing NF-κB activation. The simplest interpretation is that although PKC-theta catalytic activity is required for the response it is not the primary rate-limiting parameter. Moreover, PKC-theta phosphorylation at the turn and hydrophobic motifs may play additional roles beyond regulation of kinase activity.

DISCUSSION

Activation loop regulation of kinase activity

Our results highlight the critical role of a negative charge at the activation loop Thr^{538} in PKC-theta. It is critical in: determining catalytic activity (Figure 4); regulating phosphorylation of the hydrophobic motif (and to a lesser extent the turn motif) (Figure 5); and enabling PKC-theta to mediate CD3-induced NF-κB activation (Figure 8). The simplest interpretation is that the primary effect of Thr^{538} phosphorylation is a several log increase in catalytic activity, which in turn regulates phosphorylation of the tail, and also regulates NF-κB activation in intact T-lymphocytes.

The catalytic requirement for activation loop phosphorylation in PKC-theta is strikingly different from the phosphorylation independence of PKC-delta catalytic activity. The functional difference was not predictable based on previously proposed structural hypotheses [7]. Given the strong sequence similarity (87% in the kinase domain), the limited number of amino acid substitutions provides a favourable context in which to make predictions regarding the structural basis of the functional difference. Analysis of the location of differences in amino acid residues between them (Figure 9A and results not shown) indicates that there are: (1) no differences in the buried amino acids or the surfaces of the catalytic cleft; (2) scattered differences in surface amino acids distant from the catalytic cleft; and (3) limited differences in the activation loop and residues of subdomain VIII immediately N-terminal to the activation loop (sequence in Figure 1; model in Figure 9B). Because stabilization of the activation loop is the proposed function of the threonine phosphorylation, the changes most likely to influence it would be residues in the activation loop, or residues contacting it. Based on the analysis outlined briefly below, we propose that changes of PKC-theta Leu^{531} and His^{558} to phenylalanine residues in PKC-delta may be major contributors to the difference. Among residues contacting the activation loop, only His^{558} differs between PKC-theta and -delta. Molecular modelling of PKC-theta predicts that His^{558} is positioned closest to Leu^{531} in the N-terminus of the activation loop, but no especially favourable interaction between them is apparent. These two residues in PKC-theta (His^{558} and Leu^{531}) are replaced with phenylalanine residues in PKC-delta (Phe^{527} and Phe^{500}). With minimal realignment of their side chains, these phenylalanine residues in PKC-delta would provide a very strong hydrophobic interaction between the N- and C-termini of the activation loop in PKC-

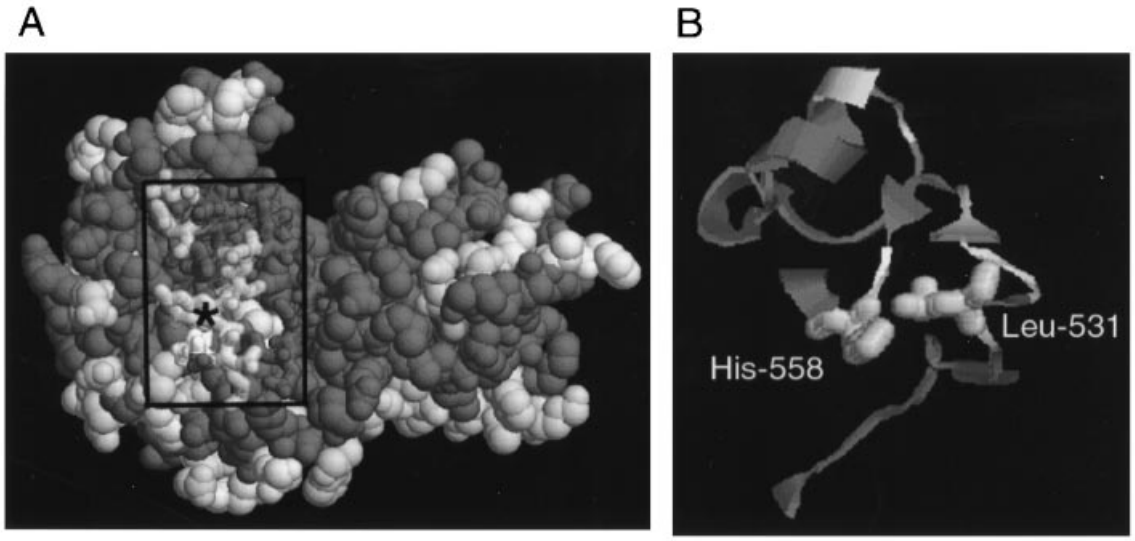

Figure 9 The region in the PKC-theta activation loop that is predicted to be most affected by changes in PKC-delta

