Inhibition of protein kinase C catalytic activity by additional regions within the human protein kinase C α -regulatory domain lying outside of the pseudosubstrate sequence

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The N-terminal pseudosubstrate site within the protein kinase C α (PKC α)-regulatory domain has long been regarded as the major determinant for autoinhibition of catalytic domain activity. Previously, we observed that the PKC-inhibitory capacity of the human PKC α -regulatory domain was only reduced partially on removal of the pseudosubstrate sequence [Parissenti, Kirwan, Kim, Colantonio and Schimmer (1998) J. Biol. Chem. 273, 8940–8945]. This finding suggested that one or more additional region(s) contributes to the inhibition of catalytic domain activity. To assess this hypothesis, we first examined the PKC-inhibitory capacity of a smaller fragment of the PKC α -regulatory domain consisting of the C1a, C1b and V2 regions [GST-R α ^{39–177}: this protein contained the full regulatory domain of human PKC α fused to glutathione S-transferase (GST), but lacked amino acids 1–38 (including the pseudosubstrate sequence) and amino acids 178–270 (including the C2 region)]. GST-R α ^{39–177} significantly inhibited PKC in a phorbol-independent manner and could not bind the peptide substrate used in our assays. These results suggested that a

region within C1/V2 directly inhibits catalytic domain activity. Providing further *in vivo* support for this hypothesis, we found that expression of N-terminally truncated pseudosubstrate-less bovine PKC α holoenzymes in yeast was capable of inhibiting cell growth in a phorbol-dependent manner. This suggested that additional autoinhibitory force(s) remained within the truncated holoenzymes that could be relieved by phorbol ester. Using tandem PCR-mediated mutagenesis, we observed that mutation of amino acids 33–86 within GST-R α ^{39–177} dramatically reduced its PKC-inhibitory capacity when protamine was used as substrate. Mutagenesis of a broad range of sequences within C2 (amino acids 159–242) also significantly reduced PKC-inhibitory capacity. Taken together, these observations support strongly the existence of multiple regions within the PKC α -regulatory domain that play a direct role in the inhibition of catalytic domain activity.

Key words: autoinhibition, protein kinase C, pseudosubstrate-independent, regulatory domain.

INTRODUCTION

Protein kinase C (PKC) is a family of phospholipid-regulated serine–threonine kinases that phosphorylates a variety of cellular proteins and plays an essential role in many signal-transduction mechanisms [1–5]. In mammalian cells, PKC isoforms are key players in cellular responses mediated by the second messenger diacylglycerol (DAG) and by phorbol ester tumour promoters [6,7] that lead to divergent cellular functions, such as proliferation and cell-cycle control, differentiation, modulation of gene expression, muscle contraction, apoptosis and tumour promotion [8–16]. PKC exists in non-stimulated cells as an inactive, autoinhibited molecule. Autoinhibition is believed to occur by a direct interaction between a highly cationic region near the N-terminus of the regulatory domain and the substrate-binding cleft of the catalytic domain [17]. This region has been termed the ‘pseudosubstrate’ site due to its similarity to the optimum PKC substrate sequence; however, it lacks a phosphorylatable serine or threonine residue [18,19]. The high-affinity binding of the pseudosubstrate sequence to the catalytic cleft blocks substrate access and, hence, catalytic activity [18,20,21]. In

the α isoform of PKC, the pseudosubstrate sequence spans amino acids 19–31 within the V1 region of the molecule [18,22]. There is considerable experimental evidence supporting the above model. House and Kemp [22] demonstrated that a small peptide, corresponding to the pseudosubstrate sequence (amino acids 19–36) of the human PKC α -regulatory domain (PKC^{19–36}), significantly inhibited PKC catalytic activity. In addition, antibodies raised against this peptide were found to activate the enzyme in the absence of cofactors, presumably by withdrawing the pseudosubstrate from the active site [23]. Finally, mutagenesis of nucleotide sequences coding for the pseudosubstrate within the PKC-regulatory domain [24] or proteolysis of pseudosubstrate site amino acids [25] results in the activation of the enzyme.

Over the past several years, new evidence has emerged suggesting that regulatory domain sequences outside the pseudosubstrate region may also play a significant role in PKC autoinhibition. Pears et al. [24] showed that complete activation of PKC could not be achieved on mutagenesis of the pseudosubstrate sequence, speculating that there may be other regulatory domain sequences that participate in the inhibition of catalytic domain

Abbreviations used: DAG, diacylglycerol; GST, glutathione S-transferase; PKC, protein kinase C; GST-R α , regulatory domain of human PKC α fused to GST; mAb, monoclonal antibody.

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activity. In our earlier studies, we compared the PKC-inhibitory capacity of the wild-type PKC α -regulatory domain and an identical regulatory domain in which the first 32 N-terminal amino acids were removed (thereby eliminating the pseudosubstrate sequence) [26]. These proteins were expressed in bacterial cells as fusion proteins linked with glutathione S-transferase (GST) and are referred to as a regulatory domain of human PKC α fused to GST (GST-R α), and GST-R α lacking the pseudosubstrate sequence (amino acids 1–32) (GST-R α^{33-270}) respectively. GST-R α was found to be a strong competitive inhibitor of the PKC catalytic activity with a K_i of approx. 40 nM [26]. Interestingly, GST-R α^{33-270} exhibited reduced (but substantial) PKC-inhibitory capacity despite the absence of the pseudosubstrate sequence ($K_i = 250$ nM) [26]. Moreover, when compared with a peptide representing solely the pseudosubstrate region of the enzyme (PKC $^{19-36}$), GST-R α and GST-R α^{33-270} were 650- and 80-fold more potent in inhibiting PKC activity respectively, suggesting that an additional PKC-inhibitory sequence(s) within the human PKC α -regulatory domain downstream of the pseudosubstrate site may also play a role in the inhibition of catalytic domain activity.

In the present study, we use the generation of further truncated and mutated PKC-regulatory domains to identify additional regions within the regulatory domain of PKC α that are involved (directly or indirectly) in the ability of the isolated regulatory domain to inhibit PKC catalytic activity. We also provide direct evidence for these additional PKC-inhibitory regions *in vivo* by showing that expression of pseudosubstrate-less N-terminally truncated bovine PKC α holoenzymes can affect yeast growth in a phorbol-dependent manner. Taken together, our results provide strong evidence for the existence of additional regions within the PKC α -regulatory domain that contribute to the inhibition of the catalytic domain activity. We, therefore, suggest that the current V1 clamp model for PKC be revised to include additional points of contact between the regulatory domain of PKC and its catalytic domain.

EXPERIMENTAL

Reagents

Isopropyl β -D-thiogalactoside was obtained from Invitrogen Laboratories (Burlington, ON, Canada). The peptide [Ser 25]-PKC $^{19-31}$ (RFARKGSLRQKNV) and fluorescein-labelled [Ser 25]PKC $^{19-31}$ (for fluorescence polarization assays) were prepared and purified by Sigma-GenoSys Laboratories (St. Louis, MI, U.S.A.), whereas γ - 32 P-labelled ATP (111 TBq/mmol) was from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Purified rat brain PKC α , PMA, peptide [Ser 25]PKC $^{19-31}$ (for PKC assays), protamine sulphate and glutathione (reduced form) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Glutathione-Sepharose was from Amersham Biosciences (Baie d'Urfé, QC, Canada), whereas bovine brain phosphatidylserine was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The isoform non-specific PKC polyclonal antibody used in the present study was obtained from Zymed Laboratories (San Francisco, CA, U.S.A.), whereas the monoclonal antibody (mAb) recognizing an epitope within the catalytic domain of PKC α (M6) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A mAb (M9), recognizing an epitope within the regulatory domain of PKC α , was a gift from Dr Susan Jaken (Eli Lilly, Indianapolis, IN, U.S.A.). Restriction endonucleases and other enzymes for molecular biology were obtained from New England Biolabs (Mississauga, ON, Canada), whereas all other chemicals were of analytical grade and obtained from Sigma-Aldrich.

