Membrane Translocation of Novel Protein Kinase Cs Is Regulated by Phosphorylation of the C2 Domain*

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Ca\textsuperscript{2+}-independent or novel protein kinase Cs (nPKCs) contain an N-terminal C2 domain of unknown function. Removal of the C2 domain of the Aplysia nPKC Apl II allows activation of the enzyme at lower concentrations of phosphatidylserine, suggesting an inhibitory role for the C2 domain in enzyme activation. However, the mechanism for C2 domain-mediated inhibition is not known. Mapping of the autophosphorylation sites for protein kinase C (PKC) Apl II reveals four phosphopeptides in the regulatory domain of PKC Apl II, two of which are in the C2 domain at serine 2 and serine 36. Unlike most PKC autophosphorylation sites, these serines could be phosphorylated in trans. Interestingly, phosphorylation of serine 36 increased binding of the C2 domain to phosphatidylserine membranes in vitro. In cells, PKC Apl II phosphorylation at serine 36 was increased by PKC activators, and PKC phosphorylated at this position translocated more efficiently to membranes. Moreover, mutation of serine 36 to alanine significantly reduced membrane translocation of PKC Apl II. We suggest that translocation of nPKCs is regulated by phosphorylation of the C2 domain.

Protein kinase Cs (PKCs)\textsuperscript{3} are a family of lipid-activated enzymes that play important roles in many cellular processes including regulation of synaptic strength in the nervous system (1). We have studied the regulation of PKC in Aplysia, an important model system for synaptic plasticity, where PKC plays several important roles in regulating synaptic strength (2–6). The structure of PKCs is conserved in Aplysia. Ca\textsuperscript{2+}-activated PKCs (cPKCs) in vertebrates (α, β, γ) and invertebrates (PKC Apl I in Aplysia) have two C1 domains and a C2 domain. Ca\textsuperscript{2+}-independent or novel PKCs (nPKCs) in vertebrates (δ, ε, η, and θ) and invertebrates (PKC Apl II in Aplysia) also have two C1 domains and a C2 domain. However, the C2 domain in these kinases is N-terminal to the C1 domains and lacks several of the critical aspartic acids necessary for the coordination of Ca\textsuperscript{2+} ions (7).

In cPKCs, the C2 domain mediates Ca\textsuperscript{2+}-dependent binding to the membrane lipid phosphatidylserine (PS) (8, 9). This binding is believed to be the primary step in kinase activation. First, it transiently recruits the enzyme to the membrane where its physiological activator, diacylglycerol (DAG), resides. Second, in conjunction with the C1 domain interacting with DAG, binding of the C2 domain to PS induces a conformational change that activates the enzyme (10, 11). Finally, the C2 domain binds to the receptor for activated protein kinase C (RACK), and this binding is important for the location of PKC translocation and for cellular functions of PKC (12).

In contrast to cPKCs, a detailed model for the role of the N-terminal C2 domain in nPKC activation is not available. C2 domains of nPKCs do not bind constitutively to lipids and are not regulated by calcium (13). However, similar to cPKCs, C2 domain-derived peptides from nPKCs act as isoform-specific inhibitors and activators in cells, implicating the C2 domain in the regulation of enzymatic activity (14–16). Removal of the C2 domain of PKC\textsubscript{η} does not perturb the substrate specificity or response of the enzyme to phosphatidylinositol 3-kinase activity (17, 18). However, removal of the C2 domain of PKC Apl II decreases the amount of PS necessary for kinase activity, implicating the C2 domain in kinase regulation (19).

In vitro, PKC Apl II is activated more poorly than PKC Apl I, even when calcium is removed from PKC Apl I, suggesting a requirement of additional activators for stimulation of PKC Apl II \textit{in vivo} (13, 19). Comparisons between PKC\textsubscript{ε} and PKC\textsubscript{α} also indicate that nPKCs require additional factors to bind efficiently to lipid membranes (20). PKC Apl II can be activated under a number of physiological conditions in cells, usually after prolonged activation of signal transduction pathways (6, 21). Prolonged activation also induces autonomous activity of PKC Apl II (22). This activation is specific, as PKC Apl I does not become autonomous under these conditions. The autonomous activity does not result from proteolysis to form a protein kinase M but probably results from post-translational modifications in the regulatory domain of PKC Apl II (22).

