The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation

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Background: A growing number of kinases are now known to be controlled by two phosphorylation switches, one on a loop near the entrance to the active site and a second on the carboxyl terminus. For the protein kinase C (PKC) family of enzymes, phosphorylation at the activation loop is mediated by another kinase but the mechanism for carboxy-terminal phosphorylation is still unclear. The latter switch contains two phosphorylation sites – one on a 'turn' motif and the second on a conserved hydrophobic phosphorylation motif – that are found separately or together in a number of other kinases.

Results: Here, we investigated whether the carboxy-terminal phosphorylation sites of a conventional PKC are controlled by autophosphorylation or by another kinase. First, kinetic analyses revealed that a purified construct of the kinase domain of PKC βII autophosphorylated on the Ser660 residue of the hydrophobic phosphorylation motif in an apparently concentration-independent manner. Second, kinase-inactive mutants of PKC did not incorporate phosphate at either of the carboxy-terminal sites, Thr641 or Ser660, when expressed in COS-7 cells. The inability to incorporate phosphate on the hydrophobic site was unrelated to the phosphorylation state of the other key phosphorylation sites: kinase-inactive mutants with negative charge at Thr641 and/or the activation-loop position were also not phosphorylated *in vivo*.

Conclusions: PKC βII autophosphorylates at its conserved carboxy-terminal hydrophobic phosphorylation site by an apparently intramolecular mechanism. Expression studies with kinase-inactive mutants revealed that this mechanism is the only one responsible for phosphorylating this motif *in vivo*. Thus, conventional PKC autoregulates the carboxy-terminal phosphorylation switch following phosphorylation by another kinase at the activation loop switch.

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Background

Most members of the kinase superfamily are precisely regulated by phosphorylation, catalyzed either by a heterologous kinase or by autophosphorylation [1–3]. The regulation of kinase function by phosphorylation is epitomized by the mitogen-activated protein (MAP) kinase cascade in which individual kinases are devoted to the specific function of sequentially activating kinases in the signaling cascade [4].

The hydrophobic phosphorylation motif, FXXSXF (in the single-letter amino-acid code, where X is any amino acid), was first noted on the carboxyl terminus of protein kinase C (PKC) [5] and p70 S6 kinase [6]. It has now also been found at the carboxyl terminus of a number of protein kinases, notably Akt (also known as protein kinase B) and PKC-related kinase (PRK). Phosphorylation at the hydrophobic motif regulates the catalytic function, stability, phosphatase sensitivity and subcellular localization of PKC [7,8]. For p70 S6 kinase, phosphorylation at this position is required for activity; this residue is the principal target for rapamycin-induced inactivation [6]. Studies with phospho-specific antibodies indicate that the presence of a phosphate group on the hydrophobic motif correlates most closely with the activity of p70 S6 kinase *in vivo* [9]. For both kinases, the phosphorylation status of the hydrophobic motif is sensitive to serum deprivation, suggesting a link with the phosphoinositide 3-kinase pathway [6,8–10]. The identification of a potential heterologous kinase that could modify the hydrophobic phosphorylation motif would provide a major breakthrough in our understanding of the cellular regulation of protein kinases involved in lipid signaling.

The PKC family of serine/threonine kinases transduces the myriad signals that promote diacylglycerol production [11,12]. Multiple isozymes exist and share the same primary structure: a single polypeptide chain containing an amino-terminal regulatory domain and a carboxy-terminal catalytic domain. For most members of the family, the diacylglycerol-promoted binding of PKC to membranes results in a conformational change that removes

the pseudosubstrate — an autoinhibitory domain — from the active site, allowing substrate phosphorylation. Membrane binding is mediated by two membrane-targeting modules, the C1 and C2 domains. For conventional PKCs, these modules are regulated by diacylglycerol and Ca^{2+}, respectively.

Before PKC can respond to second messengers, three sequential phosphorylation steps within the catalytic domain must take place [5,13,14]. These phosphorylations control the enzyme's catalytic competence and subcellular localization. Maturation of the enzyme is initiated by phosphorylation on the activation loop catalysed by a heterologous kinase. The phosphoinositide-dependent kinase, PDK-1, has recently been shown to be able to catalyze this phosphorylation *in vivo* for both conventional [15] and atypical [16,17] PKCs. This phosphorylation is required to initiate the processing of PKC: either inhibition of PDK-1 (by overexpression of kinase-inactive PDK-1) [15] or mutation of the activation-loop site to a neutral, non-phosphorylatable residue [18,19] prevents the maturation to a catalytically-competent form. Once phosphorylated in *trans* at the activation loop, PKC rapidly incorporates phosphate at two conserved sites on the carboxyl terminus, a 'turn' motif flanked by prolines and the hydrophobic phosphorylation motif. These sites correspond to the Thr641 and Ser660 residues in conventional PKC βII. The turn motif, like the hydrophobic phosphorylation motif, is conserved in a number of kinases, including protein kinase A and p70 S6 kinase. Upon stimulation by diacylglycerol, it is the carboxy-terminal phosphorylated form of the enzyme that translocates to the membrane and becomes activated.

PKC activity is thus under precise control by two different mechanisms. Phosphorylation at the activation loop is required to initiate the processing of PKC into the mature, cofactor-activatable enzyme, and ligand binding is required to remove the auto-inhibitory pseudosubstrate from the active site. This dual regulation by external signals may be important to ensure that the basal activity of the enzyme is kept to a minimum. Whether an additional point of external regulation occurs through the existence of a heterologous kinase for the hydrophobic phosphorylation motif remains to be resolved.