Preparation of GST-R α^{39-177}

A plasmid coding for a fusion protein (GST-R α^{39-177}) representing amino acids 39–177 of the human PKC α -regulatory domain fused in-frame with GST, but lacking amino acids 1–38 (including the pseudosubstrate sequence) and amino acids 178–270 (including the C2 region), was generated using standard molecular biology techniques. Briefly, a cDNA for the human PKC α -regulatory domain cloned into pBluescript SK+ [26] was used as the template to amplify, by PCR, the coding sequence for amino acids 39–177 of human PKC α . The primers used in the reaction were 5'-TCAGAGATCTGTGAAGGACCACAAATTC-3' (sense) and 5'-TCAGGAATTCTACTGTGACATGGAGCTT-3' (antisense), and contained a 5'-terminal *Bgl*III site and a 3'-terminal *Eco*RI site respectively, to facilitate cloning into the isopropyl β -D-thiogalactoside-inducible GST expression vector pGEX-2T (Amersham Biosciences). The amplified fragment was run on a 1.0% agarose gel, extracted from the gel using a Qiagen (Mississauga, ON, Canada) gel-extraction kit, digested with *Bgl*III and *Eco*RI, purified of low-molecular-mass fragments using a Qiagen PCR purification kit, and cloned into pGEX-2T at the *Bam*HI and *Eco*RI sites. The construct, after confirmation by restriction mapping and DNA sequencing (service provided by the University of Virginia Medical School, Charlottesville, VA, U.S.A.), was then introduced into *Escherichia coli* cells (strain DH5 α) using a standard Ca $^{2+}$ -mediated transfection method. Expression and purification of the GST fusion protein coded by the construct was conducted as described previously [26], except that one tablet containing a wide spectrum of protease inhibitors (CompleteTM; Roche Pharmaceuticals, Baie d'Urfé, QC, Canada) was added per 50 ml of bacterial cell lysis buffer and elution from glutathione-Sepharose columns was by several overnight incubations with 50 mM glutathione at 4 °C. The fusion proteins were then dialysed in 1 mM EDTA, 1 mM dithiothreitol and 10 mM Hepes (pH 7.5) and stored in a frozen condition at –80 °C.

Immunoblot analysis

Each purified fusion protein preparation (2 μ g) was subjected to electrophoresis on SDS/polyacrylamide gels [27] along with appropriate molecular-mass-marker proteins. The proteins were then transferred on to BA-85 nitrocellulose membranes (Mandel Scientific, Guelph, ON, Canada) and the membranes were probed with either a goat isoform non-specific PKC polyclonal antibody or a mAb (M9) using standard immunoblotting procedures.

Measurement of PKC activity

PKC catalytic activity was measured using purified rat brain PKC α and monitoring the transfer of 32 P from [γ - 32 P]ATP to the PKC substrate peptide [Ser 25]PKC $^{19-31}$ [18] or protamine in 30 min reactions at 30 °C in the absence or presence of GST or various GST fusion proteins. Reactions (100 μ l) contained 2 μ M [Ser 25]PKC $^{19-31}$ (or 2 μ M protamine), 164 μ M [γ - 32 P]ATP (0.5 μ Ci), 18 mM MgCl $_2$, 2 mM CaCl $_2$, 46.4 μ g/ml phosphatidylserine, 2.5 μ M PMA, 20 mM Tris/HCl (pH 7.5) and 5 ng of purified rat brain PKC α enzyme. The phosphatidylserine and PMA were added together as a sonicated emulsion to form small unilamellar vesicles. After the 30 min incubation, all reactions were terminated by spotting 90 μ l of sample on to 6.25 cm 2 P81 phosphocellulose filter papers. Filters were washed exhaustively with 1% phosphoric acid, transferred to liquid-scintillation vials containing 5 ml of Scintiverse E scintillation fluid (Fisher Scientific, Nepean, ON, Canada) and radioactivity was measured by liquid-scintillation counting.

Measurement of the substrate-binding affinity of PKC α -regulatory domain fragments by fluorescence polarization

Various amounts of GST-R α ^{33–270}, GST-R α ^{39–177}, a GST negative-control or an antibody raised against phosphorylated [Ser²⁵]PKC^{19–31} (positive control) were diluted serially into a buffer, containing 20 mM Hepes (pH 7.4), 10 mM MgCl₂, 1 mM CaCl₂, 0.1 mg/ml phosphatidylserine (Sigma) and 0.02 mg/ml DAG (Avanti Polar Lipids) and placed into round-bottom, black 96-well plates (Dynex Laboratories, Chantilly, VA, U.S.A.). Fluorescein-labelled [Ser²⁵]PKC^{19–31} (in either the phosphorylated or non-phosphorylated forms) was then added to a final volume of 100 μ l and the mixture incubated for 1 h at 22 °C in the absence or presence of non-fluorescent competing peptide. Anisotropy (fluorescent peptide binding) was measured on a TECAN Polarion unit with standard fluorescein filters (excitation at 485 nm and emission at 535 nm). Concentration of fluorescent peptides was determined by comparison with the intensity of 1 nM fluorescein read at 25 °C using a Beacon 2000[®] Fluorescence Polarization Instrument (Panvera Corporation, Madison, WI, U.S.A.). The instrument was calibrated with a low-polarization fluorescein standard (20 $\pm$ 5 mP, S.D. < 2) and a high-polarization standard (340 $\pm$ 8 mP, S.D. < 2) using the Beacon 2000[®] One-Step FP Standardization kit (Panvera Corporation).

Preparation of expression vectors coding for the expression of N-terminally truncated PKC holoenzymes in yeast

Yeast strains housing plasmids for the galactose-inducible expression of the bovine PKC α holoenzyme (pYECN α) or a truncated form of the enzyme lacking the first 84 or 278 N-terminal amino acids (p α ND84 and p α ND278) were kindly provided by Dr Heimo Riedel (Wayne State University, Detroit, MI, U.S.A.). To obtain a yeast expression vector coding for bovine PKC α lacking the first 32 N-terminal amino acids, the bovine PKC α coding sequence within pYECN α was amplified by PCR using sense and antisense primers, which annealed to opposite strands of the coding sequence at sites between amino acids 33 and 38 and 668 and 672 (including the stop codon) respectively. The forward primer (5'-CCGCTCGAGAAAAACCATGGAGGTGAAGAACCACCGC-3') provided a start codon, a sequence upstream of the start codon (AAAAA-ACC) to maximize translation frequency in yeast [27a] and an *Xho*I site to facilitate cloning. The reverse primer (5'-GAAGATCTTCATACCGCGCTCTGCAG-3') also included a restriction site for the endonuclease *Bgl*III. The amplified PCR product was cleaved with *Xho*I and *Bgl*III, then inserted into the *Sal*I and *Bam*HI sites of the yeast expression vector YEp73 under the control of the GAL10 promoter. The vector contains a *Leu*2 gene for selection in leucine-deficient medium. After ligation, all plasmids were used to transform *E. coli* cells (strain DH5 α) using standard Ca²⁺-mediated techniques, and clones expressing the correct recombinant vector were identified and confirmed by restriction mapping and DNA sequence analysis (DNA sequencing service at the University of Virginia Medical School). The plasmids were then introduced into *Saccharomyces cerevisiae* strain 334 [*MAT* α ; *pep*4-3; *prb*1-1122; *ura*3-52; *leu*2-3,112; *reg*1-501; *gal*1] by lithium acetate transfection [28] and clones expressing the truncated PKC holoenzymes selected on Leu⁻ plates.

Monitoring of yeast-cell proliferation

Clonal isolates of yeast cells expressing wild-type or N-terminally truncated PKC holoenzymes were grown at 30 °C in suspension cultures with vigorous shaking. The growth of these clones

was measured by diluting overnight cultures serially to a final concentration of 650 cells/ml in Leu⁻ medium containing 3% (w/v) glycerol. PKC α cDNA expression was induced in some cultures by adding 0.01% galactose in the absence or presence of 1 μ M PMA. Absorbance measurements of all cultures were taken at 600 nm at regular time intervals until saturation was reached or no growth was evident after the end of the study.

