Protein kinase C is regulated *in vivo* by three functionally distinct phosphorylations

Lisa M. Keranen, Erica M. Dutil and Alexandra C. Newton

Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0640, USA.

Background: Protein kinase Cs are a family of enzymes that transduce the plethora of signals promoting lipid hydrolysis. Here, we show that protein kinase C must first be processed by three distinct phosphorylations before it is competent to respond to second messengers.

Results: We have identified the positions and functions of the *in vivo* phosphorylation sites of protein kinase C by mass spectrometry and peptide sequencing of native and phosphatase-treated kinase from the detergent-soluble fraction of cells. Specifically, the threonine at position 500 (T500) on the activation loop, and T641 and S660 on the carboxyl terminus of protein kinase C βII are phosphorylated *in vivo*. T500 and S660 are selectively dephosphorylated *in vitro* by protein phosphatase 2A to yield an enzyme that is still capable of lipid-dependent activation, whereas all three residues are dephosphorylated by protein phosphatase 1 to yield an inactive enzyme. Biochemical analysis reveals that protein kinase C autophosphorylates on S660, that autophosphorylation on S660 follows T641 autophosphorylation, that autophosphorylation on S660 is accompanied by the release of protein kinase C into the cytosol, and that T500 is not an autophosphorylation site.

Conclusions: Structural and biochemical analyses of native and phosphatase-treated protein kinase C indicate that protein kinase C is processed by three phosphorylations. Firstly, trans-phosphorylation on the activation loop (T500) renders it catalytically competent to autophosphorylate. Secondly, a subsequent autophosphorylation on the carboxyl terminus (T641) maintains catalytic competence. Thirdly, a second autophosphorylation on the carboxyl terminus (S660) regulates the enzyme's subcellular localization. The conservation of each of these residues (or an acidic residue) in conventional, novel and atypical protein kinase Cs underscores the essential role for each in regulating the protein kinase C family.

Current Biology 1995, **5**:1394–1403

Background

Phosphorylation plays a central role in regulating the structure and function of protein kinases [1]. Since the discovery almost 40 years ago that phosphorylase kinase is activated by phosphorylation [2], a plethora of kinases have been shown to be modulated by phosphorylation. Elucidation of the kinase architecture [3] revealed the structural basis for one of these phosphorylations: first protein kinase A, and then cdk2 kinase, MAP kinase and the insulin receptor kinase were shown to have a loop near the entrance to the catalytic site whose phosphorylation was required for catalytic activity [3–6]. Biochemical and structural information have indicated that electrostatic interactions between the negative charge on the activation loop and the residues around the active site correctly align the residues that are involved in catalysis [1].

Protein kinase Cs are a family of kinases that transduce signals promoting lipid hydrolysis [7]. Pulse–chase experiments by Fabbro and coworkers [8] provided the first indication that protein kinase Cs are phosphorylated *in vivo*. They reported that protein kinase C α is first synthesized as an inactive, dephosphorylated precursor with an apparent molecular weight of 74 kDa, which is chased to a transient 77 kDa phospho-form and then to a final 80 kDa 'mature' form. Threonine-to-alanine mutagenesis [9] and molecular modeling based on the structure of the catalytic domain of protein kinase A [10] identified the activation loop of protein kinase C as a likely candidate for phosphorylation. Mutagenesis of the threonine at position 497 (T497) to alanine in protein kinase C α [11], and of the corresponding T500 to valine in protein kinase C βII [10], resulted in inactive kinase, whereas the replacement of T500 with glutamate in protein kinase C βII resulted in cofactor-activatable enzyme [10]. These results confirm the importance of negative charge at this specific position on the activation loop. (Mutation of the neighbouring T495 to glutamate in protein kinase C α resulted in detectable, but low (≤ 2 % of the mammalian-expressed wild-type enzyme) activity of bacterially-expressed enzyme [11].) However, at least one other phosphorylation was indicated from the pulse–chase experiments [8], and from peptide sequencing which had revealed that T641 on the carboxyl terminus is phosphorylated *in vivo* [12].

Most recently, differential dephosphorylation by the catalytic subunits of protein phosphatases 1 (PP1) and 2A (PP2A) has supported the modification of protein kinase C on at least two positions [13]. PP1 dephosphorylates protein kinase C to yield an inactive protein that has a significant increase in electrophoretic mobility relative to the native enzyme. PP2A dephosphorylates protein kinase C to yield a protein that displays a smaller increase in electrophoretic mobility and that can re-autophosphorylate to regain its native mobility.

Correspondence to: Alexandra C. Newton. E-mail address: anewton@ucsd.edu

This contribution reveals that protein kinase C is regulated by three functionally distinct phosphorylations *in vivo*. We show that T500 on the activation loop, and T641 and S660 on the carboxyl terminus, are phosphorylated on protein kinase C βII from the detergent-soluble fraction of cells. The analysis of phosphatase-treated samples reveals that PP1 dephosphorylates all three residues, whereas PP2A dephosphorylates only T500 and S660. Using an antibody that selectively recognizes enzyme dephosphorylated at S660, we show that S660 is an autophosphorylation site. Modification of this residue follows autophosphorylation on T641, and is accompanied by the release of protein kinase C into the cytosol. In addition, we show that T500 is not an autophosphorylation site.

Based on these results, and on the other biochemical data [8–13], we present a model for the function of each phosphorylation site. Newly synthesized protein kinase C, localized to a detergent-insoluble fraction, is phosphorylated by another kinase on T500 on the activation loop. This phosphorylation renders the kinase catalytically competent and allows a critical autophosphorylation on T641, replacing the requirement for negative charge at the activation loop. Finally, autophosphorylation on S660 releases the kinase into the cytosol, where it is ready to respond to lipid second messengers. Importantly, each phosphorylation motif is found in a number of other kinases, suggesting key roles in the regulation of the function or subcellular localization of the protein kinase superfamily.

Results and discussion

Identification of residues phosphorylated on mature protein kinase C

Rat protein kinase C βII, expressed in baculovirus and purified to homogeneity from the detergent-soluble cellular fraction, migrated as a single band on SDS polyacrylamide gel electrophoresis (PAGE) with an apparent molecular weight of 80 kDa. The enzyme was dephosphorylated with PP1 or PP2A, and native (untreated) and dephosphorylated protein were analyzed by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane and digested with trypsin; the tryptic fragments were separated by reverse-phase high-performance liquid chromatography (HPLC). Figure 1a shows a typical chromatogram for native protein kinase C, which was compared to chromatograms of the two phosphatase-treated samples to distinguish peaks whose elution time had shifted as a result of dephosphorylation (Fig. 1b,c). The absolute retention times varied slightly for each run. Thus, in comparing native and phosphatase-treated samples, we looked for peaks whose position had shifted relative to other peaks in the same sample.