PKCs are regulated by phosphorylation. Phosphorylation in the activation loop by phosphoaminoiside-dependent protein kinase I (PKD-1) is required for subsequent PKC “maturity” by autophosphorylation at two residues critical for enzyme stability and activity (8, 23, 24). Phosphorylation of PKC\textsubscript{8} on tyrosine residues is thought to be important for activation of the kinase (25–27). Upon activation, PKCs additionally phosphorylate themselves on several residues. The roles for these phosphorylations are still unknown. Autophosphorylation at a conserved site in the C-terminal domain has been associated with

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The abbreviations used are: PKC, protein kinase C; cPKC, classical PKCs; nPKC, novel PKC; PS, phosphatidylserine, PC, phosphatidylcholine; pfu, plaque-forming units; MBP, maltose-binding protein; GST, glutathione S-transferase; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin; FDBu, phosphor dibutyrate; FMA, phosphorol 12-myristate 13-acetate; RACK, receptor for activated protein kinase C; PCR, polymerase chain reaction; O5, outside 5’ primer; I3, inside 3’ primer; I5, inside 5’ primer; O3, outside 3’ primer; DAG, diacylglycerol.


Phosphorylation of the C2 Domain of PKC Apl II

| Construct name | Primers | Cloning sites | Introduced sites |
|----------------|---------|---------------|-----------------|
| MBP-C2         | I5 5'-CAGAAGGGCCCGCCAGAAGAAGAAAGG | EcoRI | Nar1 |
| S36A           | I5 5'-TTCTTTCTGTTGCGGCCCTCTGCAGT | KpnI |  |
| MBP-C2         | I5 5'-CCAGAGGTGAGCAGAAGAAGAAAGG | EcoRI | Esp31 |
| S36E           | I5 5'-TCTTCTGGTCCACCCCTGTCACGT | BanHI |  |
| MBP-C2         | I5 5'-CCACAGGGCCATTAACACAGTG | EcoRI | HaeIII |
| S68A           | I5 5'-GGTTTAAAGGCGCTTGGCTGTA | BanHI |  |
| MBP-C2         | I5 5'-GCTTCAATGGCCCAGGGCAGGCCCCATG | XmnI | BasIII |
| S2A            | I5 5'-GCCCCTGCGGCCATTGAGGCGCGCTC |  |  |
| Apl II S36A    | I5 5'-same as MBP-C2 S36A | EcoRI | Nar1 |
| Apl II S36E    | I5 5'-same as MBP-C2 S36E | EcoRI | Esp31 |
| All MBP and PKC Apl II mutants | O5 5'-GGGAATCTCCATGGTCTTCAAGGGTTCG | KpnI |  |
|                | O3 5'-CGCCAGGATTCTCCATGGTCTTCAAGGGTTCG |  |  |
| PKC<sub>e</sub> | O5 5'-GTAATACGACTCACTATAGGG | KpnI |  |
| PKC<sub>e</sub> Δ C2 | O3 5'-GGGTACCGCTGACCATGACGTCGTTCCGAGGAA | KpnI |  |

* NA, not applicable.

In this paper we identify two autophosphorylation sites, serine 2 and serine 36, in the C2 domain of the nPKC Apl II. Phosphorylation at serine 36 increases lipid binding to the C2 domain and increases translocation of PKCs in cells. We suggest that phosphorylation of C2 domains in novel PKCs is important for regulating their translocation.

**EXPERIMENTAL PROCEDURES**

Reagents—4-6-phorbol 12,13-dibutyrate was from LC Services; dioleoyl phosphatidylserine (PS) and dioleoyl phosphatidylcholine (PC) were from Avanti Polar Lipids Inc., Alabaster, AL; Triton X-100 was from Avanti; prestained molecular weight markers were from Ameri...
Pelleting of lipids was confirmed using scintillation counting of [3H]PC. C2, 50 II phosphorylation reaction contained 10 pmol of PKC Apl II or PKC Apl C2 was autophosphorylated as described (19). The phosphorylation was quenched by the addition of 5× Laemmli sample buffer and boiled.

**PKC Assays**—Protein kinase C (5 nM) activity was assayed using phosphor esters or the mixed micelle assay as described (19, 34).

**PKC and Fusion Protein in Vitro Phosphorylation**—PKCs (200–500 nM) or PKC-derived C2 domain fusion proteins (2 μM) were incubated in reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM EGTA) with 1 μM dioleoyl phosphatidylserine, 0.1 mM PMA, and 5 μCi of [γ-32P]ATP (4 μCi in 50 μM ATP) for 30 min at room temperature. Phosphorylation was quenched by the addition of 5× Laemmli sample buffer and boiled.