(**A**) Structural model of the PKC-theta kinase domain constructed by SwissModel. The C-terminal lobe is on the left-hand side and the N-terminal lobe is on the right-hand side. Residues identical between PKC-theta and -delta are shown in dark grey, and ones that differ are shown in light grey. The boxed region includes the vicinity of the activation loop (amino acids 520–560), whose residues are shown as wireframe rather then spacefill. The asterisk marks the region of His^{558}/Leu^{531}. (**B**) Isolated ribbon drawing of amino acids 520–560 of PKC-theta, which include the activation loop and residues C-terminal to it. To better reveal the proximity of His^{558}/Leu^{531} the image is rotated 60° about the *y*-axis and −60° about the *x*-axis relative to (**A**) (i.e. viewer moves down and to the left). Darker grey regions are identical between PKC-theta and -delta, whereas lighter grey regions differ between them. Side chains of two residues in PKC-theta that are substituted by phenylalanine in PKC-delta are shown with wireframe representation.

delta, thereby stabilizing its activation loop. It should be noted that a bound sulphate or phosphate ion is present at the corresponding position in other kinases that do not require phosphorylation [18,19]; this ion may also be present in PKC-delta and facilitate positioning. Strong activation loop phosphorylation dependence has been demonstrated for PKC-alpha and -betaII [20,21]; neither of them have the combination of hydrophobic residues at these positions that we postulate account for PKC-delta's lack of dependence.

Requirement for activation loop phosphorylation is context-dependent

Although the T500A mutation data for PKC-betaII demonstrate a critical role for negative charge at this position, there is at least one circumstance in which activation loop phosphorylation of PKC-betaII is not critical for catalytic activity. Baculovirus produced PKC-betaII is fully active when subjected to selective phosphatase treatment that preserves its turn motif phosphorylation but eliminates activation loop and hydrophobic motif phosphorylation [6,22]. One possible interpretation is that, for some isoforms, turn motif phosphorylation substitutes for activation loop phosphorylation to stabilize an active conformation. Thus the requirement for activation loop phosphorylation is not an absolute structural requirement, but can be modified by other post-translational modifications and potentially even environmental influences. As a result, in our studies we investigated several relevant contexts to determine how stringent the requirements are for activation loop phosphorylation for PKC-theta. The requirements are stringent for *in vitro* kinase activity; it cannot be replaced by phosphorylation at either the turn motif or at the hydrophobic motif. The requirement is also stringent for *in vivo* PKC-theta function; our studies of PKC-theta function in NF-κB-mediated transcription in T-cells affirm the critical role of negative charge at the activation loop in its physiological function. In contrast with the simplest predictions, the T538A mutant did not function efficiently as a dominant negative for NF-κB induction when transfected into Jurkat cells (Figure 8). We favour the interpretation that its lack of phosphorylation at the activation loop (prevented by T538A mutation), and/or at the hydrophobic motif (secondary to defective kinase activity) prevents interactions that are necessary to play the role of a decoy.

Since activation loop phosphorylation is important to PKC-theta function, it is important to understand whether that phosphorylation is constitutive or regulated. Our studies demonstrate that PKC-theta is constitutively phosphorylated in transfected HEK-293 T-cells; studies of PKC-theta in T-lymphocytes indicate that it is also constitutively phosphorylated in T-lymphocytes (results not shown). The term 'competence' is used to describe a kinase that is capable of catalysis, even if its catalytic activity is dormant as a result of other inhibitory interactions. PDK1 has been implicated in phosphorylating other PKCs on their activation loops, thereby making them competent [16,17]; in the present study we demonstrate physical association of PKC-theta with PDK1, suggesting that PDK1 is responsible for the constitutive activation loop phosphorylation of PKC-theta. Based on the findings presented here, we refer to PKC-theta as a 'constitutively competent kinase' in which constitutive activation loop phosphorylation confers catalytic competence. Although the three sites are generally phosphorylated on the wild-type PKC-theta construct under the conditions studied, several lines of evidence suggest that its phosphorylation will prove to be dynamically regulated under particular circumstances *in vivo*. For example, conditions have now been identified under which phosphorylation of the activation loop and hydrophobic motif of PKC-delta are dramatically regulated [11]. Furthermore, our studies with mutant PKC-theta constructs

indicate regulation of turn and hydrophobic motif phosphorylation by phosphatases (see below).