The identification of the peak that contained T500 from the activation loop was helped by the presence of tyrosine in the corresponding peptide, which was monitored by the absorbance at 277 nm. Two peaks were identified whose elution time increased by approximately 2 minutes in phosphatase-treated samples (peaks 1′ and 2′; Fig. 1b); one peak was identified whose elution time decreased by 10 minutes (peak 3′; Fig. 1b). Each of these peaks from the native and phosphatase-treated samples was analyzed

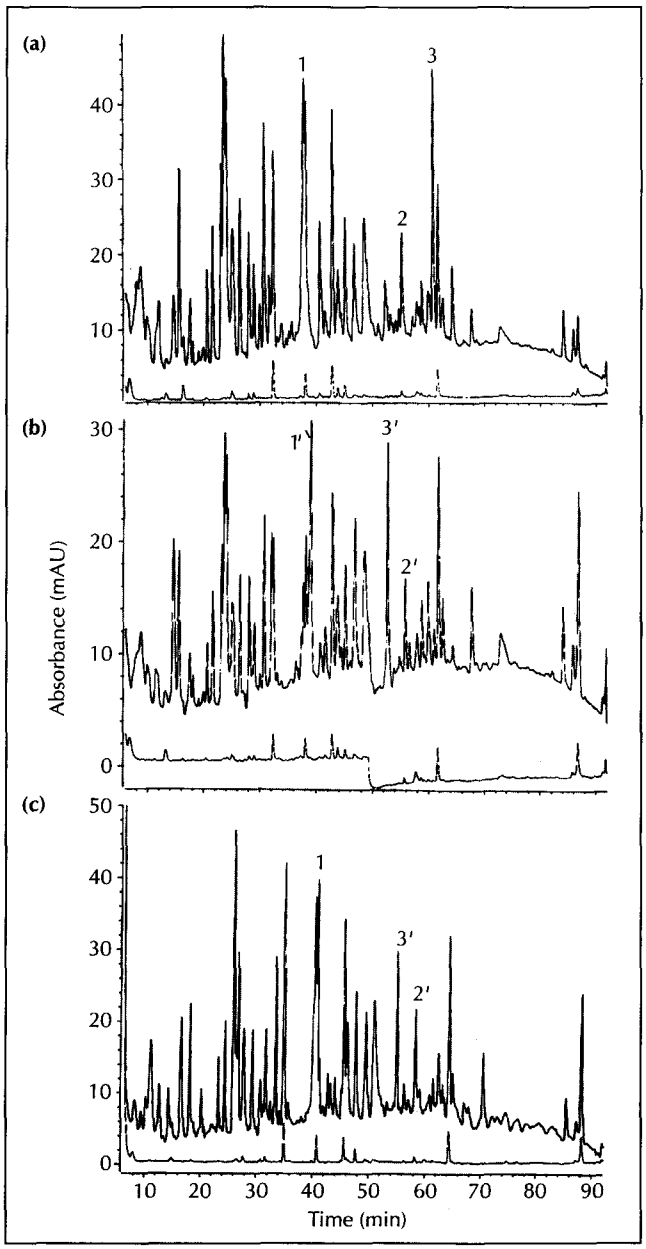

Fig. 1. Identification of phosphopeptides in native protein kinase C by comparison with peptides from phosphatase-treated enzyme. **(a)** Chromatogram of tryptic digest of native protein kinase C βII. Indicated peaks correspond to peptides containing phosphorylated T641 (peak 1), phosphorylated T500 (peak 2), and phosphorylated S660 (peak 3). **(b)** Chromatogram of tryptic digest of PP1-treated protein kinase C βII. Peaks 1′, 2′ and 3′ contain the peptides corresponding to dephosphorylated peaks 1, 2 and 3, respectively. **(c)** Chromatogram of tryptic digest of PP2A-treated protein kinase C βII. Peaks 2′ and 3′ contain the peptides corresponding to dephosphorylated peaks 2 and 3, respectively. Upper trace represents the absorbance at 210 nm; lower trace represents the absorbance at 277 nm.

Table 1. Summary of mass-spectrometric analysis of phosphorylation sites of native and phosphatase-treated protein kinase C βII.

Peak	Treatment	Mass† (predicted)	Sequence‡
1	–	1677.8 (1597.8)	^{636}HPPVLT*PPDQEVI^{649}R#
	PP1	1597.4	^{636}HPPVLT PPDQEVI^{649}R#
	PP2A	1679.7	^{636}HPPVLT*PPDQEVI^{649}R
2	–	2504.1 (2418.6)	^{500}T*FCGTPDYIAPEIIAYQPYG^{520}K
	PP1	2422.7	^{500}T FCGT/... ^{520}K
	PP2A	2429.2	^{500}T FCGT/... ^{520}K
3	–	2771.3 (2690.9)	^{650}NIDQSEFEGFS*FV/... ^{672}K
	PP1	1639.7	^{650}NIDQSEFEGFS FV^{663}N
	PP2A	1638.8	^{650}NIDQSEFEGFS FV^{663}N

†Masses were determined by laser desorption mass spectroscopy; underlined masses were determined by ES-MS. ‡Underlined amino-acid residues were not detected by Edman degradation. Phosphorylated residues are indicated by an asterisk. A backslash denotes where sequencing was terminated. #Sequenced by tandem mass spectrometry.

by mass spectrometry to obtain the mass, and by Edman degradation (phosphorylated residues appear as blanks in this analysis) or tandem mass spectrometry to obtain the amino-acid sequence. In cases where the mass was consistent with the peptide being phosphorylated, the exact mass was confirmed by elecrospray mass spectroscopy (ES-MS).

Table 1 summarizes the data from the analysis of the three peptides that contained residues phosphorylated *in vivo*: peak 1 contained the tryptic peptide H636–R649 and was phosphorylated on T641; peak 2 contained the tryptic peptide T500–K520 and was phosphorylated on T500 (activation loop); peak 3 contained the tryptic peptide N650–K672 and was phosphorylated on S660. The masses of peptides in peaks 1, 2 and 3 were 80, 86 and 80 Da greater than the predicted mass, respectively, which is consistent with the addition of one mole of phosphate per mole of peptide. The phosphate on T500 on the activation loop confirms the importance of a negative charge at this position, as indicated by mutagenesis studies [10]. The finding of phosphate on T641 on the carboxyl terminus is consistent with a report by Flint *et al.* [12], which showed that protein kinase C only weakly autophosphorylates at this position *in vitro* because the purified protein is already modified at this position. In addition, the mass spectrometry results revealed that protein kinase C is phosphorylated on a previously undescribed position *in vivo* — S660.

Peaks 1 and 3 each contained a single peptide. In contrast, mass-spectrometric analysis of peak 2 revealed one major peak (which was sequenced and was the phosphorylated activation loop peptide), a peptide whose mass was 80 Da less (2433.6; data not shown), and several other minor peaks. The ratio of phosphorylated to dephosphorylated activation-loop peptide was 6:4. A similar ratio (7:3) was obtained upon a second separate analysis of protein kinase C βII. Mass-spectrometric and sequencing analyses of corresponding peptides in protein kinase C α isolated from bovine retinas (cytosolic fraction) revealed that T497 (T500 equivalent) was approximately 60 % phosphorylated, that T638 (T641 equivalent) was greater than 90 % phosphorylated, and that S657 (S660 equivalent) was greater than 80 % phosphorylated (data not shown). Because the protein kinase C α sample was digested with endoproteinase Lys-C, we were able to determine that T494 and T495, also on the activation loop, were not significantly phosphorylated (> 70 % of residues were recovered in Edman degradation). Thus, protein kinase C from the detergent-soluble (βII) or cytosolic (α) fraction, expressed in insect cells or purified from mammalian tissue, is approximately 60 % phosphorylated on the activation loop and fully (see below) phosphorylated on two sites on the carboxyl terminus.

It is possible that protein kinase C is phosphorylated, *in vivo*, on sites additional to the three identified above. However, recent mass-spectrometric analysis of tryptic peptides that encompass > 90 % of the sequence of protein kinase C βII did not uncover any other phosphorylation sites. Rather, Koshland and coworkers [14] identified three complete, and one partial, tryptic peptides containing the three phosphorylated residues that we have identified precisely. It is unlikely, therefore, that there are additional *in vivo* phosphorylation sites on protein kinase C βII in non-stimulated cells.