PCR-mediated 'linker-scanning' mutagenesis

To localize regions within the PKC α -regulatory domain lying outside the pseudosubstrate sequence which play a role in the inhibition of PKC catalytic activity, a panel of 20 mutant pseudosubstrate-less GST-R α fusion proteins was generated by PCR-mediated mutagenesis [29]. The mutant proteins consisted of the wild-type human PKC α -regulatory domain amino acid sequence just downstream of the pseudosubstrate sequence, with each protein possessing one of 19 tandem mutations where the coding sequence for 12 amino acids was substituted by that coding for alanine residues. One additional mutant protein, GST-R α m33–38 [a GST fusion protein with the regulatory domain of human PKC α lacking the pseudosubstrate sequence (amino acids 1–32) and possessing a mutation (m) in which amino acids 33–38 were replaced with alanine residues (same notation used for all other 'linker-scanning' mutants)], contained a six-amino-acid substitution. As such, we have used the technique of PCR-mediated mutagenesis to generate a series of 'linker-scanning' mutant pseudosubstrate-less PKC-regulatory domains. The procedure involved three separate PCRs to generate the desired construct, using the plasmid pMT2 α (containing a full-length human PKC α holoenzyme cDNA) as substrate. The first PCR utilized a sense 5'-primer (5'-GGAAGATCTGAGGTGAAGGACCACAAA-3') that coded for a *Bgl*III restriction site just upstream of the coding sequence for amino acid 33, and a mutagenesis primer 1 (antisense) coding for 12 alanine residues to replace the wild-type coding sequence downstream of the site of primer annealing and ending at the site of mutagenesis. In the second PCR, mutagenesis primer 2 (sense) and an antisense 3'-primer (5'-CCGGAATTCCTCAACTGGCCGGCATCTTCAT-3') were used to amplify another fragment of the human PKC α -regulatory domain coding sequence beginning downstream of the site of mutagenesis and ending at the C-terminus of the regulatory domain (amino acid 268). Mutagenesis primer 2 also coded for 12 alanine residues upstream of the primer-binding site and is consequently complementary to mutagenesis primer 1. The two resulting PCR products thus had 100% similarity over the regions coding for alanine residues. This enabled the coding strand of the first PCR product, on denaturing, to anneal infrequently to the non-coding strand of the second PCR product. This rare hybrid was then amplified in a subsequent PCR using the 5'- and 3'-primers. PCR-mediated 'linker-scanning' mutagenesis of tandem 12-amino-acid sequences was conducted along the entire PKC α -regulatory domain. All PCRs were performed with the PerkinElmer Cetus DNA thermal cycler using the following protocol: denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s, amplification at 72 °C for 30 s (30 cycles) followed by extension at 72 °C for 10 min. Each mutant PCR product was gel-purified, digested with *Bgl*III and *Eco*RI, and ligated into the *Bam*HI and *Eco*RI sites of the GST expression vector pGEX-2T. Each construct was confirmed by restriction mapping and DNA sequencing using a DNA sequencing service located at the University of Virginia Medical School. The constructs were then transformed into *E. coli* cells (strain DH5 α) using a standard Ca²⁺-mediated technique. The fusion proteins coded by each construct

Figure 1 Structure of the fusion proteins GST-R α , GST-R α^{33-270} and GST-R α^{39-177} used in the present study

The coding sequence of the full human PKC α -regulatory domain (amino acids 1–270), a truncation composed of the sequence for amino acids 33–270, and a further truncation representing amino acids 39–177, were fused in-frame with GST to create the fusion proteins GST-R α , GST-R α^{33-270} and GST-R α^{39-177} respectively. The conserved (C1 and C2) and variable (V1 and V2) regions, if present, are indicated, as well as the phorbol ester (PMA)-, Ca²⁺- and phosphatidylserine-binding sites (PS) identified by Medkova and Cho [30] and Slater et al. [31]. Amino acid 1 represents the most N-terminal amino acid.

were purified as described above for other GST fusion proteins used in the present study.

RESULTS

Isolation and characterization of GST-R α^{39-177}

As described previously [26], we have isolated, purified and characterized GST fusion proteins from bacterial cells, corresponding to specific regions of the human PKC α -regulatory domain. One such protein contained amino acids 33–270 of the PKC α -regulatory domain sequence and was referred to as GST-R α^{33-270} [26]. To remain consistent with the nomenclature of other GST fusion proteins described in the present study, we have renamed this protein as GST-R α^{33-270} . Since GST-R α^{33-270} lacks the first 32 N-terminal amino acids, it lacks the entire V1 region, including the pseudosubstrate sequence (see Figure 1). Another GST fusion protein, prepared in the present study, contains sequences corresponding to the C1a, C1b (DAG/phorbol-binding) and V2 regions of the regulatory domain (GST-R α^{39-177}). This protein (Figure 1) lacks both the N-terminal pseudosubstrate region within V1 and the C2 region containing Ca²⁺- and phosphatidylserine-binding sites. As shown in Figure 6(B), glutathione–Sepharose-purified preparations of GST-R α^{39-177} were of expected molecular mass on SDS/polyacrylamide gels (approx. 44 kDa) and were recognized on immunoblots by a polyclonal antibody raised against rat brain PKC.

Pseudosubstrate-less fusion proteins GST-R α^{33-270} and GST-R α^{39-177} are potent inhibitors of PKC α catalytic activity

As part of our objective to localize and characterize potentially novel regions within the human PKC α -regulatory domain involved in the inhibition of catalytic domain activity, we compared the relative abilities of GST-R α^{33-270} and GST-R α^{39-177} to inhibit the activity of purified rat brain PKC α . Inhibition studies were conducted using lipid-activated holoenzyme rather than the isolated PKC catalytic domain because the catalytic activity of the latter molecule was much less stable under the conditions of our assay. As shown in Figure 2(A), both GST-R α^{33-270} and GST-R α^{39-177} were capable of inhibiting strongly the ability of PKC α to phosphorylate a PKC substrate peptide, under lipid-

Figure 2 Inhibition of PKC α catalytic activity by GST-R α^{33-270} and GST-R α^{39-177} *in vitro*

activated conditions (IC₅₀ ≈ 0.5 μM). Complete inhibition of PKC catalytic activity occurred typically at a concentration of fusion protein exceeding 10 μM, with GST-R α^{39-177} exhibiting less PKC-inhibitory capacity than GST-R α^{33-270} (Figure 2A). Inhibition of PKC activity was dose-dependent for both proteins. GST, by itself, has no PKC-inhibitory capacity [26]. Approximately 5-fold less protein was required for inhibition of catalytic activity when PMA was removed from the reactions or when phorbol ester concentrations were increased up to 20 μM (B). PKC activity was unaffected when reactions were conducted in the presence of a negative-control GST (■).