Phosphorylation at turn and hydrophobic motifs is regulated by the activation loop

The consensus view is that *cis*-autophosphorylation mediates PKC phosphorylation at its turn motif [5]. Our findings that turn motif phosphorylation is virtually lost in the 'kinase-dead' PKC-theta mutant (K409W), is fully consistent with *cis*-autophosphorylation. Since the turn motif in the T538A mutant is substantially phosphorylated (20–50 % of normal) and becomes normally phosphorylated in the presence of okadaic acid, we conclude that low-level catalytic activity (< 1 % of wild-type) allows PKC-theta to autophosphorylate at the turn motif. Phosphorylation in the presence of such poor catalytic activity is most likely explained by the easy accessibility of the turn motif to the catalytic groove resulting in an effective high local concentration driving the catalysis. For PKC-theta and PKC-delta (but not other PKCs) this is probably facilitated by having a positively charged residue (arginine) located two residues N-terminal to the turn motif (Figure 1), since this is a common feature of PKC substrates. Assuming T538A is a reasonable model for newly translated PKC-theta (i.e. unphosphorylated at Thr^{538}), these findings indicate that newly translated PKC is able to autophosphorylate its turn motif. They are consistent with findings that PKC-delta produced in bacteria is not phosphorylated at its activation loop, but is nevertheless phosphorylated at its turn motif [5]. Thus models of ordered phosphorylation of PKC-theta and PKC-delta must allow for the possibility that turn motif phosphorylation may occur before activation loop phosphorylation, which differs from some prevailing models [5]. Our findings also indicate that PKC-theta turn motif phosphorylation is subject to regulation by phosphatase activity, as has been demonstrated for other PKC isoforms [15].

Regarding phosphorylation of the hydrophobic motif of PKCs, the relative contributions of *cis*-autophosphorylation and *trans*-phosphorylation are currently debated. The ability of PKC-betaII to mediate concentration-independent autophosphorylation (pseudo zero-order kinetics) of its hydrophobic motif strongly argues for *cis*-autophosphorylation [23]; similar results are found for *cis*-autophosphorylation of the hydrophobic motif of a related kinase, protein kinase B [24]. However, Ziegler et al. [25] have demonstrated that PKC-alpha phosphorylation at the hydrophobic site can be mediated by an atypical PKC complex, and mammalian target of rapamycin ('mTOR') is involved in the regulation of phosphorylation of that site on novel PKCs [11]. Our findings regarding PKC-theta do not exclude *trans*-phosphorylation, but are more simply explained by *cis*-autophosphorylation. The finding that only the two constructs (K409W and T538A) with several log decreases in kinase activity fail to undergo phosphorylation at the hydrophobic motifs is consistent with *cis*-autophosphorylation. The partial phosphorylation in the presence of okadaic acid of the hydrophobic motif of mutant T538A (with weak catalytic activity) but not K409W (with virtually no activity) is consistent with such *cis*-autophosphorylation being balanced by phosphatase activity. It is noteworthy that the hydrophobic motif of PKC-theta has a positively charged residue at the -3 position (Figure 1), which is a rather universal preferred element in PKC substrate specificity [26]; this would be expected to increase the likelihood of *cis*-autophosphorylation compared with the majority of PKC isoforms which lack this positively charged residue.

It is noteworthy that the kinase-dead mutant K409W is unphosphorylated not only at the turn and hydrophobic motifs, but also at the activation loop (Figure 3). Our results indicate that this is not due to defective association with PDK1, but rather suggests susceptibility to endogenous phosphatases (results not shown). These results are consistent with findings regarding other isoforms that phosphorylation at the activation loop is more susceptible to phosphatase in the absence of phosphorylation at the hydrophobic motif [15].

Functions of turn and hydrophobic motif phosphorylation

Most studies of the importance of negative charge at the turn motif of PKCs indicate that it plays a limited role in the kinase activity, although it influences other properties, such as thermal stability, detergent solubility and susceptibility to proteolysis/phosphatases [5]; for example, single-site mutants of PKC-delta [10] and of PKC-alpha [15] have normal kinase activity. Our studies of the turn motif in PKC-theta, including a quadruple-alanine mutant indicate that phosphorylation plays at most a small role in the *in vitro* functions we have assessed: kinase activity, thermal stability, and regulation of phosphorylation of the activation loop and the hydrophobic motif. Thus only for PKC-beta II is there evidence for an important role of the turn motif in regulating kinase activity based on findings with a triple-alanine mutant [8].