Here, we studied the mechanism of phosphorylation of the carboxyl terminus of PKC both *in vitro* and *in vivo*. First, we found that a purified construct of the kinase domain of PKC βII autophosphorylates on Ser660 of the hydrophobic phosphorylation motif. Second, we found that phosphorylation of the carboxy-terminal sites of PKC βII in COS-7 cells depends on the catalytic competence of PKC: kinase-inactive mutants of PKC, with or without a negative charge at the activation loop phosphorylation switch, were not phosphorylated at either Thr641 or Ser660. Importantly, kinase-inactive mutants were not phosphorylated on the hydrophobic site whether or not there was a negative charge on the activation loop (Thr500) and the turn motif (Thr641). These results show that the hydrophobic phosphorylation motif in PKC βII, a conventional isozyme, is regulated by autophosphorylation and not by a heterologous kinase.

Results

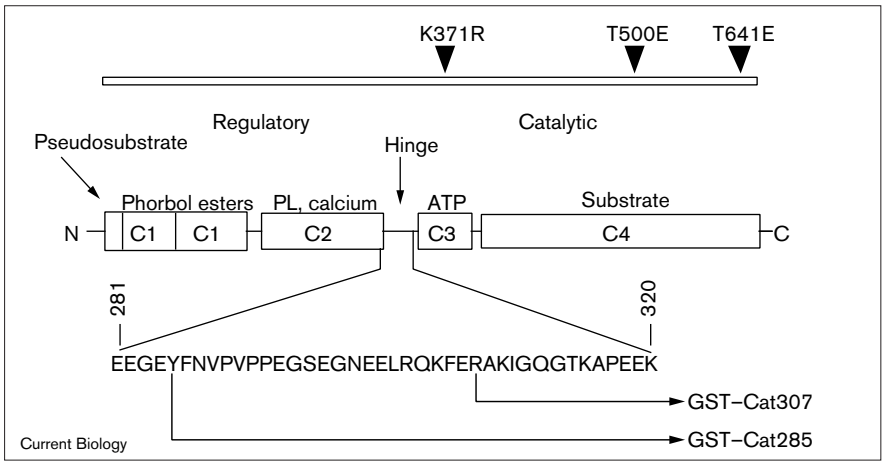
Characterization of kinase-domain constructs of protein kinase C βII

As a first step to determine the mechanism of phosphorylation of the carboxyl terminus of PKC, we examined the kinetics of autophosphorylation of the purified kinase *in vitro*. To use a dilution approach to determine whether phosphorylation was the result of intra(*cis*)-phosphorylation or inter(*trans*)-phosphorylation, we expressed and purified proteins corresponding to the PKC kinase domain that are active in the absence of membranes. Two constructs were generated with differing lengths of the hinge to test whether the hinge served a stabilizing role similar to that of the A helix in protein kinase A [20]: Cat285 and Cat307, which include 55 and 33 residues respectively, preceding the kinase [18] (Figure 1). The shorter protein corresponds to the proteolytically-generated kinase core: trypsin cleaves at K307 and K309 [21] and calpain at G309 and K318 [22].

By western blot analysis (Figure 2a), we detected Cat307 (lane 2) and Cat285 (lane 3) in the detergent-soluble (supernatant) fraction of insect cells. Using an antibody that does not discriminate between phosphorylation states of PKC, we found that the constructs for both fusion proteins were expressed equally well and to similar levels as the overexpressed wild-type (full-length) PKC βII (lane 1). Both Cat307 and Cat285 were phosphorylated on the activation loop (Figure 2a, lower panel), as detected using an antibody (P500) that is specific for the phosphorylated activation loop [15,16]. Thus, both Cat307 and Cat285 expressed well as soluble proteins and both were substrates for the heterologous kinase that modifies the activation loop.

Subcellular fractionation of the insect cell lysate (Figure 2b) revealed that the kinase-domain protein in the lysate fraction migrated as three bands (lane 4), which were also found in the detergent-soluble fraction (lane 5), whereas the detergent-insoluble fraction contained only the fastest-migrating band (lane 6). The partitioning of the kinase-domain protein was similar to that of wild-type PKC βII in that the slowest-migrating band partitioned exclusively in the detergent-soluble fraction (lane 2); for the wild-type enzyme, the fastest-migrating band partitioned exclusively in the detergent-insoluble fraction (lane 3). Mass spectrometric analysis, coupled with analysis of phosphorylation site mutants and the use of phospho-specific antibodies, previously established that the fastest-migrating

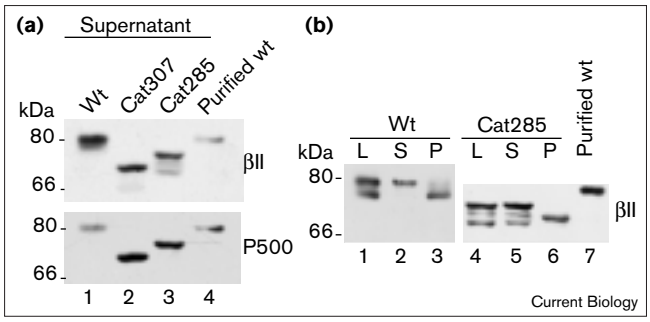
Figure 1



Schematic representation of the primary structure of PKC showing constructs used in this study. In the regulatory part of the molecule are the pseudosubstrate domain; tandem C1 domains, which bind phorbol esters; and C2 domain, which binds anionic phospholipids (PL) and calcium. The carboxy-terminal kinase part contains the ATP-binding lobe in the C3 domain and the substrate-binding lobe in the C4 domain. N, amino terminus; C, carboxyl terminus. The enlarged region shows the sequence of the hinge region of PKC βII and indicates the first residues of the two GST–PKC kinase-domain fusion proteins, Cat285 and Cat307. The location of the three point mutations in the kinase-domain are indicated at the top of the figure: K371R in the ATP-binding site, T500E on the activation loop, and T641E at the carboxyl terminus.

band represents unphosphorylated PKC, the intermediate band represents protein phosphorylated on Thr641 (not detected in Figure 2a), and the slowest-migrating form represents enzyme phosphorylated at both carboxy-terminal positions, Thr641 and Ser660 [5,7,10]. Approximately one-half of this last species is phosphorylated on Thr500 of the activation loop, a modification that does not affect the enzyme's electrophoretic mobility [5]. The data in Figure 2 show that the kinase-domain protein undergoes the same post-translational processing by phosphorylation as the wild-type enzyme, with the mature, phosphorylated enzyme partitioning in the detergent-soluble fraction. Because of the similar expression and modification of the two kinase-domain proteins, we focused on Cat285 unless otherwise stated.