Identification of residues dephosphorylated by phosphatases

The analysis of the corresponding peaks in phosphatase-treated samples enabled us to identify which amino acids are substrates for each phosphatase. Table 1 shows that the peptides identified in peaks 1, 2 and 3 were dephosphorylated after protein kinase C was treated with PP1. The mass for the peak 1 peptide was within 0.4 Da of that predicted for the non-phosphorylated peptide, and sequencing by tandem mass spectrometry revealed that T641 was not modified. The mass for the peak 2 peptide was within 4 Da of that predicted for the dephosphorylated peptide; Edman degradation detected T500, indicating that it was not modified (the sequence of this peptide was terminated after T504 because it was

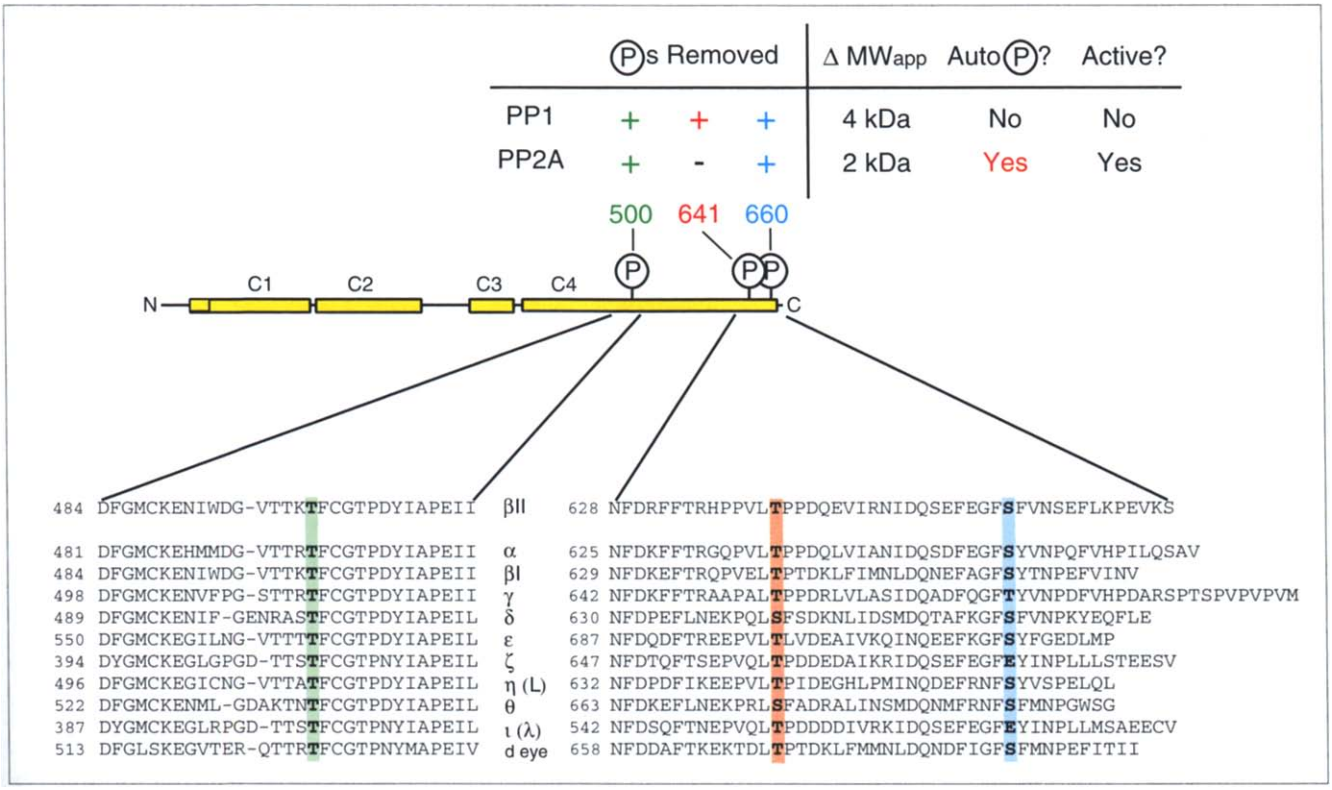

Fig. 2. Protein kinase C is phosphorylated at three sites *in vivo*. Primary structure of protein kinase C, showing conserved domains C1–C4 (for review see [26]), the sites on βII phosphorylated *in vivo* (T500, T641 and S660), and the sites dephosphorylated by PP1 and PP2A *in vitro*. The effects of dephosphorylation on the enzyme's electrophoretic mobility (apparent change in molecular weight) and enzymatic activity are described. Below the primary structure is a comparison of the amino-acid sequences of rat protein kinase C βII [27] with other protein kinase Cs, showing in bold the invariant threonine on the activation loop (T500 in protein kinase C βII; green), and the conserved hydroxyl-containing or acidic residues on the carboxyl terminus (T641(red) and S660 (blue) in protein kinase C βII). Sequences used in the alignment are: human α, ϵ, ζ, η, θ and ι; rat γ and δ [28]; rat βI [27]; mouse λ [29]; human L [30]; *Drosophila* (d) eye [31]. Amino-acid residue numbers are indicated to the left of the sequences.

clear from the mass that there were no additional modifications past this residue; the entire peptide had been sequenced in native protein kinase C, and T500 and C502 were the only residues that were not detected).

Interestingly, the mass for the peak 3 peptide was 1639.7 — considerably less than that predicted or that observed for the phosphopeptide of native enzyme. Sequencing of this peak revealed that, in contrast to native protein kinase C, it was not phosphorylated on S660. Curiously, it was truncated at N663, which accounted for the decreased mass. Given the ability of aspartate to cause the cleavage of a peptide chain (following a reaction with an adjacent backbone amide to form a cyclic succinimidyl derivative), it is possible that the asparagine was de-amidated and then cleaved. If this was the case, the reaction was inhibited when S660 was phosphorylated, perhaps because of a different conformation of the peptide chain. In summary, mass and amino-acid analysis were both consistent with PP1 dephosphorylating protein kinase C on all three residues phosphorylated *in vivo*.

PP2A-treated protein kinase C was dephosphorylated on two of the three sites. Peak 1 contained a phosphopeptide; its mass was within 2 Da of the corresponding (phosphorylated) peptide from native enzyme, and Edman degradation revealed that T641 was modified. The peptide in peak 2 had a mass that was 75 Da less than the mass of the corresponding peptide from the native enzyme and was consistent with that for the dephosphorylated peptide; the sequencing of this peak indicated that T500 was not phosphorylated. Peak 3 peptide had a mass that was consistent with truncation at N663 and dephosphorylation, as had been observed for the PP1-treated enzyme. Edman degradation confirmed the truncation and the presence of unmodified S660. Thus, PP2A dephosphorylated protein kinase C on T500 and S660, but not on T641. These results are summarized in Figure 2.

The differential dephosphorylation of protein kinase C by the two phosphatases is consistent with the different electrophoretic mobilities of PP1- and PP2A-treated protein kinase C. The removal of phosphates from T500, T641 and S660 by PP1 causes an increase in electrophoretic mobility that is equivalent to 4 kDa; the removal of T500 and S660 by PP2A causes a smaller increase in electrophoretic mobility that is equivalent to 2 kDa [13] (Fig. 2). The finding that approximately 40% of native protein kinase C is not phosphorylated on T500, yet the enzyme

migrates as a single band (80 kDa) on SDS-PAGE, reveals that the phosphorylation on T500 does not influence the electrophoretic mobility of protein kinase C.

The identification of equivalent phosphorylation sites on protein kinase C expressed in baculovirus and protein kinase C from bovine tissue indicates that the recombinant protein undergoes the same processing as the endogenous enzyme in its native environment. Furthermore, PP1 and PP2A have the same effect on the electrophoretic mobility and activity of protein kinase C from rat brain or from baculovirus [13].