Most studies of the importance of negative charge at the hydrophobic motif of PKC indicate that it plays a significant role in the regulation of kinase activity. Studies of PKC-betaII demonstrate an overall importance similar in magnitude to that which we observe with PKC-theta: reduction of maximal activity by approx. 3-fold in an alanine mutant and an intermediate phenotype of a glutamic acid mutant [14]. In contrast with the findings with PKC-betaII, our results indicate that in PKC-theta the defect in catalytic activity is not mediated primarily by thermal instability (Figure 6). The role of hydrophobic motif phosphorylation in other novel PKCs has not been analysed by site-specific mutations, but analysis of variations in the phosphorylation state of the hydrophobic motif in PKC-delta suggest that its phosphorylation is required for optimal catalytic activity [11].

No structural basis has been established for hydrophobic motif phosphorylation-mediated regulation of kinase activity. However, we have considered two structural possibilities, one of which we have disproved and the other is the subject of ongoing experiments. First, the residues in the hydrophobic motif are critical for binding of PDK1 to kinase tails, which in turn could influence activation loop phosphorylation. However, this is not the structural basis for the defect, since even when negative charge is present at the activation loop of PKC-theta (our data) or of PKC-delta [11], lack of negative charge at the hydrophobic site impairs catalytic activity. Our second structural hypothesis is based on analogy to the solved structure of protein kinase A (PKA); we propose that the PKC hydrophobic motif is involved in an intramolecular interaction with a hydrophobic pocket N-terminal lobe of its own kinase domain, analogous to that described for PKA [27–29]. Recent studies indicate that peptide binding to the analogous pocket of PDK1 regulates PDK1 kinase activity [29]. Therefore we propose that the hydrophobic motif phosphate group acts to stabilize the N-terminal lobe of the kinase domain in an optimal catalytic conformation. Such a mechanism could provide a plausible explanation for the more than additive effect of the T538E/S695A mutant (Figure 4).

An emerging theme in studies of phosphorylation of the tail of PKCs is their role in regulating localization of PKCs. In several classical PKC isoforms lack of negative charge either at the turn or hydrophobic motifs causes the protein to associate with the

detergent-insoluble fraction [6,15]. Moreover, two groups have demonstrated that specific tail phosphorylations (or mutational surrogates) inhibit membrane association in intact cells. Hydrophobic motif dephosphorylation (mimicked by alanine mutation) favours membrane association of PKC-betaII [30,31]; similarly, phosphorylation of Thr^{613} of PKC Apl I (7 amino acids N-terminal to the turn motif) inhibits membrane translocation [32]. It is therefore intriguing that our PKC-theta construct with an alanine mutation at its turn motif (T676A) shows reproducible enhancement of NF-κB activation (Figure 8). The mechanism for this enhancement is under investigation.

We thank Dr Carl June, Dr John Hansen and Professor Peter Parker for antibodies; Dr Silvio Gutkind, Dr Leonard Stephens and Dr Gerald Crabtree for constructs; and Dr Charles King and Dr John Rivera for constructive critique of the manuscript.