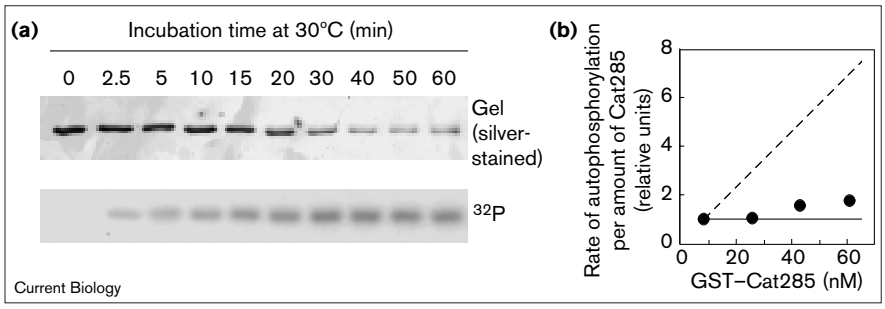
Figure 2



Subcellular localization and phosphorylation of PKC kinase-domain fusion proteins. **(a)** Western blot of the detergent-soluble supernatant fraction from Sf21 insect cells overexpressing Cat307 or Cat285, probed either with the βII antibody (Santa Cruz Biotechnology), which does not distinguish between phosphorylated or unphosphorylated forms of PKC βII, or with the phospho-specific antibody P500, which detects the phosphorylated activation loop. **(b)** Western blot of whole cell lysate (L), detergent-soluble supernatant (S) and detergent-insoluble pellet (P) from Sf21 cells overexpressing Cat285, probed with the phosphorylation-insensitive βII antibody. In (a,b), baculoviral-expressed full-length wild-type (wt) PKC βII in insect cell lysates (lane 1 in (a)) or purified to homogeneity (lane 4 in (a); lane 7 in (b)) are shown as controls.

We next characterized the biochemical properties of the kinase-domain protein. Enzyme assays (data not shown) confirmed that the activity of the kinase-domain protein was unaffected by the presence of the PKC activators, Ca^{2+}, phosphatidylserine and diacylglycerol. (Note, some inhibition of activity by anionic phospholipids was observed as described in [23]; this inhibition was observed only when assays were conducted at low ionic strength (<50 mM) and was suppressed by divalent cations. This suggests that the effect of anionic phospholipids on kinase core activity arises from non-specific electrostatic interactions that are not physiologically relevant.) All subsequent assays were performed in the absence of cofactors. To ensure that the catalytic mechanism was not impaired by removal of the regulatory half of the kinase, we examined the kinetic parameters for substrate. The K_m value for ATP was 60 ± 3 μM for Cat285, which is similar to the value for the wild-type enzyme (data not shown) [7]. The K_m value for phosphorylation of the PKC-selective peptide [24] was 15 ± 1 μM and not significantly different from the value for the full-length enzyme [7]. We also compared reaction rates for the kinase domain and wild-type enzyme under different conditions using the PKC-selective peptide as substrate. The kinase domain in the detergent-soluble cell fraction catalyzed at least 10 reactions per second, which is similar to that of wild-type enzyme (data not shown). As for wild-type enzyme, the rate of autophosphorylation was significantly slower and corresponded to less than 1 reaction per minute (data not shown). Lastly, the thermal stability of both Cat285

Figure 3



Autophosphorylation of the kinase core *in vitro*. **(a)** Silver-stained gel (upper panel) and autoradiogram (lower panel) of purified GST–Cat285 (18 nM) incubated with 100 μM [^{32}P]ATP for the indicated lengths of time at 30°C. **(b)** Increasing amounts of GST–Cat285 were allowed to autophosphorylate for 5 min at 30°C and the amount of ^{32}P incorporated was determined by liquid scintillation counting of SDS–PAGE-separated protein. Data, which represent an initial rate, were divided by the concentration of enzyme to give the rate of autophosphorylation per amount of enzyme. The data were normalized to the rate of autophosphorylation per PKC amount obtained at the lowest enzyme concentration and are thus presented as relative units. The solid line describes the kinetics in the case where enzyme activity is independent of enzyme concentration (pseudo-zero order) and the dashed line describes the kinetics in the case where enzyme activity depends on bimolecular collisions.

and Cat307 was compared and found to be the same as that of the wild-type enzyme. Thus, the additional hinge sequence in the larger construct did not provide a stabilizing role similar to that of the A helix in protein kinase A, nor was the regulatory domain required to stabilize the kinase domain of PKC. These analyses established that the kinase domain on its own shares the same biochemical properties as the full-length enzyme, differing only in its constitutive activity.