**C2 Domain Membrane-binding Assay**—Membrane binding of C2 domain constructs was determined by measuring the binding to sucrose-loaded vesicles (19, 33, 35). MBP fusion proteins on amyllose beads were phosphorylated by PKC as described above. PKC, ATP, and lipids were removed by sedimenting the beads at low speeds (3000 × g for 1 min) followed by three washes with sedimentation buffer (0.3 mg ml⁻¹ ovalbumin, 100 mM KCl, 1 mM dithiothreitol, 5 mM MgCl₂, 20 mM HEPES, pH 7.5). Fusion proteins were eluted from the beads with sedimentation buffer containing 10 μM maltose. The purified fusion proteins were prepurified to pellet aggregates for 30 min at 100,000 × g and 100,000 × g in 75% (Beckman Instruments, Palo Alto, CA). Soluble fusion proteins (1–2 μg) were incubated with sucrose-loaded vesicles (20 μM lipid) for 10 min at 15 °C. The proteins associated with the sucrose-loaded vesicles were then separated from free protein by ultracentrifugation at 100,000 × g for 30 min at 15 °C. Pelleting of lipids was confirmed using scintillation counting of [3H]PC. The percentage change between these two conditions (experimental − control/control) was determined.

**RESULTS**

The C2 Domain of PKC Apl II Contains Two Auto phosphorylation Sites—To determine whether the C2 domain of PKC Apl II is regulated by autophosphorylation, we compared tryptic phosphopeptide maps generated after autophosphorylation of purified PKC Apl II or a purified mutant lacking the C2 domain (PKC Apl IIΔC2). PKC Apl II had 5 reproducible phosphopeptides (Fig. 1A). Two of these phosphopeptides (2 and 3) were not found in the enzyme lacking the C2 domain (Fig. 1B). Removing the C2 domain generated no additional or modified sites (Fig. 1C). The absence of phosphopeptides 2 and 3 could be due to their location in the C2 domain or conformational changes due to the loss of the C2 domain. To obtain further evidence these sites were actually located in the C2 domain, we obtained tryptic phosphopeptide maps of C2 domain-containing regions of PKC Apl II after partial tryptic digests of autophosphorylated PKC Apl II and PKC Apl IIΔC2 (Fig. 2A). The C2 domain containing fragments were identified based on immunoreactivity to a C2 domain-specific antibody (Fig. 2B) and by their absence in partial tryptic digests of PKC Apl IIΔC2 (Fig. 2B, lane 2). Three bands were isolated from the gel and completely digested with trypsin for phosphopeptide mapping analysis (Fig. 2C). The largest C2 domain fragment, corresponding to the size of the intact regulatory domain contained phosphopeptides 2–5 suggesting that all four of these sites are present in the regulatory domain (Fig. 2C, upper panel). Phosphopeptide 1 was not further investigated but based on predicted mobility it may correspond to the C-terminal fragment that contains two predicted phosphorylated sites (28). The C2 domain cells were resistant to tryptic digestion (Fig. 2C). Phosphopeptide mapping of the smallest C2 domain fragment corresponding to the size of the core exhibited only phosphopeptide 3 (Fig. 2C, lower panel). Thus, phosphopeptide 3 is contained in the C2 domain, whereas phosphopeptide 2 is either near the end of the C2 domain and is sensitive to tryptic digestion or is outside the C2 domain, and its phosphorylation is decreased by C2 domain removal.

The Autonomous PKC Apl II Is Not Autophosphorylated at Site 4—Purified PKC Apl II contains both regulated and au-
A, phosphorylated, digested, and analyzed as described under "Experimental Procedures." A, PKC Apl II wild type (lane 1) and PKC Apl IIΔC2 (lane 2) were phosphorylated in vitro for 30 min in the presence of [γ-32P]ATP. The samples were partially digested with 2 μg/ml trypsin for 5 min at 30 °C, separated by SDS-PAGE, transferred to nitrocellulose, and exposed to x-ray film (Autoradiogram). B, Western blot of the membrane in A using an antibody (Ab) directed to the C2 domain of PKC Apl II (13) confirming that the bands positive for 32P incorporation in A are C2 domain-containing fragments. C, phosphopeptide maps of C2 domain containing bands from B, lane 1 (upper to lower), illustrating that peptide 3 is isolated as the C2 domain core is isolated. + and − refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; S signifies origin of sample application or coapplication.

no significant difference between oleic acid and phorbol ester stimulation (Fig. 3C). Thus, the selective requirement of the C2 domain for fatty acid-induced phosphorylation is not due to preferential autophosphorylation of this domain.