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Inhibition of PKC α catalytic activity by pseudosubstrate-less GST-R α^{33-270} and GST-R α^{39-177} fusion proteins is not through sequestration of Ca²⁺, phosphatidylserine or phorbol ester

Since GST-R α^{39-177} can strongly inhibit PKC catalytic activity, the above result suggests that one or more PKC-inhibitory regions lie between amino acids 39 and 177 of the human PKC α -regulatory domain sequence (within either the C1 and/or V2 region of the molecule). Moreover, since Ca²⁺- and phosphatidylserine-binding sites in PKC α lie within the C2 region [30], GST-R α^{39-177} appeared capable of inhibiting PKC catalytic activity despite lacking binding sites for Ca²⁺ and phosphatidylserine, suggesting that inhibition of PKC by GST-R α^{33-270} (and probably GST-R α^{33-270}) was not by sequestration of these PKC activators in our reactions. GST-R α^{39-177} , however, possesses both the C1a and C1b phorbol-binding sites [31]. Thus PKC inhibition by GST-R α^{39-177} could be potentially by the ability of this protein to bind phorbol ester, thereby reducing the amount available for activation of PKC in our reactions. To test this hypothesis, we examined the ability of GST-R α^{39-177} and GST-R α^{33-270} to inhibit PKC in the presence of phosphatidylserine and Ca²⁺, but under varying concentrations of phorbol ester. As shown in Figure 2(B), both GST-R α^{39-177} and GST-R α^{33-270} were capable of inhibiting PKC activity in the absence of phorbol ester; moreover, addition of phorbol ester (at concentrations up to 20 μM) had no effect on the ability of the fusion protein to inhibit PKC. Taken together, these findings suggest that both GST-R α^{33-270} and GST-R α^{39-177} inhibit PKC by a mechanism not involving the sequestration of Ca²⁺, phosphatidylserine or phorbol ester. This conclusion is further supported by our recent observation that GST-R α^{33-270}

Figure 3 Measurement of the affinity of GST-R α^{33-270} and GST-R α^{39-177} for [Ser²⁵]PKC¹⁹⁻³¹ by fluorescence polarization

[Ser²⁵]PKC¹⁹⁻³¹ was labelled with fluorescein at its N-terminus and was phosphorylated subsequently (A) or left unphosphorylated (B). The fluorescently labelled peptides were then assessed for their ability to bind to increasing concentrations of GST ($\square$), GST-R α^{33-270} ($\circ$), GST-R α^{39-177} ($\bullet$) or an antibody raised against the phosphorylated peptide ($\blacksquare$). Affinity was measured by fluorescence polarization, whereby binding of the fluorescently labelled peptides decreases their ability to rotate in solution. This results in increased polarization of the emitted fluorescence light (greater 'anisotropy').

could inhibit the activity of protamine-activated PKC α *in vitro*, where no Ca²⁺, phosphatidylserine or phorbol ester was added to the reactions (Figure 7B and [26]). Protamine acts as both an activator and a substrate for PKC [32,33].

GST-R α^{33-270} and GST-R α^{39-177} do not bind [Ser²⁵]PKC¹⁹⁻³¹

Some biological PKC substrates called Sticks (substrates that interact with C-kinases) are known to bind to PKC-regulatory domains [34]. For example, Dekker and Parker [35] have shown that the PKC substrate GAP-43 binds to the V0/C2 region of PKC δ . PKC-regulatory domains bind to Sticks at or near their PKC phosphorylation sites and phosphorylation of these proteins reduces their interaction with PKC [34]. Hence, it is possible that the inhibition of PKC by GST-R α^{33-270} and GST-R α^{39-177} was the result of their ability to bind the peptide substrate used in our PKC assays ([Ser²⁵]PKC¹⁹⁻³¹). This would result in less substrate being available for binding to PKC and a concomitant reduction in reaction velocity. To test this hypothesis, we assessed whether GST-R α^{33-270} or GST-R α^{39-177} could bind fluorescently labelled phosphorylated (Figure 3A) or non-phosphorylated (Figure 3B) [Ser²⁵]PKC¹⁹⁻³¹ in a fluorescence polarization assay. In this assay, binding of the fluorescently labelled peptides to a receptor

molecule reduces the ability of the peptides to tumble freely in solution, thus 'polarizing' the emitted light. The degree of polarization (anisotropy) is directly proportional to the amount of bound peptide [36,36a]. A phospho-specific mAb raised against phosphorylated [Ser²⁵]PKC¹⁹⁻³¹ (the positive control) increased strongly the observed anisotropy in a concentration-dependent manner when phosphorylated [Ser²⁵]PKC¹⁹⁻³¹ was used as the fluorescently labelled substrate peptide (Figure 3A). Even fluorescein-labelled non-phosphorylated [Ser²⁵]PKC¹⁹⁻³¹, which binds to the antibody with greatly reduced affinity, induced a dose-dependent increase in anisotropy (Figure 3B). In contrast, no change in anisotropy was observed for the phosphorylated or non-phosphorylated fluorescently labelled peptide in the presence of GST-R α^{33-270} or GST-R α^{39-177} , even at concentrations as high as 10 μ M. Thus it appears that these GST fusion proteins inhibit PKC by a mechanism not involving the sequestration of the peptide substrate.

In vivo evidence of PKC-inhibitory regions within the PKC α -regulatory domain lying outside the pseudosubstrate sequence

Yeast cells expressing wild-type bovine PKC α exhibit dramatically reduced growth on treatment with phorbol ester compared with non-expressing cells [37,38]. This dramatic growth inhibition by PKC appears to occur by an apparent arrest of the cell cycle at cytokinesis or at an early bud formation [39]. The growth-inhibitory phenotype is clearly PKC-dependent, since cells transfected with an identical expression vector lacking the PKC cDNA insert grow normally and since growth inhibition is not observed unless galactose is added to the medium to induce PKC α expression via a galactose-inducible promoter [37,38]. The requirement of phorbol ester to induce the growth-inhibitory phenotype and the lack of response of endogenous yeast PKCs to phorbol ester [40,41] further indicates that the activated form of the mammalian enzyme is responsible for this phenotype.

Underscoring the PKC dependence of this growth-inhibitory phenotype, C-terminal truncation of the PKC cDNA abolished both the catalytic activity of the expressed PKC and the ability of the expressed PKC to inhibit cell growth [42]. Moreover, co-expression of a PKC-regulatory domain with the PKC β -1 holoenzyme blocked the ability of PKC β -1 to induce the growth-inhibitory phenotype [43]. Taken together, the above results indicate that mammalian PKC expression in yeast strongly inhibits cell growth, and that the growth-inhibitory phenotype requires activation of the enzyme and is directly related to the catalytic activity of the expressed PKC.

The above phenotypic assay serves as an ideal *in vivo* system to assess our hypothesis that additional regions within the PKC α -regulatory domain, lying outside the pseudosubstrate region, contribute to the inhibition of catalytic domain activity. If this hypothesis is true, one would expect that PKC holoenzymes truncated from the N-terminus to remove the pseudosubstrate region would exhibit partial activation, but some autorepression of the enzyme would remain which could be alleviated by addition of phorbol ester. Expression of these pseudosubstrate-less holoenzymes in yeast would then be expected to induce some growth inhibition (due to partial activation of the enzyme). However, further activation of the enzyme by the addition of phorbol ester would augment this growth inhibition. To test this hypothesis, constructs for the galactose-induced expression of PKC holoenzymes truncated from the N-terminus by 32, 84 and 278 amino acids (p α ND32, p α ND84 and p α ND278 respectively) were prepared as described in the Experimental section. These plasmids were introduced into yeast cells by lithium acetate

Figure 4 Expression level of various wild-type and N-terminally truncated bovine PKC α holoenzymes in yeast cells as determined by immunoblotting experiments

Yeast cells were transfected with constructs coding for the wild-type bovine PKC α holoenzyme, or N-terminally truncated forms of the enzyme lacking 32, 84 or 278 amino acids (strains PKC α , α ND32, α ND84 or α ND278 respectively). Equivalent numbers of galactose-treated yeast cells (as determined by absorbance measurements) were lysed by boiling in SDS sample buffer. The lysates were then centrifuged to remove debris and equal volumes of each extract loaded on to an SDS/polyacrylamide gel. After electrophoresis, the proteins in the gel were electrophoretically transferred on to a nitrocellulose membrane and probed with a mAb specific for the catalytic domain of PKC α (M6). The molecular masses of reference proteins are indicated on the left-hand side of the immunoblot.

transfection. As shown in Figure 4, the expressed truncated PKC holoenzymes were of correct molecular mass (as determined by SDS/PAGE) and were recognized by a mAb directed against the catalytic domain of rat brain PKC α . However, the level of expression of these truncated proteins did vary substantially. When a defined number of cells from each strain (grown in identical levels of galactose) were lysed by boiling in SDS sample buffer, it was found that recombinant protein expression was highest in cells expressing full-length protein, followed by strains of yeast cells expressing a bovine PKC α holoenzyme lacking either amino acids 1–84 or 1–278 from the N-terminus of the protein (α ND84 and α ND278 respectively). These proteins were expressed at one-third of the expression level of PKC α -expressing cells. The strain of yeast cells expressing a bovine PKC α holoenzyme lacking amino acids 1–32 from the N-terminus of the protein (α ND32) had expression levels approx. 5–10 % of that for PKC α -expressing cells (Figure 4). This suggested that sequences immediately downstream of the translational start site (where the cDNAs were inserted into the yeast expression vector) strongly affect the expression level of the recombinant proteins.