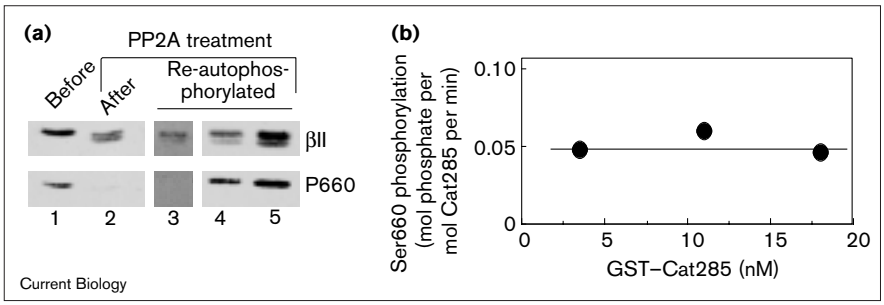
We next explored whether the kinase domain was able to autophosphorylate upon incubation with Mg^{2+}/ATP. The autoradiogram in Figure 3a (lower panel) shows the time-dependent incorporation of ^{32}P into purified GST–Cat285. Silver staining (upper panel) revealed that the increasing phosphate incorporation impaired detection of PKC by this method, thus serving as an additional indicator for phosphorylation. (Western blot analysis using an antibody that does not discriminate between phosphorylation states of PKC revealed constant staining for all lanes. Additionally, no proteolytic products were detected by silver staining.) Quantitative analysis of these data revealed a half-time ($t_{1/2}$) of approximately 15 minutes, with phosphate incorporation linear with time up to at least 5 minutes. This time was chosen for the kinetic analysis to ensure determination of an initial rate.

Mechanism of autophosphorylation

We next examined the dependence of the rate of autophosphorylation on the concentration of the enzyme. PKC was diluted over a fivefold concentration range and allowed to autophosphorylate for 5 minutes, so that phosphate incorporation represented the initial rate of autophosphorylation. The rate of phosphate incorporation normalized to a constant amount of PKC was independent of the total enzyme concentration (Figure 3b). If phosphate incorporation resulted from bimolecular collisions, then the data would have been best described by a line with a slope of one (dashed line) rather than the line with a slope of zero (solid line). Thus, the rate of autophosphorylation per molecule of PKC was independent of enzyme concentration. These data reveal that the rate-limiting step in the autophosphorylation of the kinase domain is intramolecular.

Lastly, we examined the mechanism of phosphorylation of the *in vivo* phosphorylation site, Ser660, which is phosphorylated in the mature enzyme used in the above experiments. For this study, we selectively dephosphorylated Cat285 on Ser660 by incubation of the GST-purified protein with the catalytic subunit of protein phosphatase 2A (PP2A). This phosphatase dephosphorylates Thr500 and Ser660 on the full-length protein to yield a protein that retains full catalytic activity and is able to re-autophosphorylate on Ser660 [5,13]. Importantly, this phosphatase does not dephosphorylate Thr641, the residue that is essential for catalysis in the full-length, mature enzyme [10]. Figure 4a shows that treatment of the catalytic domain with PP2A (lane 2) resulted in generation of a species with a faster electrophoretic mobility than the starting material (lane 1). This species corresponds to the intermediate band in Figure 2b and is consistent with dephosphorylation of Ser660 but not Thr641, as occurs for the full-length enzyme. Under the conditions of the assay, approximately 50% of the catalytic-domain protein was dephosphorylated. Addition of Mg^{2+}/ATP resulted in the rephosphorylation of Ser660 as assessed by the increase in the fraction of PKC co-migrating with fully phosphorylated enzyme (upper panel) and the renewed immunoreactivity with an antibody to phosphorylated Ser660 (P660, lower panel). Importantly, the ratio of the upper to the intermediate band (3:1) was the same whether 3.5 nM or 18 nM enzyme was present in the autophosphorylation mixture. Thus, the rate of re-autophosphorylation of the

Figure 4



Autophosphorylation at Ser660 *in vitro*. **(a)** Purified GST–Cat285 was dephosphorylated at Ser660 by treating the enzyme with the catalytic subunit of PP2A and then various concentrations of fusion protein were incubated with 100 μM ATP for 5 min at 30°C (lane 3, 3.5 nM; lane 4, 11 nM ; lane 5, 18 nM). Reaction products were separated by SDS–PAGE (7.5% gel), and phosphorylation at Ser660 was determined by western blot analysis using either the phosphorylation-insensitive antibody βII (upper panel) or the phospho-specific antibody P660 (lower panel). Lane 1, starting material (45 ng); lane 2, PP2A-treated sample (45 ng); lanes 3–5, re-autophosphorylated material (20, 60 and 100 ng, respectively); all lanes are from the same gel. Note that the lower protein amount in lane 3 necessitated a threefold longer exposure of the chemiluminescence blot compared with the exposure used in the other lanes. Also, the weaker sensitivity of the P660 antibody (compared with the βII antibody) did not allow detection of phosphorylated protein in lanes 2 and 3 (< 30 ng protein). **(b)** The rate of Ser660 phosphorylation normalized to a constant amount of Cat285 was plotted as a function of Cat285 protein concentration. Data are presented as the number of moles of phosphate incorporated per mole of Cat285 per min; these were obtained from scanning the upper and lower bands in the upper panel in (a), and revealed that 5% of the protein was being phosphorylated per min at each of the three protein concentrations. The solid line describes the kinetics in the case where enzyme activity is independent of enzyme concentration (pseudo-zero order). Data are the average of two separate experiments; the range (± 2%) is smaller than the symbols.

hydrophobic site (approximately 0.05 mole phosphate per mole PKC per minute) was independent of the total enzyme concentration (Figure 4b). Both the kinase domain and phosphatase used in this experiment were homogeneously pure, as assessed by silver staining of samples analyzed by SDS–PAGE (see [13]). Nonetheless, we addressed the possibility that a trace amount of a putative heterologous kinase could be associated with the phosphatase preparation and could be causing the phosphorylation of Ser660. This was found not to be the case: thermally-inactivated PKC βII did not become phosphorylated on Ser660 following the phosphatase treatment and the subsequent incubation with Mg^{2+}/ATP described in Figure 4 (data not shown). It is also noteworthy that pseudo-zero order kinetics would not have been observed had a contaminating kinase been present either in the PKC or phosphatase preparation (unless this kinase had the remarkable property of binding PKC with an affinity below the nM range). Thus, the observed re-autophosphorylation on Ser660 required the catalytic competence of PKC. These data reveal that Ser660 is autophosphorylated by PKC, with the kinetics being most consistent with intramolecular autophosphorylation.