Mechanism of activation loop phosphorylation

In order to determine whether T500 is autophosphorylated, protein kinase C was incubated with [^{32}P]-ATP under *in vitro* conditions that promote maximal autophosphorylation and then treated with endoproteinase Lys-C, which cleaves protein kinase C βII at K320 (L.M.K. and A.C.N., unpublished observations). Importantly, this cleavage site is carboxy-terminal to the major *in vitro* autophosphorylation sites in the regulatory domain (S16 and T17) and in the hinge region (T314) [12]. Under the conditions of our assays, no significant autophosphorylation on T324 was observed, although autophosphorylation on this position has been reported [12].

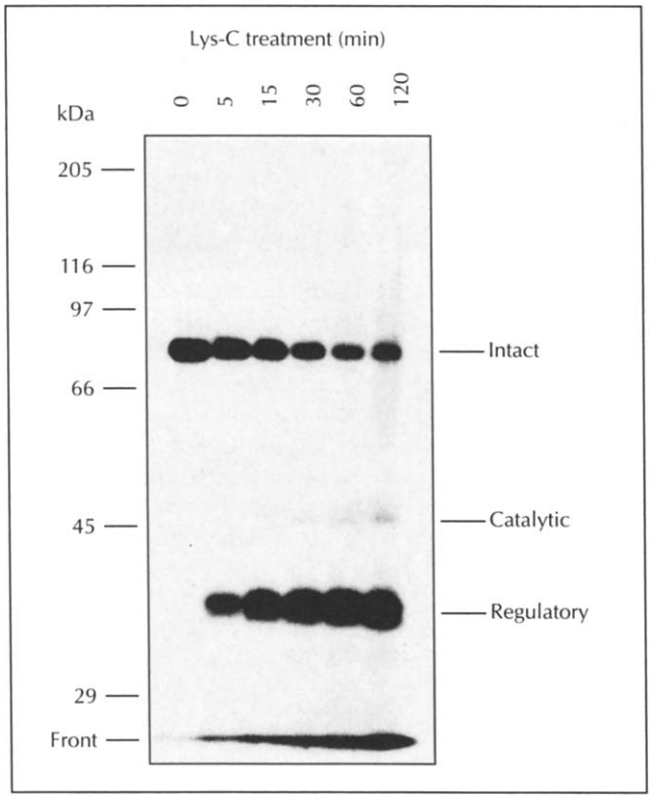

Fig. 3. Protein kinase C does not autophosphorylate on T500. Protein kinase C (10 nM) was maximally autophosphorylated, as described in Materials and methods, and then treated with endoproteinase Lys-C (0.3 U ml^{-1}) for the indicated times. Samples were analyzed by SDS-PAGE (8 %) followed by autoradiography. The positions of native protein kinase C and the proteolytically-generated catalytic and regulatory domains are indicated.

Figure 3 shows that the cleaved regulatory domain was labelled with ^{32}P, but that no detectable label was associated with the catalytic domain. Silver-stain analysis revealed that, after 120 minutes of proteolysis, although approximately the same molar amount of cleaved catalytic domain was present as starting intact enzyme, less than 2 % of the radioactivity was associated with this domain. No phosphorylation of the catalytic domain was observed whether the assay contained phosphatidylserine and diacyglycerol dispersed in detergent micelles (Fig. 3) or presented as sonicated lipid vesicles (data not shown). Thus, even though mass spectrometry established that approximately half the enzyme had no phosphate on position 500, the pure protein was not able to incorporate phosphate at this position. This would suggest that another kinase phosphorylates protein kinase C on its activation loop.

Mechanism and order of carboxy-terminal phosphorylations

The differential recognition of enzyme dephosphorylated on S660 by an antibody generated against a carboxy-terminal peptide of protein kinase C βII (residues 645–673) allowed the characterization of the phosphorylation on S660. Figure 4a shows a western blot of equal amounts of pure protein kinase C βII treated with or without PP1 or PP2A, and then probed with an antibody against the catalytic domain that recognizes both dephosphorylated and fully phosphorylated protein kinase C (a silver stain of the samples in Fig. 4 confirmed that equal amounts of protein were present in all lanes).

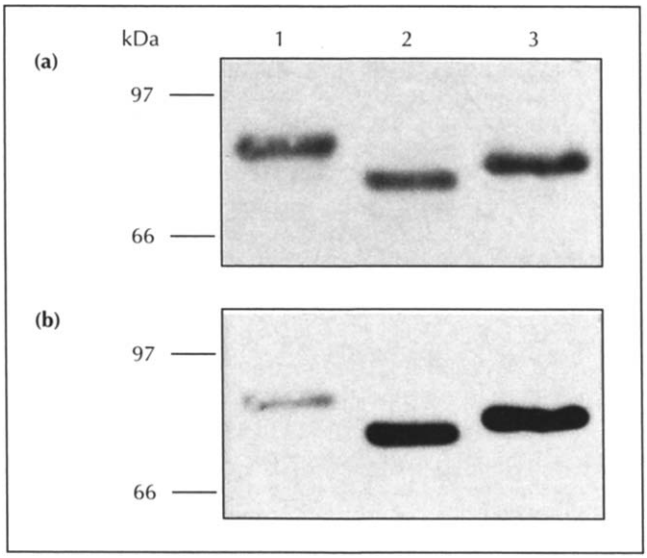

Fig. 4. An antibody directed against a carboxy-terminal peptide of protein kinase C βII selectively recognizes protein kinase C dephosphorylated on S660. **(a)** Western blot of native (lane 1), PP1-treated (lane 2), or PP2A-treated (lane 3) protein kinase C βII labelled with a polyclonal antibody against the bacterially-expressed catalytic domain. A silver stain confirmed that equal amounts of protein were present in all lanes (data not shown). Protein was analyzed on a 7 % polyacrylamide gel; 97 kDa and 66 kDa molecular weight standards are indicated. **(b)** The same blot shown in (a), reprobed with a polyclonal antibody directed against residues 645–673 of protein kinase C βII.

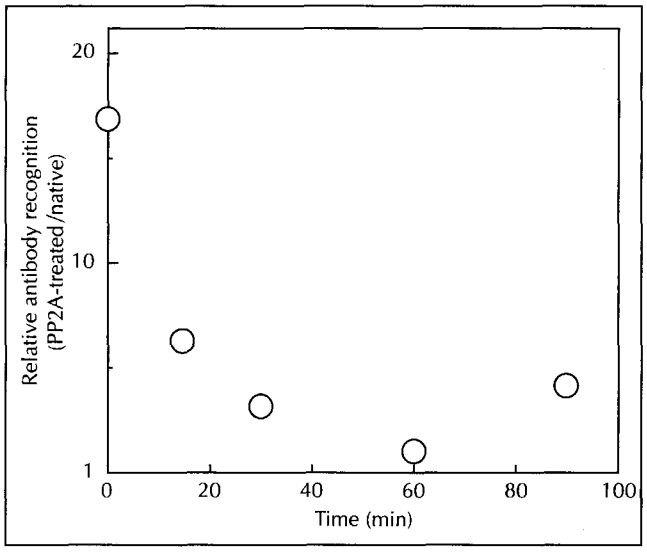

Fig. 5. Protein kinase C autophosphorylates on S660. Protein kinase C βII was incubated with or without PP2A (3 U ml^{-1}, 45 min at 22 °C) to dephosphorylate position 660, and was then incubated with ATP (50 μM) and $MgCl_2$ (5 mM) in the presence of Ca^{2+} (350 μM), phosphatidylserine (200 μM), diacylglycerol (5 μM) and microcystin (3 μM) for the indicated times. Samples were analyzed by SDS-PAGE (7 %), electrophoretically transferred to nitrocellulose, and probed with the antibody directed against residues 645–673 of protein kinase C βII. Data are presented as the amount of immunoreactivity of the phosphatase-treated sample relative to that of sample treated identically except that phosphatase was absent in the initial incubation (native).