We then examined the effect of expression of wild-type or various truncated PKC holoenzymes on the growth of yeast cells in the absence or presence of the phorbol ester PMA. As shown in Figure 5(A), addition of galactose to control yeast cells (transfected with the galactose-inducible yeast expression vector without the PKC cDNA insert) induced a significant increase in growth rate. Growth was elevated in a manner directly proportional to the amount of galactose added to the medium (results not shown). PMA had no significant effect on the growth of control cells. Addition of galactose to PKC α -expressing yeast cells also stimulated growth (Figure 5B). However, in contrast with control cells, PKC α -expressing yeast cells treated with both galactose and PMA exhibited virtually no growth, consistent with our previous findings [42]. The degree of growth inhibition for these cells in the presence of phorbol ester was directly proportional to the amount of galactose added to

the medium and the amount of PKC protein induced by galactose (results not shown). This further confirms the PKC dependence of the growth-inhibitory phenotype. Interestingly, galactose-treated cells expressing an N-terminally truncated form of PKC α lacking the pseudosubstrate sequence (α ND32; Figure 5C) grew at a slower rate than wild-type PKC α -expressing cells in the absence of PMA. This is consistent with previous reports of activation of PKC by removal/mutagenesis of the inhibitory pseudosubstrate site [23,24]. [This was in spite of the strongly reduced expression for the α ND32 construct compared with PKC α (Figure 4).] Moreover, we also observed that growth inhibition of cells expressing the α ND32 protein could be further augmented by the addition of 1 μ M PMA. These findings suggest that the α ND32 protein was not activated fully on removal of the pseudosubstrate region and that some autoinhibition of the protein appeared to remain, which could be alleviated by treatment with PMA.

Another mutant PKC α holoenzyme was expressed in yeast, which lacked the first N-terminal 84 amino acids, including the V1 (pseudosubstrate) region and the first phorbol-binding region, C1a. On addition of galactose, yeast cells expressing this protein (α ND84) also grew more slowly than control cells in the absence of PMA (Figure 5D), consistent with the activation of PKC α by removal of the pseudosubstrate region. Moreover, addition of 1 μ M PMA to these cells also enhanced growth inhibition, despite the deletion of the pseudosubstrate sequence and the C1a phorbol-binding region. This suggested that, even for the α ND84 protein, catalytic activity was not maximal and that autoinhibitory forces within C1b and/or C2 remained that could be relieved by the addition of PMA.

When a truncated PKC holoenzyme lacking 278 N-terminal amino acids was expressed in yeast (thereby removing the entire regulatory domain), even greater growth inhibition was observed on incubation of the cells with galactose in the absence of PMA (Figure 5E) compared with that observed for the α ND84 protein (Figure 5D). Since expression of the α ND278 protein was similar to that of the α ND84 protein (Figure 4), the enhanced growth inhibition for the α ND278 protein was probably due to the loss of additional PKC-inhibitory sites and, consequently, a greater catalytic activity for the molecule. Consistent with this latter argument is the reduced saturation density for cells transfected with the α ND278 construct compared with cells expressing the α ND84 construct in the absence of both galactose and phorbol ester. PMA was unable to augment further growth inhibition in these cells, consistent with the removal of the coding sequences for the two phorbol-binding domains in the expressed proteins and the expression of a fully, constitutively active, PKC catalytic domain.

Generation of a panel of 20 'linker-scanning' mutant GST-R α ^{33–270} fusion proteins by PCR-mediated mutagenesis

To identify the locations of PKC-inhibitory domains lying outside the pseudosubstrate sequence, a panel of 'linker-scanning' mutant GST-R α ^{33–270} proteins was constructed by PCR-mediated mutagenesis [29], as described in the Experimental section. These proteins contain the full PKC α -regulatory domain but lack the N-terminal pseudosubstrate region (amino acids 1–32). In addition, tandem 12-amino-acid stretches within GST-R α ^{33–270} have been replaced with alanine residues using PCR-mediated mutagenesis. One exception was the protein GST-R α 33–38, where only six amino acids (residues 33–38) were replaced with alanine residues. All fusion proteins used in the present study were purified as described in the Experimental section and equal quantities were loaded on SDS/polyacrylamide gels and

Figure 5 Effect of galactose and/or phorbol ester on the growth of control yeast cells expressing wild-type or various truncated forms of bovine PKC α

Control yeast cells (**A**), or yeast cells expressing full-length PKC α (**B**), or yeast cells expressing PKC α holoenzymes truncated from the N-terminus by 32 (**C**), 84 (**D**) or 278 (**E**) amino acids were monitored for growth by taking absorbance readings at 600 nm for the liquid cultures at selected time points. Expression of the wild-type and truncated PKC α enzymes in yeast was under the control of a galactose-inducible promoter. Cells were grown in the absence of any additional agent ($\circ$), in the presence of 1 μ M PMA ($\bullet$), in the presence of 0.01% galactose ($\square$) or in the presence of both galactose and PMA ($\blacksquare$). The growth of five clonal isolates of each construct was monitored as described in the Experimental section. The mean absorbance of the clones at various times in culture is depicted for each construct ($\pm$ S.E.M.). The depicted experiment is representative of three independent experiments with similar findings.

probed with anti-PKC α antibodies. As shown in Figure 6(B), all proteins were of similar molecular mass and were recognized on immunoblots probed with a PKC α polyclonal antibody. In contrast, only a select number of these proteins were recognized by a mAb (M9) raised against the regulatory domain of PKC α (Figure 6A). This is not surprising, since the epitope for this antibody has not been defined and several of the generated mutations may destroy the epitope or block the ability of the M9 antibody to recognize it.

Inhibitory capacities of a panel of 20 pseudosubstrate-less mutant GST-R α^{33-270} proteins

To localize additional PKC-inhibitory sequences within the regulatory domain of PKC α lying outside the pseudosubstrate sequence, all 20 of the 'linker-scanning' mutant proteins described above were examined for their PKC-inhibitory capacity relative to wild-type GST-R α^{33-270} . It was theorized that those

stretches of amino acids which, when mutated, blocked or reduced the ability of GST-R α^{33-270} to inhibit the activity of purified PKC α probably define regions which play an important role in the ability of the PKC-regulatory domain to inhibit the catalytic domain activity. Figure 7 depicts the ability of GST, GST-R α^{33-270} and 'linker-scanning' mutants of GST-R α^{33-270} to inhibit the activity of purified rat brain PKC α holoenzyme using protamine as substrate. Interestingly, mutagenesis of a region just downstream of the pseudosubstrate site (amino acids 33–74) had a very dramatic effect on PKC-inhibitory capacity. This region may represent an extension of the PKC pseudosubstrate region (see the Discussion section below). However, amino acids 39–62 appear to play the greatest role in the ability of GST-R α^{33-270} to inhibit PKC α activity. These observations are consistent with our observation above that a PKC α holoenzyme lacking 32 N-terminal amino acids can induce cell growth inhibition in a phorbol-dependent manner, suggesting that the truncated enzyme remains partially repressed and activatable by phorbol ester. In addition, although mutagenesis of regions throughout the

Figure 6 Immunoblotting analysis of GST, GST-R α^{33-270} , GST-R α^{39-177} and various 'linker-scanning' mutant forms of GST-R α^{33-270} , where specific amino acids within the regulatory domain primary sequence were changed to alanine residues

Equimolar preparations of each protein were run on SDS/polyacrylamide gels, transferred electrophoretically on to nitrocellulose and probed with a mAb to the PKC α -regulatory domain (M9) (A) or an isoform-non-specific PKC polyclonal antibody (B). The number range following the letter 'm' in the name of each mutant protein indicates which amino acids were replaced with alanine residues (where 1 represents the most N-terminal amino acid).