Sites in the C2 Domain Can Be Trans-phosphorylated—Incubation of PKC Apl II or PKC Apl I (data not shown) with fusion proteins containing the C2 domain led to trans-phosphorylation of the C2 domain (Fig. 4A). Phosphopeptide mapping results of both trans-phosphorylated GST- and MBP-C2 domain fusion proteins indicated two major C2 domain phosphorylation sites (Fig. 4, B and C). In contrast, phosphorylation of GST or MBP alone did not generate these sites (data not shown). Coapplication of phosphopeptides derived from autophosphorylated PKC Apl II and the trans-phosphorylated GST-C2 domain fusion protein demonstrated that phosphopeptide 3 was identical in the autophosphorylated kinase and in the trans-phosphorylated C2 domain (Fig. 4D). An additional phosphopeptide (2') was also observed in both fusion proteins, whose migration was slightly altered from phosphopeptide 2 in autophosphorylated PKC Apl II.

Site 2 Is Serine 2—We used a series of C2 domain deletion constructs (19) to attempt to delineate the sites of trans-phosphorylation that corresponded to the various phosphopeptides. However, deleting any area of the C2 domain prevented phosphorylation of peptide 3 suggesting that phosphorylation of this site is sensitive to conformation of the C2 domain (data not shown). In contrast, the site that produced phosphopeptide 2' was removed by an N-terminal deletion but not a C-terminal deletion (data not shown). If site 2 was at the N terminus of the C2 domain, the tryptic peptide is predicted to be altered by deletion of two amino acids from the linker region of the fusion protein (MSR → ASMSR) consistent with the change from site 2 to the new site 2'. An N-terminal location would also be consistent with the loss of phosphopeptide 2' from the C2 domain.
domain by the partial tryptic digestion (Fig. 2C, lower panel). Indeed, site 2 was not observed when serine 2 was mutated to alanine in the MBP-C2 domain fusion protein (data not shown).

The Major Autophosphorylation Site of PKC Apl II Is Serine 36—Phosphoamino acid analysis of phosphopeptides 2, 3, and 5 revealed them all to be serines (data not shown). By using a hypothetical phosphopeptide map of regulatory domain tryptic peptides, and assuming phosphopeptide 2 is M(pS)R, there were only two possible serines (serine 36 and serine 68) that were consistent with the migration of phosphopeptide 3 (Fig. 5). Conversion of serine 68 to alanine did not affect peptide 3 phosphorylation (data not shown). In contrast conversion of serine 36 to alanine prevented peptide 3 phosphorylation in both the trans-phosphorylated fusion protein (Fig. 5, B–D) and the intact kinase (Fig. 5, E–G).

C2 Domain Phosphorylation at Serine 36 Promotes Lipid Binding—The C2 domain of PKC Apl II does not bind to phosphatidylserine membrane preparations using vesicles (19), lipid micelles (13), or extruded sucrose-loaded vesicles (Fig. 6A; quantitated in Fig. 6B). In contrast, the C2 domain showed significant binding to extruded sucrose-loaded vesicles (60% PS, 40% PC) after trans-phosphorylation by PKC Apl II (Fig. 6A; quantitated in Fig. 6B). This result was also observed using PS vesicles (data not shown). In these experiments less than 5% of the fusion protein was phosphorylated (see “Experimental Procedures”), and thus phosphorylated protein did not con-
Phosphorylation of the C2 Domain of PKC Apl II