When the same blot was stripped and reprobed with the antibody directed against the carboxy-terminal peptide, the dephosphorylated bands were labelled preferentially (Fig. 4b). Densitometric analysis of blots from three separate experiments revealed that the antibody reacted 15-times more strongly with the phosphatase-treated samples than with the native enzyme. This antibody, specific to the carboxyl terminus, therefore provides a tool to determine the phosphorylation state of S660. It should be noted that the use of antibodies directed against the carboxyl terminus of protein kinase C is wide-spread (for example, [11]), and that a re-evaluation of results may be warranted as this carboxy-terminal phosphorylation site is conserved in all isozymes except ζ and ι, which have a glutamate at the corresponding position (Fig. 2).

We have shown previously that protein kinase C dephosphorylated by PP2A can re-autophosphorylate and regain native mobility upon incubation with ATP [13]. Western-blot analysis revealed that the antibody specific to the carboxyl terminus had a markedly reduced affinity for this re-autophosphorylated form compared with the dephosphorylated, faster-migrating form (Fig. 5). This result establishes that S660 is an autophosphorylation site, as would be predicted from the increase in electrophoretic mobility that accompanies autophosphorylation of PP2A-treated enzyme. Thus, both T641 [12] and S660 are autophosphorylation sites.

To determine the order of the two carboxy-terminal autophosphorylations, we examined the subcellular fractions of Sf-9 cells expressing protein kinase C βII. Western-blot analysis showed that two faster-migrating forms of protein kinase C were present in the detergent-insoluble fraction, whereas only the mature 80 kDa form was present in the detergent-soluble fraction (Fig. 6a). (Note that mass-spectrometric analysis of this 80 kDa soluble form revealed that it is quantitatively phosphorylated on the two carboxy-terminal sites.) The faster-migrating forms co-migrated with enzyme treated with PP2A (intermediate-migrating species; phosphate on T641) and PP1 (fastest-migrating species; no phosphates). The faster-migrating forms were shown not to be proteolytic products of native protein kinase C, because they were recognized both by the antibody directed against the carboxyl terminus (see below) and by a polyclonal antibody whose epitope lies within the first 18 amino acids of protein kinase C βII (L.M.K. and A.C.N., unpublished observations).

Figure 6b shows that both faster-migrating forms were labelled preferentially by the antibody against the carboxyl terminus relative to the antibody against the catalytic domain (which does not discriminate between the phosphorylated forms). This result indicates that neither the 76 kDa nor the 78 kDa precursor form is phosphorylated on S660. Phosphorylation on S660 must

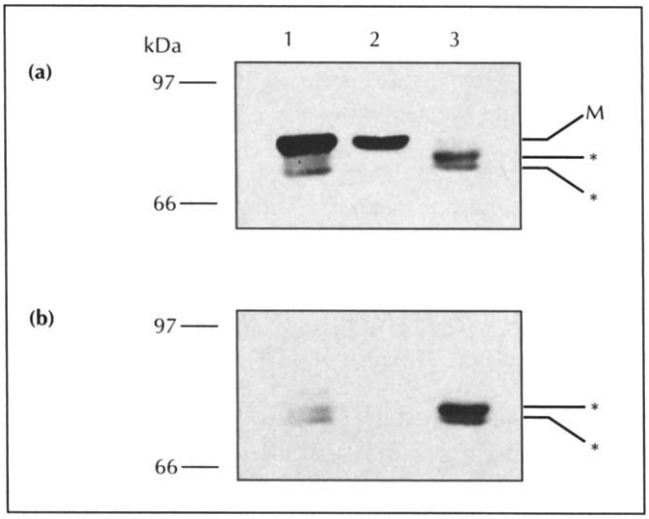

Fig. 6. Phosphorylation on S660 correlates with the release of protein kinase C from the detergent-insoluble fraction of cells. Western blot of subcellular fractions of Sf-9 cells expressing protein kinase C βII, lysed in buffer containing 0.2 % Triton X-100: whole cell lysate (lane 1), detergent-soluble fraction (lane 2), and detergent-insoluble fraction (lane 3). **(a)** Western blot probed with an antibody against the catalytic domain that reacts equally with dephosphorylated and fully phosphorylated protein kinase C. Protein was analyzed on a 7 % polyacrylamide gel; 97 kDa and 66 kDa molecular weight standards are indicated. **(b)** Same blot shown in (a), reprobed with a polyclonal antibody against residues 645–673 of protein kinase C βII that preferentially recognizes enzyme with no phosphate on S660 (see Fig. 4). Note that both faster-migrating forms in the detergent-insoluble fraction are preferentially labelled by this antibody. Mature (80 kDa) protein kinase C is indicated with 'M'; faster-migrating forms (78 kDa and 76 kDa) are indicated with asterisks.

therefore follow phosphorylation on T641 — the fastest-migrating form has either no phosphates or one phosphate on T500 (this phosphorylation does not alter the electrophoretic mobility of the protein), and the intermediate form is phosphorylated on T641 (causing the first shift in electrophoretic mobility) and presumably on T500. This order of phosphorylation is consistent with the electrophoretic mobility shifts reported by Fabbro and coworkers [8], who noted that the fastest-migrating form was chased to the intermediate-migrating form and then to the mature form. In summary, these data reveal that neither of the two faster-migrating precursor forms has phosphate on position 660.

Figure 6 also shows that phosphorylation on S660 is accompanied by the release of protein kinase C from the detergent-insoluble cell fraction into the cytosol. The two faster-migrating forms are not phosphorylated on S660 and partition with the detergent-insoluble fraction, whereas the mature form is quantitatively phosphorylated on S660 and partitions in the detergent-soluble fraction. Lysis without detergent confirmed that the 80 kDa form partitioned in the cytosol. Consistent with this, two faster-migrating forms associate with cytoskeletal, but not detergent-soluble, fractions from bovine retinas [15].

Our data are consistent with phosphorylation on S660 regulating the subcellular localization of protein kinase C. Alternatively, the correlation between phosphorylation and localization could be coincidental. For example, the protein kinase C that is bound to the detergent-insoluble fraction could be a substrate for a cytoskeletal-associated phosphatase with specificity for S660 — whenever mature enzyme bound the cytoskeleton, it would become dephosphorylated. However, this would seem unlikely as pulse–chase experiments have established that the faster-migrating forms are chased to the mature 80 kDa form. In particular, the faster-migrating species do not reappear after formation of the mature enzyme, even after stimulation with phorbol esters [8].

Assignment of function to each phosphorylation

We have shown previously that PP1-treated protein kinase C was inactive and could not re-autophosphorylate, even in the presence of native protein kinase C [13]. This led us to propose that PP1 dephosphorylated a residue that was modified by a different kinase. A likely candidate was position 500 on the activation loop, because mutagenesis supported a requirement for negative charge at this position for maximal activity [10]. How can this be reconciled with the finding that the T500/S660 dephosphorylated enzyme is active, whereas the T500/T641/S660 dephosphorylated enzyme is not? One possibility is that T641 is the trans-phosphorylation site. However, this would be inconsistent with the report by Flint *et al.* [12], who showed that protein kinase C autophosphorylates on T641 *in vitro*. Neither is the site likely to be S660, because this is also an autophosphorylation site. A more likely candidate is T500, which is not autophosphorylated by protein kinase C *in vitro* (see Fig. 3).