Figure 7 Effect of mutagenesis of specific regions within GST-R α^{33-270} on the ability of the protein to inhibit rat brain PKC α

Equimolar (4 μ M) preparations of GST, GST-R α^{33-270} or various mutant forms of GST-R α^{33-270} (labelled 'm'; see Figure 6) were added to rat brain PKC α and assessed for their ability to inhibit its catalytic activity using protamine as substrate (B). A schematic drawing of the primary structure of GST-R α^{33-270} , including the positions of specific amino acids and the location of the C1a, C1b, V2 and C2 regions, is provided in (A). All data points represent the mean activity ($\pm$ S.E.M.) detected for PKC in three experiments in the presence of the protein indicated relative to a GST control.

regulatory domain appears to have some impact on the PKC-inhibitory capacity of GST-R α^{33-270} , a broad region within V2/C2 also appears to play a significant role in PKC inhibition.

DISCUSSION

To assess whether additional PKC-inhibitory regions outside the pseudosubstrate sequence exist within the PKC α -regulatory

domain and to rule out possible sequestration of Ca²⁺ or phosphatidylserine as the mechanism of inhibition, we generated a GST fusion protein (GST-R α^{39-177}) containing only the C1 and V2 regions of the enzyme (Figure 1). We found that this protein inhibited strongly lipid-activated rat brain PKC α *in vitro* (Figure 2A) and that this inhibition was not due to sequestration of PKC activators or the substrate used in the assay of PKC catalytic activity. Our findings are consistent with the recent work of Slater et al. [44], where a similar C1-containing fusion protein, at micromolar concentrations, was found to be a potent inhibitor of baculovirus-expressed rat brain PKC α activity. This protein was also capable of inhibiting the constitutive activity of the catalytic subunit of PKC, consistent with our previous findings for GST-R α^{33-270} [26]. Although these authors also showed that the protein could activate PKC at nanomolar concentrations [44], we were not able to replicate this latter finding using GST-R α^{39-177} , purified rat brain PKC α and [Ser²⁵]PKC¹⁹⁻³¹ as substrate. Nevertheless, our observations and those of Slater et al. [44] suggest that regions within the C1 region of PKC play a role in the inhibition of catalytic domain activity.

Consistent with the involvement of the C1 region in the inhibition of PKC catalytic activity are our findings that mutagenesis of the coding sequence for amino acids 33–86 within the C1a region of PKC resulted in a substantial loss in the PKC-inhibitory capacity of GST-R α^{33-270} . In this region, mutagenesis of amino acids 39–62, in particular, had the greatest effect on protein function. Additional studies have been published which further support the role of the C1a region in the inhibition of catalytic domain activity. For example, Rotenberg et al. [45] have shown that a purified PKC α holoenzyme lacking the first 84 N-terminal amino acids (ND84) exhibited significantly higher catalytic activity *in vitro* when compared with the wild-type PKC α holoenzyme in the absence of PKC activators (although this could have been the result of loss of the pseudosubstrate-containing V1 region rather than C1a). Others have revealed, by analysis of the PKC α C1 domain, that several charged residues appear to play an important role in maintaining PKC in an inactive conformation [46]. Models of the folding patterns of the C1a and C1b domains presented by these researchers revealed a polarized distribution of hydrophobic and ionic residues. Only one anionic surface residue, aspartate, was present. The authors demonstrated that mutation of Asp⁵⁵ to alanine (D⁵⁵ $\rightarrow$ A) in human PKC α resulted in the generation of an enzyme 10-fold

more active than its wild-type counterpart in the absence of activators and displaying a significant reduction in the specificity of the enzyme for phosphatidylserine. They concluded that the C1a domain was involved in maintaining the enzyme in an inactive conformation by tethering to another part of the PKC α molecule. It was also noted that phosphatidylserine was capable of disrupting this intramolecular binding by competing with the Asp⁵⁵ residue for binding to the C2 region, thereby activating the molecule. The authors proposed that, in the resting state, Asp⁵⁵ maintains PKC in an inactive conformation by interacting with an amino acid within C2 (probably Asn¹⁸⁹). On localization of PKC to phosphatidylserine-abundant membranes, phosphatidylserine would compete with Asp⁵⁵ for binding to the C2 region and disrupt this tethering. Mutation of Asp⁵⁵ would also abolish this tethering, thereby releasing the enzyme from the inactive conformation, and reducing the need for specific phosphatidylserine binding. The enzyme would thus have the enhanced conformational flexibility necessary to allow activation much more easily than for wild-type PKC α . It is interesting to note that in our study mutagenesis of amino acids 51–62 had one of the strongest effects on the ability of the pseudosubstrate-less PKC α R domain to inhibit PKC catalytic activity. Thus in addition to its role in tethering to the C2 domain, our results suggest that this region of PKC may play a direct role in the inhibition of catalytic domain activity.

In addition to the C1 region, PCR-mediated 'linker-scanning' mutagenesis studies presented in this paper also suggest that a broad stretch of amino acids within the C2 region (amino acids 159–242) also contribute to the PKC-inhibitory capacity of GST-R α ^{33–270}. Several published studies support this hypothesis. For example, removal of the C2-like domain within a novel isoform of PKC resulted in the ability of the enzyme to be activated at lower concentrations of phosphatidylserine, suggesting an inhibitory role for the C2 domain [47]. Moreover, as stated previously, residues within the C2 region of PKC may participate in interdomain interactions with both the C1a and C1b regions, helping to stabilize the closed conformation of PKC α . In a study by Slater et al. [44], the authors demonstrate that the D⁵⁵ → A mutant of PKC α is somewhat potentiated by phorbol ester, despite the loss of C1a–C2 tethering, indicating that dissociation of other interactions may be involved. Finally, experiments described in this paper (Figure 5) illustrate that the maximum effect of PKC expression on yeast growth in the absence of phorbol ester occurs for that construct in which the coding sequence for the entire regulatory domain was removed (construct ND278), considerably greater than the effect of loss of just V1 (ND32) or both V1 and C1a (ND84). Residues within C2 may be involved in tethering between C1a and C2, as suggested by Bittova et al. [46]. This is further supported by the conclusion that the conformational change involved in PKC activation (pseudosubstrate sequence exposure) is independent of the conformational change that accompanies membrane binding (hinge exposure) [21]. Since the C2 domain is involved in phosphatidylserine binding, it is possible that the proposed Asp⁵⁵–C2 interaction [46] might also prevent DAG/phorbol binding to the C1 domain until phosphatidylserine activation had occurred. Consistent with this notion, Pepio and Sossin [47] have illustrated that C2 domain peptides are able to block phorbol binding to a single C1 domain of PKC γ . Moreover, others have shown that the addition of a C2 domain fusion protein to a C1 domain fusion protein led to a decrease in C1 phorbol binding, whereas a fusion protein containing both C1 and C2 domains had a lower affinity for phorbol ester than a fusion protein containing only C1 domains [48]. Binding of phosphatidylserine to the C2 region would release the C1a domain from tethering to C2, allowing further activation by DAG/phorbol.

Figure 8 Classic 'V1 clamp' model and our proposed 'revised model' for the autoinhibition and activation of PKC α

The classic 'V1 clamp' model for PKC α (upper left) proposes that only the V1 region (containing the pseudosubstrate sequence) interacts with the catalytic cleft of the catalytic domain (C α) to block substrate access and catalytic activity. We propose that there are probable multiple points of interaction between the regulatory domain of PKC α and the catalytic cleft of the enzyme, particularly involving the C1 and C2 regions of the molecule (lower left). These interactions help to facilitate autoinhibition of the enzyme. Activation of the enzyme by agents, such as phosphatidylserine (PS), PMA or Ca²⁺, disrupts such interactions (bottom right) such that the catalytic cleft is available to bind and phosphorylate substrate molecules (shown as black squares).