Serine 36 is the primary phosphorylated residue in the C2 domain of PKC Apl II. A, hypothetical phosphopeptide mapping of the regulatory domain of PKC Apl II identifies probable peptide 3. The regulatory region of PKC Apl II was divided into constituent tryptic peptides and their mass/charge ratio calculated based on singular phosphorylations. The relative hydrophobicity of each peptide was then calculated in the phospho-chromatography buffer (36). The resulting values for regulatory domain peptides (black circles), C2 domain-specific peptides (gray circles), and C2 domain peptide TTTK (white circle) were plotted against each other to generate a phosphopeptide “map” where the location of each peptide is relative. B–D, the residue within peptide 3 responsible for its heavy phosphorylation is serine 36. Proteins were phosphorylated, digested, and analyzed as described under “Experimental Procedures.” B, phosphopeptide analysis of the wild type (wt) MBP-C2 domain. C, phosphopeptide analysis of the MBP-C2 domain fusion protein with a Ser-36 → Ala mutation (MBP-C2 S36A). D, phosphopeptide analysis of coapplied wild type MBP-C2 domain and MBP-C2 S36A. E–G, PKC Apl II autophosphorylates at serine 36. E, phosphopeptide analysis of wild type PKC Apl II. F, phosphopeptide map of the mutant PKC Apl II containing the Ser-36 → Ala mutation (PKC Apl II S36A). G, phosphopeptide map of coapplied PKC Apl II wild type and PKC Apl II S36A. + and − refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; S signifies origin of sample application or coapplication.

Fig. 5. Serine 36 is the primary phosphorylated residue in the C2 domain of PKC Apl II. A, hypothetical phosphopeptide mapping of the regulatory domain of PKC Apl II identifies probable peptide 3. The regulatory region of PKC Apl II was divided into constituent tryptic peptides and their mass/charge ratio calculated based on singular phosphorylations. The relative hydrophobicity of each peptide was then calculated in the phospho-chromatography buffer (36). The resulting values for regulatory domain peptides (black circles), C2 domain-specific peptides (gray circles), and C2 domain peptide TTTK (white circle) were plotted against each other to generate a phosphopeptide “map” where the location of each peptide is relative. B–D, the residue within peptide 3 responsible for its heavy phosphorylation is serine 36. Proteins were phosphorylated, digested, and analyzed as described under “Experimental Procedures.” B, phosphopeptide analysis of the wild type (wt) MBP-C2 domain. C, phosphopeptide analysis of the MBP-C2 domain fusion protein with a Ser-36 → Ala mutation (MBP-C2 S36A). D, phosphopeptide analysis of coapplied wild type MBP-C2 domain and MBP-C2 S36A. E–G, PKC Apl II autophosphorylates at serine 36. E, phosphopeptide analysis of wild type PKC Apl II. F, phosphopeptide map of the mutant PKC Apl II containing the Ser-36 → Ala mutation (PKC Apl II S36A). G, phosphopeptide map of coapplied PKC Apl II wild type and PKC Apl II S36A. + and − refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; S signifies origin of sample application or coapplication.

Contribute significantly to the measured translocation of total fusion protein. The binding was reduced if the vesicles contained only PC (Fig. 6A; quantitated in Fig. 6B). Conversion of serine 36 to alanine significantly decreased binding of the phosphorylated fusion protein to the lipid vesicles (Fig. 6A; quantitated in Fig. 6B), whereas conversion of serine 68 to alanine did not decrease binding (data not shown). In contrast, conversion of serine 36 to glutamic acid significantly increased binding of the fusion protein to lipids, even in the absence of phosphorylation. These results suggest that phosphorylation of serine 36 increased lipid binding. Phosphorylation of serine 2 may also be involved in binding since the phosphorylated form of both the S36E and S36A fusion proteins bound lipids better than did their nonphosphorylated form (Fig. 6A, quantitated in Fig. 6B).

The conversion of serine 36 to glutamic acid caused a small shift in the position of the protein on SDS-PAGE gels (Fig. 6A). This band comigrated with a phosphorylated form of the wild type fusion protein (Fig. 6A). The form of the fusion protein migrating at this position bound better to the lipid vesicles than did the lower band (Fig. 6A). These results suggest that phosphorylation caused a conformational change involved in lipid binding. However, phosphorylation was neither necessary nor sufficient to cause the shift, since (i) phosphorylated wild type protein incubated with PC vesicles alone rarely exhibited the shift in molecular weight (Fig. 6A) and (ii) in experiments using higher concentrations of fusion proteins and sucrose-loaded vesicles, the shifted band was seen in the nonphosphorylated wild type protein and even in the nonphosphorylated S36A fusion protein (data not shown). These results are consistent with a model whereby a conformational shift is required for lipid binding, and phosphorylation of the fusion protein enhances the stability of this conformation.