Kinase-inactive PKC is not phosphorylated *in vivo*

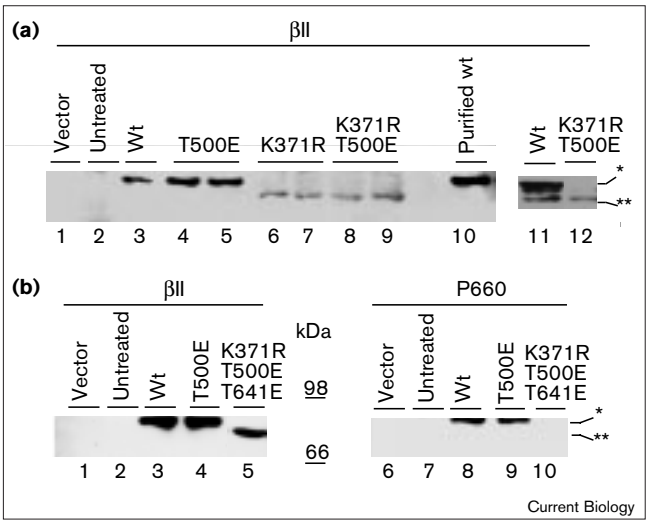
The foregoing data show that PKC autophosphorylates at one of the key sites involved in the post-translational processing of PKC. To test whether this is the mechanism responsible for the modification of the carboxy-terminal sites *in vivo*, we examined the phosphorylation of kinase-inactive versions of full-length PKC expressed in COS-7 cells.

Figure 5 shows a western blot of COS-7 cell lysates overexpressing kinase-inactive mutants or wild-type full-length PKC βII. Both wild-type enzyme (Figure 5a, lane 3) and the T500E activation-loop mutant (Figure 5a; lanes 4,5) migrated as a major band at 80 kDa (single asterisk), representing protein phosphorylated at both carboxy-terminal sites [5]. Both these species co-migrated with mature PKC βII purified from baculovirus (Figure 5a, lane 10). In contrast, the catalytically-inactive mutant K371R (Figure 5a; lanes 6,7) migrated as a single band with an apparent molecular weight of 76 kDa (double asterisks). No upper, 80 kDa band (mature enzyme) or intermediate band was observed. Thus, the catalytically-inactive mutant is not phosphorylated at Thr641 and Ser660, or any other sites on the carboxyl terminus that cause changes in the electrophoretic mobility of PKC.

One possibility for the lack of phosphorylation of the carboxy-terminal sites of the kinase-inactive mutants is that a negative charge is required on the activation loop site in order for a putative heterologous kinase to phosphorylate the carboxy-terminal sites. We tested this possibility by examining the phosphorylation state of the double mutant K371R/T500E. Figure 5a shows that this protein was also not phosphorylated when expressed in COS-7 cells (Figure 5a; lanes 8,9); it co-migrated with unphosphorylated PKC. A similar result was obtained upon analysis of the samples with a second antibody against PKC βII; with this antibody, the minor fastest-migrating species in the wild-type protein was apparent (Figure 5a; lane 11, double asterisks) and the T500E/K371R protein clearly co-migrated with this fully-dephosphorylated form (Figure 5a; lane 12, double asterisks).

We addressed one last possibility: whether negative charge at both the activation loop and Thr641 might be

Figure 5



Catalytically-inactive PKC βII is not phosphorylated at the carboxy-terminal sites *in vivo*. Shown here are western blots of whole cell lysates from COS-7 cells transfected with the DNA for full-length wild-type enzyme (wt), activation-loop mutant (T500E), kinase-inactive mutant (K371R), double mutant (K371R/T500E), or the triple mutant K371R/T500E/T641E. **(a)** The blot was probed with two phosphorylation-insensitive antibodies against PKC βII: a monoclonal antibody from RBI (lanes 1–10) or a polyclonal antibody from Santa Cruz Biotechnology (lanes 11,12). For each mutant indicated, the results of two independent experiments are shown in adjacent lanes. The results of a transfection with vector alone (lane 1) and a mock transfection with no DNA (lane 2) are also shown. Purified PKC βII (20 ng) was loaded as a positive control for immunoblotting (lane 10). **(b)** The blot was probed with an antibody that does not discriminate between phosphorylation states of protein kinase C (βII from RBI; lanes 1–5) or an antibody that specifically labels phosphorylated Ser660 (P660; lanes 6–10). Lanes 1,6, lysates from cells transfected with vector alone; lanes 2,7, untreated cells. In (a,b), a single asterisk indicates the position of the mature (carboxy-terminal-phosphorylated) 80 kDa PKC; the double asterisks mark the position of the unphosphorylated 76 kDa PKC precursor.

required for recognition of the hydrophobic site by a putative heterologous kinase. Figure 5b shows that a kinase-inactive mutant with a negative charge at both Thr500 and Thr641 (K371R/T500E/T641E) migrated faster than the wild-type enzyme (lane 3) or the T500E mutant (lane 4) when expressed in COS-7 cells. To confirm that this increased electrophoretic mobility resulted from lack of phosphate at Ser660, the only position of the three *in vivo* sites available for phosphorylation, the blot was probed with the P660 antibody. Figure 5b shows that the wild-type enzyme (lane 8) and T500E mutant (lane 9) were strongly labeled with the P660 antibodies, whereas comparable amounts of the triple mutant were not labeled with this antibody (lane 10). Thus, the hydrophobic site is not phosphorylated *in vivo* unless PKC has catalytic competence; this result is independent of the phosphorylation state of the activation loop or Thr641.