Why is a negative charge on position 500 required for expression of functional enzyme, but not required once mature enzyme has been made? One explanation is that trans-phosphorylation on the activation loop by another kinase is essential to allow the critical autophosphorylation on T641 and then on S660. If so, it is the phosphate on T641 that is critical for function, and when this is removed (for example, by PP1) the enzyme is inactive. However, T641 cannot be phosphorylated unless the kinase has first been phosphorylated on the activation loop; this phosphorylation of the activation loop might correctly align residues for catalysis, or might place the carboxyl terminus in a position for autophosphorylation. When the phosphate on T500 is removed, the enzyme may retain activity because the phosphate on T641 maintains the enzyme in a catalytically competent conformation. If this is the case, then mutants with non-phosphorylatable neutral residues at the activation loop may be catalytically inactive [10,11] because T641 (or the equivalent) has not been autophosphorylated. In this model, therefore, trans-phosphorylation on T500 promotes autophosphorylation on T641, but is not sufficient to maintain catalytic competence alone.

The loss of enzymatic activity upon the dephosphorylation of T500, T641 and S660, but not upon the dephosphorylation of T500 and S660, suggests a key role for a phosphate on T641 in maintaining an active conformation. Consistent with this, Bishop and coworkers [16] reported that the mutation of T642 to alanine in protein kinase C βI (corresponding to T641 in protein kinase C βII) resulted in an insoluble, inactive enzyme. Our results would suggest that the mutant protein was insoluble because it was catalytically incompetent and could not autophosphorylate on S661 (corresponding to S660 in protein kinase C βII). The proximity of the carboxyl terminus to the substrate-binding cavity, and its flexibility as indicated by proteolytic sensitivity (L.M.K. and A.C.N., unpublished observations), suggest that this domain may cover the substrate-binding cavity. Phosphorylation on T641 may play a crucial role in anchoring the carboxyl terminus away from the substrate-binding cavity.

Phosphatase experiments reveal that autophosphorylation on S660 is not required for activity — PP2A-treated enzyme displays the same V_{max} towards substrate phosphorylation under conditions where the enzyme cannot re-autophosphorylate on S660 [13]. Rather, phosphorylation at this position appears to regulate the subcellular distribution of protein kinase C, as the protein kinase C that is quantitatively phosphorylated on the two carboxy-terminal positions partitions in the cytosol, whereas protein that is not phosphorylated on S660 associates with the detergent-insoluble fraction of cells.

Other kinases have similar phosphorylation motifs

The general importance of the activation loop and carboxy-terminal phosphates is suggested by the high degree of conservation of hydroxyl-containing residues at these positions among protein kinase C isozymes; all

conventional, novel and atypical protein kinase Cs have a serine or threonine at these positions, except for a glutamate at the equivalent of position 660 in protein kinase C ζ and ι (Fig. 2). Phosphorylation on the activation loop is also central to the regulation of other serine/threonine kinases [1] and tyrosine kinases [6]. In addition, the structure of protein kinase A provides a hint that at least one of the carboxy-terminal phosphorylation sites in protein kinase C may be conserved outside the protein kinase C family. The *in vivo* phosphorylation site of protein kinase A, S338 [17], is located at the very top of the ATP-binding lobe on a random coil that stretches from the lower to upper lobe [3]; molecular modeling predicts that T641 in protein kinase C βII would occupy an analogous position at the top of the kinase [18]. As suggested above, it is possible that phosphorylation on T641 prevents the carboxy-terminal tail from blocking the active site.

The previously unknown autophosphorylation site on S660 is also present in other kinases: the motif of a serine surrounded by hydrophobic residues is present in kinases as diverse as the G-protein-coupled receptor kinases [19] and the S6 kinase family [20]. In the case of rhodopsin kinase, autophosphorylation at this carboxy-terminal position modulates the enzyme's substrate specificity [19], which is consistent with phosphorylation at this motif regulating macromolecular interactions. In the case of S6 kinase, phosphorylation on the equivalent of S660 appears to regulate sensitivity to the immunosuppressant rapamycin [20]. In addition, at least one kinase is regulated by two functionally distinct autophosphorylations on the carboxy-terminal outskirts of the kinase core: Ca^{2+}/calmodulin-dependent kinase II autophosphorylates on T286, which is a similar distance in primary sequence from the kinase core as T641, and is also regulated by phosphorylations on T305 and S314, which have surrounding hydrophobic residues similar to the environment of S660 [21,22].

A model for the regulation of protein kinase C by three functionally distinct phosphorylations

Figure 7 presents a tentative model for the regulation of protein kinase C by phosphorylation and by lipid second messengers that is consistent with these and previously published data. As demonstrated by pulse–chase experiments, protein kinase C is first synthesized as an inactive precursor that is associated with the detergent-insoluble fraction of cells and that migrates with an electrophoretic mobility 4 kDa less than the mature form (Fig. 6) [8]. Our data suggest that the enzyme is phosphorylated by a putative protein kinase C kinase on its activation loop, as protein kinase C cannot autophosphorylate at this position (Fig. 3). The electrophoretic mobility of the enzyme is unaffected by phosphorylation on T500 (the native-migrating band in Fig. 4 is 40 % dephosphorylated on T500). A negative charge on the activation loop is likely to align residues correctly for catalysis [1,9–11], and the primary consequence is autophosphorylation on T641 [12]. This autophosphorylation causes the first shift in electrophoretic mobility [8] — PP2A-treated enzyme, which is phosphorylated only on T641, migrates with an intermediate mobility relative to mature and completely dephosphorylated enzyme (Fig. 4, lane 3), and co-migrates with the intermediate-migrating form in the detergent-insoluble fraction of cells, which is not phosphorylated on S660 (Fig. 6, lane 3). Phosphorylation on T641 replaces the requirement for phosphate on the activation loop (PP2A-treated enzyme, with phosphate only on T641, is fully activatable).

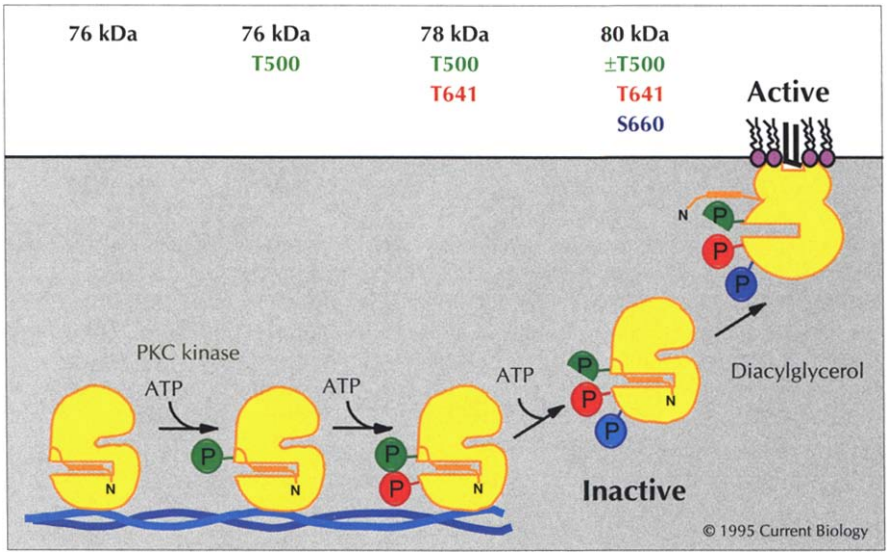

Fig. 7. A model, consistent with the biochemical data, for the functions of the three *in vivo* phosphorylation sites of protein kinase C. Newly synthesized, catalytically-incompetent protein kinase C (yellow) associates with a detergent-insoluble structure (light blue) [8]. The enzyme is phosphorylated on T500 (green), on its activation loop, by a putative protein kinase C kinase (PKC kinase), which renders it catalytically competent (protein kinase C does not autophosphorylate on this position; mutant protein kinase C with a negative charge at this position is activatable [10], whereas enzyme with neutral, non-phosphorylatable residues at this position is inactive [10,11]). The first consequence of this trans-phosphorylation on T500 is autophosphorylation on T641 (red) at the top of the catalytic core; phosphate at this position replaces the requirement for negative charge at the activation loop (PP2A-treated enzyme, with phosphate only on T641, is fully activatable); a second autophosphorylation, on S660 (blue), releases mature protein kinase C into the cytosol (only the 80 kDa form, which is quantitatively phosphorylated on the two carboxy-terminal sites, is in the detergent-soluble fraction of cells; note that T500 is only partially phosphorylated in the mature form and is not required for function once T641 is phosphorylated). The generation of diacylglycerol (black) results in membrane translocation, interaction with phosphatidylserine (purple), pseudosubstrate (orange) release from the active site, and substrate phosphorylation (for review see [26]). The apparent molecular weight of each form of protein kinase C is indicated above the protein; note that phosphorylation on T500 does not appear to alter electrophoretic mobility.