Conversion of Serine 36 to Alanine or Glutamic Acid Does Not Affect Enzyme Activation—Removal of the C2 domain allows kinase activation at lower concentrations of PS using the mixed micelle assay. To determine whether mutations at serine 36 could mimic this effect, we assayed PKC Apl II S36A and S36E and wild type PKC Apl II in the mixed micelle assay. No difference was seen in the concentration of PS required for activation of PKC Apl II S36A or S36E compared with the wild type control (Fig. 7). The lack of an effect of S36E suggests that phosphorylation of this site is not important for removing C2 domain inhibition or that the glutamic acid does not mimic phosphorylation of serine 36. Whereas the conversion to glutamic acid does allow for lipid binding of the isolated C2 domain, there may be differences between the phosphorylated residue and the glutamic acid in the context of the whole protein.

Characterization of a Phosphospecific Antibody to Serine 36—To examine phosphorylation of serine 36 in cells, we generated a phosphopeptide antibody to serine 36 (phospho-Ser-36). We tested the specificity of the antibody using autophosphorylated, purified PKC Apl II and PKC Apl II S36A (Fig. 8A). Purified PKC Apl II showed some reactivity with the phosphopeptide antibody, and stimulation of PKC Apl II by either PS-PMA or oleic acid resulted in a large increase in immuno-
Protein phosphorylation is an important post-translational modification that regulates cellular functions. Autophosphorylation of the C2 domain of PKC Apl II is serine 36 in the C2 domain, and this phosphorylation is required for proper translocation of PKC. However, only a small percentage of wild type enzyme may have been assisted in translocation by transient phosphorylation, and this would lead to an increased translocation of wild type protein compared with the Ser → Ala mutation.

**DISCUSSION**

**Autophosphorylation of PKC Apl II—** A major site for autophosphorylation in PKC Apl II is serine 36 in the C2 domain, and PKC Apl II is probably also autophosphorylated at serine 2 in the C2 domain. Although these autophosphorylations are likely due to cis-autophosphorylation (initial studies suggest single order kinetics), unlike many PKC phosphorylations, serines 2 and 36 can be phosphorylated in trans. Furthermore, serine 36 is contained in a consensus PKC phosphorylation site (Fig. 9A). At this time, we do not know whether serine 2 and serine 36 are cis- or trans-phosphorylated in cells. The site was trans-phosphorylated by PKC Apl I in vitro; however, we did not see phosphorylation of a kinase-dead mutant of PKC Apl II when coexpressed in Sf21 cells with PKC Apl I (data not shown).

The autonomous kinase was not autophosphorylated at peptide 4. We have not directly determined the sequence of peptide 4, but based on partial tryptic digests it is likely to be in the regulatory domain. Furthermore, based on predicted tryptic phosphopeptide mobilities, both peptides 4 and 5 are in the hinge domain. Phosphorylation of the C2 domain was observed by immunoreactivity increased after addition of PDBu to the cells (Fig. 8). Immunoreactivity increased after addition of PDBu to the cells (150 ± 30%, n = 3, S.E.), consistent with serine 36 being autophosphorylated in cells (Fig. 8B). PDBu also translocated serine 36-phosphorylated PKC Apl II (Fig. 8B). The relative percent translocation of serine 36-phosphorylated PKC Apl II was increased compared with total PKC Apl II (690 ± 300% compared with 90 ± 30%, n = 3, S.E.), consistent with a role for phosphorylation in translocation. However, this large increase in percentage translocation is probably also related to a pool of misfolded PKC after overexpression in Sf21 cells. Misfolded PKC would neither translocate nor be autophosphorylated at serine 36, thus decreasing the relative translocation of the nonphosphorylated pool of PKC Apl II. Phosphorylation was not sufficient for translocation, since in the absence of PDBu phosphorylated protein was found mainly in the supernatant. Similarly, the distribution of Apl II S36E was not different than that of the wild type enzyme (data not shown), and translocation of Apl II S36E was similar to wild type PKC Apl II (60 ± 18%, n = 3, S.E.). In contrast, the relative percent translocation of PKC Apl II S36A was impaired compared with wild type PKC Apl II (20 ± 20% compared with 90 ± 30%, n = 3, S.E.), suggesting that phosphorylation of the wild type protein is required for proper translocation of PKC. Thus, even though only a small percentage of wild type enzyme is phosphorylated at any one point in time, a larger percentage of the translocated wild type protein may have been assisted in translocation by transient phosphorylation.