Discussion

This study establishes that the two carboxy-terminal phosphorylation sites of conventional PKC that are involved in the maturation of the enzyme — Thr641 and Ser660 in the case of PKC βII — are modified by autophosphorylation and not by a heterologous kinase. Studies *in vitro* showed that purified enzyme that had been selectively dephosphorylated on the Ser660 residue in the hydrophobic phosphorylation motif rapidly re-autophosphorylated at this position, most likely by an intramolecular mechanism. Studies *in vivo* revealed that autophosphorylation was the only mechanism involved in modifying Thr641 and Ser660 because neither residue became phosphorylated in kinase-inactive PKC mutants. Thus, the only point of control by a heterologous kinase in the maturation of PKC is at the activation loop.

The PKC kinase domain autophosphorylates *in vitro*

Mature PKC βII has previously been shown to be modified by intramolecular autophosphorylation upon activation *in vitro* [25]. The sites modified have been mapped to three separate regions in the primary sequence: two immediately preceding the amino-terminal pseudosubstrate (Ser16 and Thr17), two in the hinge region (Thr314 and Thr324), and one (Thr634) in the carboxyl terminus [26]. An intramolecular mechanism was shown to account for the rate-limiting step in autophosphorylation of the full-length enzyme [25,26]; the mechanism of phosphorylation of specific sites, however, has not been dissected.

Here, we used a recombinant kinase domain construct to address the mechanism of autophosphorylation of *in vitro* phosphorylation sites within the kinase domain and, more specifically, the *in vivo* phosphorylation site, Ser660. Previous efforts to demonstrate autophosphorylation of a proteolytically-generated catalytic fragment had not been successful, possibly as a result of cleavage of autophosphorylation sites in the amino and carboxyl termini of the domain [25,27]. We used a GST-purified protein to demonstrate that the kinase core by itself is able to autophosphorylate and does so by the same intramolecular reaction mechanism as described for the full-length protein [25]. Thus, the autophosphorylation of PKC does not depend on the presence of the regulatory domain or any cofactors such as phospholipids and, additionally, does not require a higher order protein–phospholipid aggregate as proposed previously [28,29].

It should be noted that the kinase used in these studies was GST-tagged and that the tag was not removed for the kinetic analysis to minimize the thermal inactivation of the purified protein. Because GST is a dimer [30], with an equilibrium dissociation constant below the nM range, the enzyme used in this study was most likely a two-enzyme complex. If the two active sites were positioned so as to access one another, pseudo-zero order kinetics would be

observed in the dilution studies, even though the phosphorylation could be intermolecular. Interestingly, the autophosphorylation of Thr634, a phosphorylation site *in vitro* [25,31], was concentration-dependent (data not shown). This would suggest that the active sites of the two GST-linked enzymes do not access one another, because the rate-limiting reaction in the phosphorylation of Thr634 occurs by collision of PKC on one complex with PKC on another complex. Thus, the concentration independence of the whole kinase core, and that of Ser660, is consistent with an intrapeptide reaction.

The hydrophobic phosphorylation motif is modified by intramolecular autophosphorylation

The question of whether the hydrophobic phosphorylation motif is recognized by a heterologous kinase or whether it is modified by autophosphorylation is central to understanding the regulation of the many kinases containing this phosphorylation switch. For the PKCs, phosphorylation at this position by a heterologous kinase would add a third regulatory switch in addition to the regulation of the activation loop by PDK-1 and the regulation of the pseudosubstrate by cofactors.

We found that, when the fully-processed kinase domain is treated with PP2A to dephosphorylate Ser660, the enzyme re-incorporates phosphate at this position upon incubation with Mg^{2+}/ATP. Dilution studies showed that rate of re-phosphorylation at residue 660 was the same over a five-fold concentration range of the protein, consistent with intramolecular autophosphorylation.

Carboxy-terminal sites are modified by autophosphorylation *in vivo*

The finding that the hydrophobic phosphorylation motif is modified by autophosphorylation suggests that PKC itself, and not a heterologous kinase, modifies this position *in vivo*. Especially if intramolecular, the high local concentration of the carboxyl terminus near the active site of PKC would make it unlikely for another kinase to effectively compete for phosphorylation unless tethered right next to PKC.

Expression of kinase-inactive mutants of PKC revealed that autophosphorylation is the only mechanism accounting for the modification of the two carboxy-terminal phosphorylation sites of PKC *in vivo*. The kinase-dead mutant of PKC βII, K371R, does not become phosphorylated at the carboxy-terminal phosphorylation sites. One possibility is that phosphorylation of the carboxy-terminal sites by a putative heterologous kinase requires phosphorylation of the activation loop. Although the activation loop of the K371R mutant is available for phosphorylation by PDK-1, or a related kinase, this site is extremely susceptible to dephosphorylation in the absence of phosphates on the carboxyl terminus ([15]; our unpublished data). Thus, to ensure a negative charge at the activation loop, we generated a kinase-dead, T500E double mutant. We found that this mutant did not incorporate phosphate at either of the two carboxy-terminal phosphorylation sites. Neither was the triple mutant K371R/T500E/T641E phosphorylated on Ser660 *in vivo*. Thus, the carboxyl terminus is not modified by a heterologous kinase regardless of whether the activation loop is phosphorylated. Importantly, the hydrophobic site is not phosphorylated unless PKC has catalytic competence independently of the phosphorylation state of the two other key sites on the enzyme.