The enzyme then autophosphorylates on S660, resulting in the second and final shift in electrophoretic mobility [8,13]. This autophosphorylation does not affect the enzyme's activity (PP2A-treated enzyme displays the same V_{max} towards substrate phosphorylation under conditions where the enzyme cannot re-autophosphorylate [13]). Instead, phosphorylation on S660 appears to release the kinase from the detergent-insoluble fraction into the cytosol — the two faster-migrating forms that partition with the detergent-insoluble fraction are not phosphorylated on S660, whereas the mature form found in the cytosolic fraction is phosphorylated on this residue (Fig. 6).

Lastly, phosphatases dephosphorylate the activation loop so that about half the protein kinase C in the cytosol is not phosphorylated on the activation loop (but is quantitatively phosphorylated on the two carboxy-terminal sites). Whether specific dephosphorylation/phosphorylation on T500 regulates the soluble enzyme, and whether such regulation depends on membrane-binding, remains to be established. The generation of diacylglycerol results in membrane translocation, pseudosubstrate release from the substrate-binding cavity, and substrate phosphorylation.

Conclusions

Three functionally distinct phosphorylations of protein kinase C are necessary before the enzyme is competent to respond to second messengers. Dual regulation by phosphorylation and by lipid second messengers provides exquisite control for protein kinase C function, perhaps by ensuring low background noise in the absence of specific stimulation.

Materials and methods

Bovine brain L-α-phosphatidylserine and *sn*-1,2-dioleoylglycerol were obtained from Avanti Polar Lipids. PVDF membranes were purchased from Bio-Rad Laboratories, nitrocellulose (Schleicher and Schuell) from Midwest Scientific, Triton X-100 from Pierce, and peroxidase-conjugated goat anti-rabbit antibodies and endoproteinase Lys-C from Boehringer Mannheim. Protein kinase C βII was purified to homogeneity from the baculovirus expression system as described ([23]; note that enzyme was purified from the Triton X-100 soluble fraction); protein kinase C α was purified to homogeneity from the cytosolic fraction of bovine retinas following the same procedure. Protein kinase C was stored at −20 °C in 10 mM Tris buffer, pH 7.5 (4 °C), 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 150 mM KCl and 50 % glycerol. Protein phosphatase 1 catalytic subunit α isoform (PP1) and protein phosphatase 2A catalytic subunit (PP2A) were generous gifts from Anna DePaoli-Roach [13]. PP1 was stored at −20 °C in 50 mM imidazole, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 2 mM DTT, 2 mM $MnCl_2$ and 50 % glycerol (45 μg ml^{-1}; 20 000 nmol min^{-1} mg^{-1} using phosphorylase a, at 1 mg ml^{-1}, as a substrate in the presence of 5 mM caffeine); PP2A was stored at −20 °C in 20 mM Tris, pH 7.2, 5 mM EDTA, 2 mM EGTA and 10 % glycerol (10 μg ml^{-1}; 10 000 nmol min^{-1} mg^{-1} using phosphorylase a, at 2 mg ml^{-1}, as a substrate in the presence of 5 mM caffeine). The activity of the phosphatases is presented in U ml^{-1}, where 1 U is 1 nmol phosphate removed from phosphorylase a in 1 min under the conditions described above for each phosphatase. A polyclonal antibody against bacterially-expressed catalytic domain, and one against bacterially-expressed regulatory domain of protein kinase C βII, were a gift of Andrew Flint and Daniel E. Koshland, Jr.; a polyclonal antibody against a peptide comprising residues 645–673 of protein kinase C βII was provided by Eli Lilly. All other chemicals were reagent-grade.

Dephosphorylation of protein kinase C

Protein kinase C (150 pmol) was incubated with PP1 (16 U ml^{-1}) in 20 mM HEPES buffer, pH 7.5, containing 200 μM $MnCl_2$, 1 mM DTT, 20 μM EDTA, 20 μM EGTA, 250 μM phosphatidylserine and 4 μM diacylglycerol at 22 °C. Dephosphorylation was allowed to proceed for 6 h, with fresh aliquots of PP1 added after 2 h and 4 h to yield a final concentration of 48 U ml^{-1}. Dephosphorylation of protein kinase C (300 pmol) by PP2A (6 U ml^{-1}, final concentration) was similar except 400 μM EDTA and 250 μM EGTA were present. Dephosphorylation reactions were quenched by the addition of 1/5 volume of SDS-PAGE sample buffer [23]. Proteins were separated by SDS-PAGE (6 % polyacrylamide) and electrophoretically transferred to PVDF membrane as described [24].

Analysis of protein kinase C by mass spectrometry

Native (80 kDa), PP1-dephosphorylated (76 kDa) and PP2A-dephosphorylated (78 kDa) protein kinase C bands were excised from PVDF membrane and digested with trypsin as described [24]. Peptides were isolated by reverse-phase HPLC and detected by monitoring the absorbances at 210, 277 and 292 nm. Masses of peptides in peaks of interest were determined by laser desorption mass spectroscopy. Peptides from native protein kinase C with masses corresponding to those predicted for phosphorylated tryptic fragments were sequenced by automated Edman degradation [25] or tandem mass spectrometry; corresponding peptides in phosphatase-treated samples were sequenced similarly. Masses of phosphopeptides were confirmed by ES-MS.

Subcellular fractionation of Sf-9 cells and western-blot analysis

Sf-9 cells (4×10^6) infected with protein kinase C βII in baculovirus were harvested 4 days post-infection, centrifuged, and resuspended in 750 μl lysis buffer containing 50 mM HEPES (pH 7.5 at 4 °C), 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 40 μg ml^{-1} leupeptin, 2 mM benzamidine, 0.2 mM PMSF and 0.2 % Triton X-100. An aliquot was removed for analysis (whole-cell lysate). The remaining lysate was centrifuged at 350 000 × *g* for 15 min at 4 °C. The supernatant was removed (detergent-soluble fraction), and the pellet was resuspended in an equal volume of lysis buffer (detergent-insoluble fraction). 5 μl aliquots of each fraction were analyzed by SDS-PAGE (7 % acrylamide), electrophoretically transferred to nitrocellulose, probed with the indicated primary antibodies, and incubated with peroxidase-conjugated secondary antibody. Labelling was detected using Chemi-luminescence (Pierce). For some experiments, pure protein kinase C βII was treated with indicated phosphatases, as described [13], and analyzed by silver staining or western-blot analysis. Intensity of protein or antibody staining, from gels or blots developed in the linear range, was quantified using a Molecular Dynamics scanning densitometer.