**Mechanistic Model for C2 Domain Phosphorylation—** The C2
domains of nPKCs contain an extended region in what would be the calcium binding region loop 1 as defined in cPKCs. In PKCα this region forms an α-helix (40), but there is little sequence conservation in this region in the ε/η family (7), and without a crystal structure of an ε-like C2 domain the organization of the loops is still speculative. Serine 36 is contained in the extended C-terminal region of loop 1 (Fig. 9A). Phosphorylation of serine 36 increases in vitro lipid binding and cellular translocation of PKC Apl II. However, since phosphorylation increases negative charge, it would not be expected to increase binding to acidic lipids by electrostatic interactions directly. We propose a model (Fig. 9B) where phosphorylation of this residue leads to a conformational shift in the loops of the C2 domain that expose a cryptic lipid-binding site. The N-terminal portion of loop 1 is highly conserved in PKC-like PKCs and contains a peptide used as a specific inhibitor of PKCe binding to RACK (14–16; Fig. 9A). This region might act as the cryptic lipid binding/RACK-binding domain for these nPKCs. In contrast, the extended region of the loop that contains serine 36 is not as well conserved. However, all isoforms contain a phosphorylatable site in this region (Fig. 9A), and PKCs contains at least one autophosphorylation site in the C2 domain.2

This hypothesis is consistent with earlier results measuring lipid binding to C2 domain deletion constructs of PKC Apl II. In these studies, removal of the C terminus of the C2 domain allows for lipid binding, whereas removal of the N terminus of the C2 domain (containing the phosphorylation site and proposed lipid-binding site) does not (19). This suggests that the N-terminal region of the C2 domain contains a site for lipid binding that is normally inhibited by the C-terminal of the C2 domain. A model for the actions of the PKCe-activating peptide also predicts intra-C2 domain interactions (16). This peptide leads to increased isoform-specific membrane translocation of PKCe (16). The peptide is made from a conserved sequence around loop 3 (Fig. 9A) and is believed to interact with loop 1 to increase access of PKC to RACKS (16). We suggest that phosphorylation of the C2 domain serves the same purpose as the peptide, inhibiting intra-loop interactions to expose a cryptic lipid or RACK-binding site. Indeed loop 3 is negatively charged suggesting that introducing a negative charge in loop 1 would inhibit loop 1-loop 3 interactions. Intra-C2 domain interactions have also been shown to be important in regulating the C2-B domain of synaptotagmin (39). Different synaptotagmin isoforms show distinct abilities to bind to inositol polyphosphates through a region in the C2 domain. However, differences in binding are not due to the actual inositol polyphosphate binding sequence, which is well conserved in all isoforms. Instead isoform-specific differences map to the C terminus of the C2 domain where some isoforms have residues that interact with the inositol polyphosphate binding domain and inhibit binding, whereas other isoforms lack these residues and exhibit binding (39). Deletion of the C terminus of the C2 domain of synaptotagmin allows inositol polyphosphate binding to all the isoforms (39).

Role of C2 Domain Phosphorylation in Translocation by PDBu—Phorbol esters translocate PKC Apl II to the membrane in cells, and this translocation may be partially dependent on C2 domain phosphorylation at serine 36. Although one might expect PDBu-mediated translocation to depend only on the C1 domain, mutations in the C2 domain of cPKCs can

Fig. 7. Substrate phosphorylation is unaffected by the S36A or S36E mutations. Protein kinase C activity assays were performed as described under “Experimental Procedures.” PKC assays were performed for wild type (wt) PKC Apl II (white circles), PKC Apl II S36A (black circles), and PKC Apl II S36E (white squares) illustrating no significant change in phosphatidylserine-dependent substrate phosphorylation in the mutant kinases.

Fig. 8. Serine 36 is phosphorylated in vivo and is necessary for PKC membrane translocation. Proteins were phosphorylated and kinase assays performed as described under “Experimental Procedures.” A, purified wild type (wt) PKC Apl II and PKC Apl II S36A were autophosphorylated in vitro. Enzymes were stimulated by no activators (BL), 50 μg/ml dioleoyl phosphatidylserine and 20 nM PMA (PS/TPA), or 20 μM oleic acid (Oleic) in the absence (−) or presence (+) of 50 μM ATP and [γ-32P]ATP. A, upper panel, immunoblot using the phospho-Ser-36 antibody illustrating the increase in immunoreactivity with PS/TPA and oleic acid stimulation of the wild type PKC that is absent in the mutant, PKC Apl II S36A. A, middle panel, autoradiogram of the upper panel illustrating an increase in 32P incorporation for the stimulated wild type PKC that parallels that seen in the anti-phospho-Ser-36 Western blot. A, lower panel, immunoblot of the upper panel using the PKC Apl II antibody indicating that approximately equal protein is loaded in each lane and between PKC constructs. B, cells were fractionated as described under “Experimental Procedures.” Anti-PKC Apl II immunoblot of fractionated SF-21 cells infected with baculovirus coding for wild type PKC Apl II (1st to 4th lanes) and PKC Apl II S36A (5th to 8th lanes) after treatment in the absence (−) or presence (+) of 4-μg-PDBu. C, anti-phospho-Ser-36 immunoblot of the experiment in B illustrating serine 36 phosphorylation in vivo of only wild type PKC Apl II and the preferential PDBu-induced membrane translocation of serine 36-phosphorylated PKC.