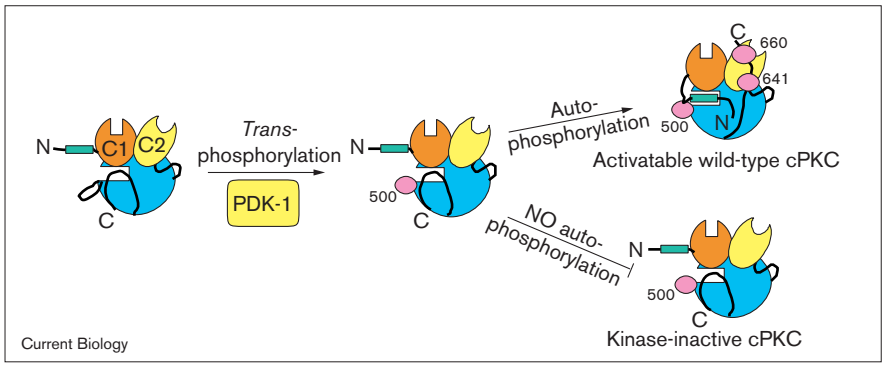
Phosphorylation of the activation loop is required to permit autophosphorylation of carboxy-terminal sites

We have previously proposed that phosphorylation at the activation loop is the first step in the processing of PKC [5]. This event, by analogy with protein kinase A, was proposed to structure the active site for catalysis, with the immediate consequence being two sequential autophosphorylations at the carboxyl terminus. Consistent with this model, activation-loop mutants in which the phosphorylatable position has been replaced by neutral, non-phosphorylatable residues do not become phosphorylated on the carboxy-terminal phosphorylation sites and, hence, are inactive ([18,19]; A.S. Edwards and A.C.N., unpublished observations). Further support for this model was recently provided by the finding that overexpression of a kinase-inactive PDK-1 results in the accumulation of unphosphorylated PKC [15]. Thus, phosphorylation of the activation loop appears to be a necessary, and first, event in the processing of PKC. Here, we show that the requirement for activation-loop phosphorylation is not to allow recognition of the carboxy-terminal phosphorylation sites by a heterologous kinase, but rather to activate the enzyme so that it can catalyze the autophosphorylations at the carboxyl terminus. In other words, phosphorylation of the activation loop is required for phosphorylation of the carboxyl terminus because it imparts the catalytic competence required for the carboxy-terminal autophosphorylations (Figure 6).

Conclusions

The hydrophobic phosphorylation motif found in PKC βII is conserved not only among the PKCs, but also in a number of other kinases, such as Akt and S6 kinase, that are linked to lipid signaling [6,32]. Phosphorylation at this motif is sensitive to serum, leading to the proposal that it is regulated by a phosphoinositide-dependent kinase, tentatively referred to as 'PDK-2' [33]. Nonetheless, such a kinase has remained refractory to identification. Indeed, it has recently been reported that PDK-2 is actually PDK-1 that has switched substrate specificity upon binding a peptide based on the hydrophobic phosphorylation motif of the PKC-related kinase, PRK [34]. Because this report did not examine the phosphorylation of kinase-inactive mutants of the substrate kinase, PKB, it is not clear whether the reported phosphorylation was mediated by

Figure 6



Model for the processing of conventional PKC (cPKC) by phosphorylation. The domain structure of PKC is shown with the membrane-targeting modules (C1,C2) linked to the kinase-domain (blue circle). In the unphosphorylated conformation (left), the carboxyl terminus (C, black line) is proposed to bind the active site, displacing the pseudosubstrate (green rectangle). The first, and rate-limiting, step in the maturation of cPKC is the phosphorylation at the exposed activation loop (black loop on kinase domain) by the heterologous kinase, PDK-1 [14] (middle). This phosphorylation (Thr500 in PKC βII) appears to structure the active site for catalysis, with the immediate consequence being the rapid autophosphorylation of the two carboxy-terminal phosphorylation sites, one in the turn motif (Thr641 in PKC βII) and the second in the hydrophobic phosphorylation motif (Ser660 in PKC βII) (top right). Phosphorylation of the carboxyl terminus locks the active site in a catalytically-competent conformation [7,10] and promotes the binding of the pseudosubstrate to the substrate-binding cavity; the enzyme remains inactive until second-messenger-dependent membrane recruitment provides the energy to release the pseudosubstrate [12]. Phosphorylation at both carboxy-terminal sites requires the catalytic competence of PKC: kinase-inactive constructs are not phosphorylated at the carboxy-terminal sites regardless of the phosphorylation state of the activation loop and, for the hydrophobic site, the phosphorylation state of Thr641 (bottom right).

the 'converted' activity of PDK-1, or whether it was an autophosphorylation reaction that became possible because of allosteric or steric constraints relieved in the presence of the activating peptide.

Alternatively, the hydrophobic phosphorylation site may provide a regulatory switch by being a target for stimulus-regulated phosphatase activity (rather than heterologous kinase activity). In this regard, Schreiber and coworkers have recently reported that the serum and rapamycin sensitivity of the hydrophobic site in S6 kinase may arise from stimulation of phosphatase activity rather than from the previously hypothesized inhibition of kinase activity [35].

Here, we have established that, at least for a conventional PKC, the conserved hydrophobic site is modified exclusively by autophosphorylation *in vivo*. Thus, phosphorylation at this position does not provide a point for external regulation. Nor is this a point of regulation for the atypical PKCs, as these kinases have a constitutive negative charge (Glu) at the position of the phosphorylatable residue. Therefore, neither conventional nor atypical PKCs are regulated by the hypothetical PDK-2. Rather, regulation through phosphatases provides an attractive model to account for the changes in phosphorylation of the hydrophobic site *in vivo* for mature PKC.