Autophosphorylation of protein kinase C

Protein kinase C (10 nM) was maximally autophosphorylated as described [23] in the presence of lipid mixed micelles (15 mol % phosphatidylserine, 5 mol % diacylglycerol, 0.1 % Triton X-100) or 140 μM phosphatidylserine and 4 μM diacylglycerol. Autophosphorylated enzyme was analyzed directly by SDS-PAGE (8 % acrylamide) or first incubated with endoproteinase Lys-C (0.3 U ml^{-1}) to separate the regulatory and catalytic domains. In one experiment, protein kinase C (20 nM) was first dephosphorylated with protein phosphatase 2A (3 U ml^{-1}) as described [13], and then incubated with 3 μM microcystin, 50 μM ATP and 5 mM $MgCl_2$. Samples were analyzed by SDS-PAGE (7 % acrylamide).

Acknowledgements: We thank John Neveu and colleagues at the Harvard Microchemistry Facility for performing the mass spectrometry and peptide sequencing, and for helpful discussions. We thank Sherry Cai for purifying protein kinase C βII, Michelle Greene for purifying protein kinase C α, Amy Edwards for valuable technical assistance, and Susan Tsutakawa, Dan Koshland and George Thomas for sharing results prior to publication. We are grateful to Anna DePaoli-Roach for a generous gift of the phosphatases, Eli Lilly for the polyclonal antibody against residues 645–673 of protein kinase C βII, Andrew Flint and Dan Koshland for the antibodies against bacterially-expressed domains of protein kinase C βII, and CalBiochem and Avanti Polar Lipids for participating in the National Science Foundation Young Investigator program. This work was supported by National Institutes of Health Grant GM 43154 and a National Science Foundation Young Investigator Award (to A.C.N.).

References

1. Taylor SS, Radzio-Andzelm E: **Three protein kinase structures define a common motif.** *Structure* 1994, **2:**345–355.
2. Krebs EG, Graves DJ, Fischer EH: **Factors affecting the activity of muscle phosphorylase *b* kinase.** *J Biol Chem* 1959, **234:**2867–2873.
3. Knighton DR, Zheng J, Ten Eyck LF, Ashford VA, Xuong N-H, Taylor SS, Sowadski JM: **Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase.** *Science* 1991, **253:**407–425.
4. De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim SH: **Crystal structure of cyclin-dependent kinase 2.** *Nature* 1993, **363:**595–602.
5. Zhang F, Strand A, Robbins D, Cobb MH, Goldsmith EJ: **Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution.** *Nature* 1994, **367:**704–711.
6. Hubbard SR, Wei L, Ellis L, Hendrickson WA: **Crystal structure of the tyrosine kinase domain of the human insulin receptor.** *Nature* 1994, **372:**746–754.
7. Nishizuka Y: **Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C.** *Science* 1992, **258:**607–614.
8. Borner C, Filipuzzi I, Wartmann M, Eppenberger U, Fabbro D: **Biosynthesis and posttranslational modifications of protein kinase C in human breast cancer cells.** *J Biol Chem* 1989, **264:**13902–13909.
9. Cazaubon SM, Parker PJ: **Identification of the phosphorylated region responsible for the permissive activation of protein kinase C.** *J Biol Chem* 1993, **268:**17559–17563.
10. Orr JW, Newton AC: **Requirement for negative charge on 'activation loop' of protein kinase C.** *J Biol Chem* 1994, **269:**27715–27718.
11. Cazaubon S, Bornancin F, Parker PJ: **Threonine-497 is a critical site for permissive activation of protein kinase C α.** *Biochem J* 1994, **301:**443–448.
12. Flint AJ, Paladini RD, Koshland DE, Jr: **Autophosphorylation of protein kinase C at three separated regions of its primary sequence.** *Science* 1990, **249:**408–411.
13. Dutil EM, Keranen LM, DePaoli-Roach AA, Newton AC: ***In vivo* regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation.** *J Biol Chem* 1994, **269:**29359–29362.
14. Tsutakawa SE, Medzihradszky KF, Flint AJ, Burlingame AL, Koshland DE: **Determination of *in vivo* phosphorylation sites in protein kinase C.** *J Biol Chem,* in press.
15. Williams DS, Park S, Schlamp CL, Newton AC: **Protein kinase C association with the retinal cytoskeleton and phosphorylation of vimentin.** *Exp Eye Res* 1994, **58:**747–759.
16. Zhang J, Wang L, Schwartz J, Bond RW, Bishop WR: **Phosphorylation of Thr^{642} is an early event in the processing of newly synthesized protein kinase $C\beta_1$ and is essential for its activation.** *J Biol Chem* 1994, **269:**19578–19584.
17. Shoji S, Titani K, Demaille JG, Fischer EH: **Sequence of two phosphorylated sites in the catalytic subunit of bovine cardiac muscle adenosine 3':5'-monophosphate-dependent protein kinase.** *J Biol Chem* 1979, **254:**6211–6225.
18. Orr JW, Newton AC: **Intrapeptide regulation of protein kinase C.** *J Biol Chem* 1994, **269:**8383–8387.
19. Palczewski K, Ohguro H, Premont RT, Inglese J: **Rhodopsin kinase autophosphorylation: characterization of site-specific mutations.** *J Biol Chem* 1995, **270:**15294–15298.
20. Pearson RB, Dennis PB, Han J-W, Williamson NA, Kozma SC, Wettenhall REH, Thomas G: **Rapamycin induces $p70^{s6k}$ inactivation by dephosphorylation of an essential site within a novel hydrophobic motif.** *EMBO J,* **14:**5279–5287.
21. Patton BL, Miller SG, Kennedy MB: **Activation of type II calcium/calmodulin-dependent protein kinase by Ca^{2+}/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin-binding domain.** *J Biol Chem* 1990, **265:**11204–11212.
22. Colbran RJ, Soderling TR: **Calcium/calmodulin-independent autophosphorylation sites of calcium/calmodulin-dependent protein kinase II.** *J Biol Chem* 1990, **265:**11213–11219.
23. Orr JW, Keranen LM, Newton AC: **Reversible exposure of the pseudosubstrate domain of protein kinase C by phosphatidylserine and diacylglycerol.** *J Biol Chem* 1992, **267:**15263–15266.
24. Fernandez J, DeMott M, Atherton D, Mische SM: **Internal protein sequence analysis: enzymatic digestion for less than 10 μg of protein bound to polyvinylidene difluoride or nitrocellulose membranes.** *Anal Biochem* 1992, **201:**255–264.
25. Tempst P, Riviere L: **Examination of automated polypeptide sequencing using standard phenyl isothiocyanate reagent and subpicomole High-Performance Liquid Chromatographic analysis.** *Anal Biochem* 1989, **183:**290–300.
26. Newton AC: **Protein kinase C: Structure, function, and regulation.** *J Biol Chem* 1995, **270:**28495–28498.
27. Ono Y, Kurokawa T, Fujii T, Kawahara K, Igarashi K, Kikkawa U, *et al.*: **Two types of complementary DNAs of rat brain protein kinase C.** *FEBS Lett* 1986, **206:**347–352.
28. Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ: **Molecular cloning and characterization of PKCι, an atypical isoform of protein kinase C derived from insulin-secreting cells.** *J Biol Chem* 1993, **268:**24296–24302.
29. Akimoto K, Mizuno K, Osada S-I, Hirai S-I, Tanuma S-I, Suzuki K, Ohno S: **A new member of the third class in the protein kinase C family, PKCλ, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells.** *J Biol Chem* 1994, **269:**12677–12683.
30. Bacher N, Zisman Y, Berent E, Livneh E: **Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin and heart.** *Mol Cell Biol* 1991, **11:**126–133.
31. Schaeffer E, Smith D, Mardon G, Quinn W, Zuker C: **Isolation and characterization of two new *Drosophila* protein kinase C genes, including one specifically expressed in photoreceptor cells.** *Cell* 1989, **57:**403–412.

Received: **31 July 1995**; revised: **22 September 1995**.
Accepted: **23 October 1995**.