2 A. M. Pepio and W. Sossin, unpublished data.
FIG. 9. Sequence alignment of novel C2 domains and model of phosphorylation-induced lipid binding. A, comparison of the C2 domains of PKC Apl II and novel vertebrate PKCs. Loop positions are based on those described previously (7, 41). Shaded residues are conserved in all isoforms. Darkly shaded residues represent known phosphorylation sites in PKC Apl II (serine 2 and 36) and phosphorylatable residues in loop 1 of C2 domains of other novel PKCs. Sites of the Rack inhibitory peptide and pseudo-Rack peptide are from Ref. 16. B, model of phosphorylation-induced lipid binding. B, left panel, a hypothetical representation of the C2 domain of PKC Apl II with a positively charged lipid binding surface on loop 1 whose access is restricted by the position of loop 3. B, right panel, phosphorylation of serine 36 in the putative α-helix of loop 1 shifts the position of loop three to expose the lipid binding surface on loop 1. C2 domain structure adapted from Ref. 41.

FIG. 10. Model of the role for C2 domain phosphorylation in novel PKC membrane translocation and activation. A, fully mature PKC Apl II (phosphorylated on its activation loop Thr-561 by PDK1 and autophosphorylated on its two C-terminal residues resides in the cytosol awaiting activation. The pseudosubstrate (PS) is occupying the catalytic pocket, and the C2 domain restricts activator binding to the C1 domain. B, PKC Apl II can become activated through production of DAG by PLCγ or PLCβ and high levels of phosphatidylserine binding to its C1 domain. Solely the C1 domain of PKC Apl II mediates this membrane translocation and as a result its membrane affinity would be low and lead to only transient kinase activation. C, alternatively, PKC Apl II can become autophosphorylated (in cis or perhaps in trans) on serine 36 in loop 1 of its C2 domain. D, this phosphorylation may expose a cryptic C2 domain lipid binding site allowing phosphatidylserine binding, and now both the C1 and C2 domains are responsible for recruiting the kinase to the membrane. This C1- and C2-mediated activation produces a high membrane affinity and may be responsible for persistent activation of novel PKCs like PKC Apl II.

decrease PDBu-mediated translocation, suggesting that both domains are required for translocation (11, 42). Moreover, the C2 domain is required for translocation of cPKCs and phosphorylatable residues in loop 1 of C2 domains of other novel PKCs. Sites of the Rack inhibitory peptide and pseudo-Rack peptide are from Ref. 16. B, model of phosphorylation-induced lipid binding. B, left panel, a hypothetical representation of the C2 domain of PKC Apl II with a positively charged lipid binding surface on loop 1 whose access is restricted by the position of loop 3. B, right panel, phosphorylation of serine 36 in the putative α-helix of loop 1 shifts the position of loop three to expose the lipid binding surface on loop 1. C2 domain structure adapted from Ref. 41.

Phosphorylation of the C2 Domain of PKC Apl II
mediated by the binding of the C2 domain of the soluble kinase to the membrane in the presence of Ca\textsuperscript{2+} ions (11). However, a more stable and prolonged translocation was associated with the DAG binding to the C1 domain in the presence of C2 domain binding. Thus, there exist two distinct states of cPKC membrane association as follows: a low affinity state mediated by the C2 domain alone and a high affinity state mediated by both the C1 and C2 domains acting together. We propose that where calcium recruits the aid of conventional C2 domains to translocate cPKCs, phosphorylation may recruit the aid of novel C2 domains to bind lipids and perpetuate PKC membrane association (Fig. 10). This requirement of the C2 domain to perpetuate PKC membrane association would act to help rather than hinder kinase activity and may indeed be a mechanism for persistent kinase activation.

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