The current model for PKC activation involves induction by PKC activators of a change in protein conformation such that the pseudosubstrate region of PKC is dislodged from the catalytic cleft to allow binding and phosphorylation of PKC substrates [11]. Numerous studies (including this one) clearly show the importance of the pseudosubstrate sequence in the autoinhibition of PKC. However, several recent studies suggest the existence of additional intramolecular interactions that help maintain PKC in its inactive conformation. These include multiple interactions between the C1 and C2 domains of PKC α , many of which have been described above [31,46,48–51]. In addition, an association of regions within the C2 domain has been documented which may contribute to PKC autoinhibition, namely between the receptors for activated C-kinase 1 binding site and the pseudoreceptors for activated C-kinase site [52]. Finally, interactions between the C2 domain and other regions of PKC probably involving V5 [53] have been reported. These interdomain interactions are proposed to help maintain the enzyme in an inactive state by stabilizing the closed conformation of PKC α , allowing the pseudosubstrate site in the V1 region to occupy the catalytic cleft [depicted in Figure 8 ('V1 clamp' model)]. Dissociation of these interactions within PKC α then results in the release of the pseudosubstrate sequence from the catalytic cleft and the formation of the active conformation of PKC [44]. In the present study, we present evidence for the existence of additional regions within the human PKC α -regulatory domain that play a role in the ability of the regulatory domain to inhibit PKC catalytic activity. We propose that these regions inhibit PKC either indirectly, by providing the appropriate conformation for blocking catalysis at the active site by the pseudosubstrate sequence or additional regions, or directly by interacting with the active site to inhibit its activity. Our observation that a truncated PKC α holoenzyme lacking the pseudosubstrate sequence can still affect growth in a phorbol-dependent manner when expressed in yeast cells supports the

existence of at least one site (in addition to the pseudosubstrate sequence) which directly inhibits PKC catalysis. Moreover, since PKC holoenzymes truncated by as much as 84 amino acids were still responsive to phorbol (Figure 4), the results obtained for yeast are consistent with the findings *in vitro* and suggest that there are regions downstream of amino acid 84 (probably within C1b and/or C2), which play a direct role in the inhibition of catalytic domain activity by the PKC-regulatory domain. Results obtained for yeast also indicate that autoinhibition of PKC by domains outside the pseudosubstrate sequence can occur *in vivo*, thus helping to rule out potential artifacts *in vitro* to account for our findings.

What could be the mechanism by which these additional site(s) within the regulatory domain of PKC inhibit PKC catalytic activity? To help address this question, we examined the amino acid sequences surrounding alanine residues within the regulatory domains for human and bovine PKC α and assessed whether they might define additional PKC pseudosubstrate sites as defined by the criteria developed by Nishikawa et al. [19]. Interestingly, no additional pseudosubstrate-like sequences were found, suggesting that the additional sites are novel, and that the mechanism of inhibition does not appear to involve the occupation of the catalytic cleft by a PKC pseudosubstrate. Perhaps these additional sites inhibit PKC by binding close to (but not in) the catalytic cleft, such that they may still inhibit PKC by sterically hindering substrate access.

In summary, our study identifies additional regions within the regulatory domain of human PKC α that appear to play a role in the inhibition of catalytic domain activity. Since these regions contribute to PKC inhibition without involvement of the pseudosubstrate region, it would appear that the identified sites do not simply provide the appropriate conformation for high-affinity interaction between the pseudosubstrate sequence and the catalytic cleft. Rather, based on our results obtained for yeast, it appears that one or more of these newly identified regions bind close to the catalytic cleft of the enzyme thereby inhibiting PKC activity directly. It is conceivable that these regions block catalytic activity by hindering substrate binding sterically, even when the pseudosubstrate region (which occupies the substrate-binding site) is removed. Thus we propose that the classic 'V1 clamp' model for PKC autoinhibition [54,55] and other more recently devised models [46] be amended to include additional points of contact between the regulatory domain of PKC and its catalytic domain (Figure 8). These probably reside in the C1a and C2 regions of the molecule. It will be interesting to assess, through binding and structural studies, whether these newly identified regions bind within or near the catalytic cleft of PKC and which simply provide the appropriate conformation for PKC inhibition.

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REFERENCES

- 1 Stabel, S. and Parker, P. J. (1991) Protein kinase C. *Pharmacol. Ther.* **51**, 71–95
- 2 Hug, H. and Sarre, T. F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291**, 329–343
- 3 Jaken, S. (1996) Protein kinase C isozymes and substrates. *Curr. Opin. Cell Biol.* **8**, 168–173
- 4 Parker, P. J., Kour, G., Marais, R. M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Protein kinase C – a family affair. *Mol. Cell Endocrinol.* **65**, 1–11
- 5 Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607–614
- 6 Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257**, 7847–7851
- 7 Blumberg, P. M. (1988) Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. *Cancer Res.* **48**, 1–8
- 8 Ashendel, C. L. (1985) The phorbol ester receptor: a phospholipid-regulated protein kinase. *Biochim. Biophys. Acta* **822**, 219–242
- 9 Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (London)* **308**, 693–698
- 10 Weinstein, I. B. (1988) The origins of human cancer: molecular mechanisms of carcinogenesis and their implications for cancer prevention and treatment – twenty-seventh G. H. A. Clowes memorial award lecture. *Cancer Res.* **48**, 4135–4143
- 11 Newton, A. C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* **270**, 28495–28498
- 12 Azzi, A., Boscoboinik, D. and Hensey, C. (1992) The protein kinase C family. *Eur. J. Biochem.* **208**, 547–557
- 13 Huang, K. P. (1990) Role of protein kinase C in cellular regulation. *BioFactors* **2**, 171–178
- 14 House, G. M., Johnson, M. D., Hsiao, W. L., O'Brian, C. A. and Weinstein, I. B. (1988) Structural and functional studies of protein kinase C. *Adv. Exp. Med. Biol.* **234**, 127–140
- 15 Murray, N., Thompson, L. and Fields, A. (1997) The role of protein kinase C in cellular proliferation and cell cycle control. In *Molecular Biology Intelligence Unit* (Parker, P. and Dekker, L., eds.), pp. 97–120, Landes, Austin, TX
- 16 Lucas, M. and Sanchez-Margalet, V. (1995) Protein kinase C involvement in apoptosis. *Gen. Pharmacol.* **26**, 881–887
- 17 Dekker, L. V., McIntyre, P. and Parker, P. J. (1993) Mutagenesis of the regulatory domain of rat protein kinase C- η . A molecular basis for restricted histone kinase activity. *J. Biol. Chem.* **268**, 19498–19504
- 18 House, C. and Kemp, B. E. (1987) Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* **238**, 1726–1728
- 19 Nishikawa, K., Tokar, A., Johannes, F. J., Songyang, Z. and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem.* **272**, 952–960
- 20 Orr, J. W. and Newton, A. C. (1992) Interaction of protein kinase C with phosphatidylserine. 2. Specificity and regulation. *Biochemistry* **31**, 4667–4673
- 21 Orr, J. W. and Newton, A. C. (1994) Requirement for negative charge on 'activation loop' of protein kinase C. *J. Biol. Chem.* **269**, 27715–27718
- 22 House, C. and Kemp, B. E. (1990) Protein kinase C pseudosubstrate prototope: structure–function relationships. *Cell. Signalling* **2**, 187–190
- 23 Makowski, M. and Rosen, O. M. (1989) Complete activation of protein kinase C by an antipeptide antibody directed against the pseudosubstrate prototope. *J. Biol. Chem.* **264**, 16155–16159
- 24 Pears, C. J., Kour, G., House, C., Kemp, B. E. and Parker, P. J. (1990) Mutagenesis of the pseudosubstrate site of protein kinase C leads to activation. *Eur. J. Biochem.* **194**, 89–94
- 25 Hashimoto, E., Tanaka, Y. and Yamamura, H. (1990) Mechanism of proteolytic activation of rat liver protein kinase C generating Ca²⁺/phospholipid-independent form with apparent molecular mass of 80,000. *Int. J. Biochem.* **22**, 865–871
- 26 Parissenti, A. M., Kirwan, A. F., Kim, S. A., Colantonio, C. M. and Schimmer, B. P. (1998) Inhibitory properties of the regulatory domains of human protein kinase C α and mouse protein kinase C ϵ . *J. Biol. Chem.* **273**, 8940–8945
- 27 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 27a Cigan, A. M. and Donahue, T. F. (1987) Sequence and structural features associated with translational initiator regions in yeast. *Gene* **59**, 1–18
- 28 Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163–168
- 29 Vallejo, A., Pogulis, R. and Pease, L. (1995) Mutagenesis and synthesis of novel recombinant genes using PCR. In *PCR Primer: A Laboratory Manual* (Dieffenback, C. and Dveksler, G., eds.), pp. 603–612, Cold Spring Harbor Laboratory Press, Plainview, NY
- 30 Medkova, M. and Cho, W. (1998) Mutagenesis of the C2 domain of protein kinase C- α . Differential roles of Ca²⁺ ligands and membrane binding residues. *J. Biol. Chem.* **273**, 17544–17552
- 31 Slater, S. J., Ho, C., Kelly, M. B., Larkin, J. D., Taddeo, F. J., Yeager, M. D. and Stubbs, C. D. (1996) Protein kinase C α contains two activator binding sites that bind phorbol esters and diacylglycerols with opposite affinities. *J. Biol. Chem.* **271**, 4627–4631
- 32 Leventhal, P. S. and Bertics, P. J. (1993) Activation of protein kinase C by selective binding of arginine-rich polypeptides. *J. Biol. Chem.* **268**, 13906–13913
- 33 Ferrari, S., Marchiori, F., Marin, O. and Pinna, L. A. (1987) Ca²⁺ phospholipid-dependent and independent phosphorylation of synthetic peptide substrates by protein kinase C. *Eur. J. Biochem.* **163**, 481–487