Materials and methods

Materials

Bovine brain L-α-phosphatidylserine and *sn*-1,2-dioleoylglycerol were purchased from Avanti Polar Lipids; Triton X-100 and peroxidase-conjugated anti-rabbit IgG antibodies were obtained from Calbiochem; chemiluminescence SuperSignal substrates were from Pierce; polyvinylidene difluoride membrane (Immobilon-P) was purchased from Millipore; [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was from DuPont NEN; ATP, DTT, HEPES, EGTA and protamine sulfate were from Sigma; restriction enzymes and other DNA modifying enzymes were obtained from New England Biolabs, Stratagene, or Boehringer Mannheim; the pEGFP-C1 vector was from Clontech; pAcHLT-B was from Pharmingen; and SuperFect transfection reagent was obtained from Qiagen. The PKC-selective peptide substrate (Ac-FKKSFKL-NH_2) was kindly synthesized by E. Komives (University of California at San Diego). The cDNA for rat PKC βII [36] was a gift from Daniel E. Koshland Jr. All other chemicals were reagent grade.

Antibodies

A polyclonal antibody directed against the carboxyl terminus of the catalytic domain of PKC βII (PKC C-18; epitope amino acids 657–673) was purchased from Santa Cruz. A monoclonal antibody against PKC βII (clone PK-B26; epitope amino acids 660–673) was purchased from RBI. A polyclonal antibody directed against the phosphorylated activation loop of PKC βII (P500) was generated by immunizing rabbits with a phosphopeptide based on the sequence D_{494}GVTTKpTFCGTPD$_{506}$ (where pT corresponds to phospho-threonine) [16]. An antibody that selectively labels phosphorylated Thr634 (P634) [31] was provided by David Sweatt (Baylor College of Medicine), and one directed against phosphorylated Ser660 (P660) was provided by Michael Comb (New England Biolabs).

PKC constructs

DNA encoding residues 307–673 and 285–673 of PKC βII were cloned into the pAcHLT-B vector (which provides a GST tag), and recombinant baculovirus was generated according to the 'Baculovirus Expression Vector System – Instruction Manual' by Pharmingen. DNA encoding the K371R and K371R/T500E mutants was generated by PCR using wild-type PKC βII or T500E mutant as the template ([18]; L.M. Keranen and A.C.N., unpublished data); the construct for the triple mutant K371R/T500E/T641E was prepared by cutting and pasting the T641E construct into the K371R/T500E construct.

Electrophoresis and western blots

Samples of cell extracts or purified protein were analyzed by SDS–PAGE (7.5% gels) and subsequent transfer to polyvinylidene difluoride membrane. Blots were probed with antibodies to the catalytic domain of PKC, and labeling was detected by chemiluminescence. Antibody staining was quantified using a Molecular Dynamics scanner under conditions where antibody reactivity was linear with antigen.

Kinase activity assay

PKC activity was assayed by measuring the rate of ^{32}P incorporation from [γ-^{32}P]ATP into either PKC-selective peptide [24] or protamine sulfate as a substrate in a paper assay as described [37]. The standard reaction mix (80 μl) contained 20 mM HEPES pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 100 μM ATP, 50 μM peptide substrate or 1 mg ml^{-1} protamine sulfate. In some experiments, 0.15 mM $CaCl_2$ and sonicated dispersions of phosphatidylserine (140 μM) and diacylglycerol (4 μM) were included in the reaction mixture. Samples were incubated at 30°C for the indicated amount of time and stopped by the addition of 25 μl of a solution containing 0.1 M ATP and 0.1 M EDTA or 4× sample buffer. Aliquots (85 μl) were spotted onto P81 ion-exchange chromatography paper, washed four times with 0.4% phosphoric acid and finally rinsed in 95% ethanol. Incorporation of ^{32}P was detected by liquid scintillation counting in 5 ml BiosafeII (Research Products International Corp.).

Autophosphorylation assay

The assay for autophosphorylation of the kinase domain was carried out essentially as described for substrate phosphorylation except that the substrate was omitted and reactions were quenched by addition of 4× sample buffer. Phosphorylation was detected by measuring ^{32}P incorporation in kinase-domain bands excised from gels following SDS–PAGE or by western blot analysis using phosphorylation-specific antibodies.

Expression of the kinase core in Sf21 cells

Sf21 cells infected with baculoviral constructs of PKC were harvested after 3 days at 27°C by homogenization in lysis buffer containing 50 mM HEPES pH 7.5, 0.2% Triton X-100, 1 mM EDTA, 1 mM DTT, 85 μM leupeptin, 2 mM benzamidine and 0.2 mM phenylmethanesulfonyl fluoride as described [10]. After removing an aliquot of the lysate, the remainder was centrifuged at 100,000 *g* for 20 min at 4°C, the pellet was resuspended in lysis buffer, and lysate, supernatant and pellet were stored in 50% glycerol at −20°C. For some experiments, the kinase core was purified from the supernatant according to the GST purification method [38].

Expression of PKC constructs in COS-7 cells

PKC constructs were transiently transfected into COS-7 cells using SuperFect transfecting reagent. Transfection of 6-well plates were carried out according to the manufacturer's protocol. Cells were harvested in lysis buffer, either sonicated or snap frozen, and subjected to the same procedure as described above for expression in Sf21 cells [10].

Phosphatase treatment of purified kinase core

The purified catalytic domain of PKC βII (2–4 pmol) was incubated with PP2A (6 U ml^{-1}) in 20 mM HEPES buffer pH 7.5, 1 mM DTT, 40 μg ml^{-1} leupeptin, and 10 μg ml^{-1} BSA for 15 min at room temperature. The reaction was stopped by adding microcystin to a final concentration of 1 μM.

Data analysis

The dependence of enzymatic activity on ATP or substrate concentrations was analyzed by Michaelis–Menten kinetics using the program SigmaPlot. Protein concentration estimations were carried out by densitometric scanning (densitometer by Molecular Dynamics) of either western blots or silver-stained gels following SDS–PAGE.

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