REFERENCES

1 Altman, A., Isakov, N. and Baier, G. (2000) Protein kinase C-theta: a new essential superstar on the T-cell stage. Immunol. Today **21**, 567–573
2 Monks, C. R., Kupfer, H., Tamir, I., Barlow, A. and Kupfer, A. (1997) Selective modulation of protein kinase C-theta during T-cell activation. Nature (London) **385**, 83–86
3 Coudronniere, N., Villalba, M., Englund, N. and Altman, A. (2000) NF-kappa B activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-theta. Proc. Natl. Acad. Sci. U.S.A. **97**, 3394–3399
4 Bauer, B., Krumbock, N., Ghaffari-Tabrizi, N., Kampfer, S., Villunger, A., Wilda, M., Hameister, H., Utermann, G., Leitges, M., Uberall, F. and Baier, G. (2000) T cell expressed PKCtheta demonstrates cell-type selective function. Eur. J. Immunol. **30**, 3645–3654
5 Parekh, D. B., Ziegler, W. and Parker, P. J. (2000) Multiple pathways control protein kinase C phosphorylation. EMBO J. **19**, 496–503
6 Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995) Protein kinase C is regulated *in vivo* by three functionally distinct phosphorylations. Curr. Biol. **5**, 1394–1403
7 Stempka, L., Girod, A., Muller, H. J., Rincke, G., Marks, F., Gschwendt, M. and Bossemeyer, D. (1997) Phosphorylation of protein kinase Cdelta (PKCdelta) at threonine 505 is not a prerequisite for enzymatic activity. Expression of rat PKCdelta and an alanine 505 mutant in bacteria in a functional form. J. Biol. Chem. **272**, 6805–6811
8 Edwards, A. S., Faux, M. C., Scott, J. D. and Newton, A. C. (1999) Carboxyl-terminal phosphorylation regulates the function and subcellular localization of protein kinase C betaII. J. Biol. Chem. **274**, 6461–6468
9 Li, W., Zhang, J., Bottaro, D. P. and Pierce, J. H. (1997) Identification of serine 643 of protein kinase C-delta as an important autophosphorylation site for its enzymatic activity. J. Biol. Chem. **272**, 24550–24555
10 Stempka, L., Schnolzer, M., Radke, S., Rincke, G., Marks, F. and Gschwendt, M. (1999) Requirements of protein kinase Cdelta for catalytic function. Role of glutamic acid 500 and autophosphorylation on serine 643. J. Biol. Chem. **274**, 8886–8892
11 Parekh, D., Ziegler, W., Yonezawa, K., Hara, K. and Parker, P. J. (1999) Mammalian TOR controls one of two kinase pathways acting upon nPKCdelta and nPKCepsilon. J. Biol. Chem. **274**, 34758–34764
12 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13 Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T. and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. Nature (London) **379**, 466–469
14 Edwards, A. S. and Newton, A. C. (1997) Phosphorylation at conserved carboxyl-terminal hydrophobic motif regulates the catalytic and regulatory domains of protein kinase C. J. Biol. Chem. **272**, 18382–18390
15 Bornancin, F. and Parker, P. J. (1996) Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase Calpha. Curr. Biol. **6**, 1114–1123
16 Vanhaesebroeck, B. and Alessi, D. R. (2000) The PI3K–PDK1 connection: more than just a road to PKB. Biochem. J. **346**, 561–576
17 Toker, A. and Newton, A. C. (2000) Cellular signaling: pivoting around PDK-1. Cell (Cambridge, Mass.) **103**, 185–188
18 Xu, R. M., Carmel, G., Sweet, R. M., Kuret, J. and Cheng, X. (1995) Crystal structure of casein kinase-1, a phosphate-directed protein kinase. EMBO J. **14**, 1015–1023
19 Skamnaki, V. T., Owen, D. J., Noble, M. E., Lowe, E. D., Lowe, G., Oikonomakos, N. G. and Johnson, L. N. (1999) Catalytic mechanism of phosphorylase kinase probed by mutational studies. Biochemistry **38**, 14718–14730
20 Cazaubon, S., Bornancin, F. and Parker, P. J. (1994) Threonine-497 is a critical site for permissive activation of protein kinase C alpha. Biochem. J. **301**, 443–448
21 Orr, J. W. and Newton, A. C. (1994) Intrapeptide regulation of protein kinase C. J. Biol. Chem. **269**, 8383–8387
22 Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A. and Newton, A. C. (1994) *In vivo* regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. J. Biol. Chem. **269**, 29359–29362
23 Behn-Krappa, A. and Newton, A. C. (1999) The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation. Curr. Biol. **9**, 728–737
24 Toker, A. and Newton, A. C. (2000) Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. J. Biol. Chem. **275**, 8271–8274
25 Ziegler, W. H., Parekh, D. B., Le Good, J. A., Whelan, R. D., Kelly, J. J., Frech, M., Hemmings, B. A. and Parker, P. J. (1999) Rapamycin-sensitive phosphorylation of PKC on a carboxy-terminal site by an atypical PKC complex. Curr. Biol. **9**, 522–529
26 Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z. and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. J. Biol. Chem. **272**, 952–960
27 Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science (Washington, D.C.) **253**, 414–420
28 Batkin, M., Schvartz, I. and Shaltiel, S. (2000) Snapping of the carboxyl terminal tail of the catalytic subunit of PKA onto its core: characterization of the sites by mutagenesis. Biochemistry **39**, 5366–5373
29 Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M., Currie, R. A. and Alessi, D. R. (2000) Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. EMBO J. **19**, 979–988
30 Feng, X., Becker, K. P., Stribling, S. D., Peters, K. G. and Hannun, Y. A. (2000) Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation. J. Biol. Chem. **275**, 17024–17034
31 Feng, X. and Hannun, Y. A. (1998) An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. J. Biol. Chem. **273**, 26870–26874
32 Nakhost, A., Dyer, J. R., Pepio, A. M., Fan, X. and Sossin, W. S. (1999) Protein kinase C phosphorylated at a conserved threonine is retained in the cytoplasm. J. Biol. Chem. **274**, 28944–28949

Received 27 June 2001/20 September 2001; accepted 5 November 2001