- 34 Jaken, S. and Parker, P. J. (2000) Protein kinase C binding partners. *Bioessays* **22**, 245–254
- 35 Dekker, L. V. and Parker, P. J. (1997) Regulated binding of the protein kinase C substrate GAP-43 to the V0/C2 region of protein kinase C- δ . *J. Biol. Chem.* **272**, 12747–12753
- 36 Checovich, W. J., Bolger, R. E. and Burke, T. (1995) Fluorescence polarization – a new tool for cell and molecular biology. *Nature (London)* **375**, 254–256
- 36a Checovich, W. J., Bolger, R. E. and Burke, T. (1995) Erratum. *Nature (London)* **375**, 520
- 37 Riedel, H., Su, L. and Hansen, H. (1993) Yeast phenotype classifies mammalian protein kinase C cDNA mutants. *Mol. Cell. Biol.* **13**, 4728–4735
- 38 Riedel, H., Hansen, H., Parissenti, A. M., Su, L., Shieh, H. L. and Zhu, J. (1993) Phorbol ester activation of functional rat protein kinase C β -1 causes phenotype in yeast. *J. Cell. Biochem.* **52**, 320–329
- 39 Parissenti, A. M., Villeneuve, D., Kirwan-Rhude, A. and Busch, D. (1999) Carbon source-dependent regulation of cell growth by murine protein kinase C ϵ expression in *Saccharomyces cerevisiae*. *J. Cell. Physiol.* **178**, 216–226
- 40 Iwai, T., Fujisawa, N., Ogita, K. and Kikkawa, U. (1992) Catalytic properties of yeast protein kinase C: difference between the yeast and mammalian enzymes. *J. Biochem. (Tokyo)* **112**, 7–10
- 41 Ogita, K., Miyamoto, S., Koide, H., Iwai, T., Oka, M., Ando, K., Kishimoto, A., Ikeda, K., Fukami, Y. and Nishizuka, Y. (1990) Protein kinase C in *Saccharomyces cerevisiae*: comparison with the mammalian enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5011–5015
- 42 Su, L., Parissenti, A. M. and Riedel, H. (1993) Functional carboxyl terminal deletion map of protein kinase C α . *Receptors Channels* **1**, 1–9
- 43 Parissenti, A. M., Kim, S. A., Colantonio, C. M., Snihura, A. L. and Schimmer, B. P. (1996) Regulatory domain of human protein kinase C α dominantly inhibits protein kinase C β -I-regulated growth and morphology in *Saccharomyces cerevisiae*. *J. Cell. Physiol.* **166**, 609–617
- 44 Slater, S. J., Seiz, J. L., Cook, A. C., Buzas, C. J., Malinowski, S. A., Kershner, J. L., Stagliano, B. A. and Stubbs, C. D. (2002) Regulation of PKC α activity by C1–C2 domain interactions. *J. Biol. Chem.* **277**, 15277–15285
- 45 Rotenberg, S. A., Zhu, J., Hansen, H., Li, X. D., Sun, X. G., Michels, C. A. and Riedel, H. (1998) Deletion analysis of protein kinase C α reveals a novel regulatory segment. *J. Biochem. (Tokyo)* **124**, 756–763
- 46 Bittova, L., Stahelin, R. V. and Cho, W. (2001) Roles of ionic residues of the C1 domain in protein kinase C- α activation and the origin of phosphatidylserine specificity. *J. Biol. Chem.* **276**, 4218–4226
- 47 Pepio, A. M. and Sossin, W. S. (1998) The C2 domain of the Ca²⁺-independent protein kinase C *ApIII* inhibits phorbol ester binding to the C1 domain in a phosphatidic acid-sensitive manner. *Biochemistry* **37**, 1256–1263
- 48 Quest, A. F. and Bell, R. M. (1994) The regulatory region of protein kinase C λ . Studies of phorbol ester binding to individual and combined functional segments expressed as glutathione S-transferase fusion proteins indicate a complex mechanism of regulation by phospholipids, phorbol esters, and divalent cations. *J. Biol. Chem.* **269**, 20000–20012
- 49 Pepio, A. M., Fan, X. and Sossin, W. S. (1998) The role of C2 domains in Ca²⁺-activated and Ca²⁺-independent protein kinase Cs in aplysia. *J. Biol. Chem.* **273**, 19040–19048
- 50 Pepio, A. M. and Sossin, W. S. (2001) Membrane translocation of novel protein kinase Cs is regulated by phosphorylation of the C2 domain. *J. Biol. Chem.* **276**, 3846–3855
- 51 Pak, Y., Enyedy, I. J., Varady, J., Kung, J. W., Lorenzo, P. S., Blumberg, P. M. and Wang, S. (2001) Structural basis of binding of high-affinity ligands to protein kinase C: prediction of the binding modes through a new molecular dynamics method and evaluation by site-directed mutagenesis. *J. Med. Chem.* **44**, 1690–1701
- 52 Ron, D. and Mochly-Rosen, D. (1995) An autoregulatory region in protein kinase C: the pseudoanchoring site. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 492–496
- 53 Banci, L., Cavallaro, G., Kheifets, V. and Mochly-Rosen, D. (2002) Molecular dynamics characterization of the C2 domain of protein kinase C β . *J. Biol. Chem.* **277**, 12988–12997
- 54 Newton, A. C. (1997) Regulation of protein kinase C. *Curr. Opin. Cell Biol.* **9**, 161–167
- 55 Keranen, L. M. and Newton, A. C. (1997) Ca²⁺ differentially regulates conventional protein kinase Cs' membrane interaction and activation. *J. Biol. Chem.* **272**, 25959–25967

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