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

Angie F. KIRWAN*, Ashley C. BIBBY†, Thierry MVILONGO‡, Heimo RIEDEL§, Thomas BURKES§, Sherri Z. MILLIS§ and Amadeo M. PARISSENTI*†‡†

*Tumor Biology Research Program, Northeastern Ontario Regional Cancer Centre, 41 Ramsey Lake Road, Sudbury, ON, Canada P3E 5J1, †Department of Chemistry and Biochemistry, Laurentian University, Sudbury, ON, Canada P3E 2C6, §Department of Biological Sciences, Wayne State University, Detroit, MI 48202, U.S.A., and ‡Fluorescence Polarization Group, Panvera Corporation, Madison, WI 53711, U.S.A.

The N-terminal pseudosubstrate site within the protein kinase Cα (PKCα)-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 PKCα-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 PKCα-regulatory domain consisting of the C1a, C1b and V2 regions [GST-Rα19–39]: this protein contained the full regulatory domain of human PKCα 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-Rα19–39 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 PKCα 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-Rα19–39 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 PKCα-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 PKCα-regulatory domain (PKCα9–36), 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-Rα, regulatory domain of human PKCα fused to GST; mAb, monoclonal antibody.

1 To whom correspondence should be addressed at Tumor Biology Research Program, Northeastern Ontario Regional Cancer Centre, 41 Ramsey Lake Road, Sudbury, ON, Canada P3E 5J1 (e-mail aparissenti@neorcc.on.ca).
activity. In our earlier studies, we compared the PKC-inhibitory capacity of the wild-type PKCa-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 PKCa fused to GST (GST-Rα), and GST-Rα lacking the pseudosubstrate sequence (amino acids 1–32) (GST-Rα 33–270) respectively. GST-Rα was found to be a strong competitive inhibitor of the PKC catalytic activity with a Kᵦ of approx. 40 nM [26]. Interestingly, GST-Rα 33–270 exhibited reduced (but substantial) PKC-inhibitory capacity despite the absence of the pseudosubstrate sequence (Kᵦ = 250 nM) [26]. Moreover, when compared with a peptide representing solely the pseudosubstrate region of the enzyme (PKC 19–36), GST-Rα and GST-Rα 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 PKCa-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 PKCa 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 PKCa 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 PKCa-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 [Ser²⁵]-PKCa 19–31 (RFARKGSLRKNV) and fluorescein-labelled [Ser²⁵]PKCa 19–31 (for fluorescence polarization assays) were prepared and purified by Sigma-GenoSys Laboratories (St. Louis, MO, U.S.A.), whereas γ-[³²P]-labelled ATP (111 TBq/mmol) was from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Purified rat brain PKCa, PMA, peptide [Ser²⁵]PKCa 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 phosphatidylinositol 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 PKCa (M6) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A mAb (M9), recognizing an epitope within the regulatory domain of PKCa, 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-Rα 39–177

A plasmid coding for a fusion protein (GST-Rα 39–177) representing amino acids 39–177 of the human PKCa-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 PKCa-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 PKCa. The primers used in the reaction were 5'-TCAGAGATCTGTGAAGGACCACAAATTC-3' (sense) and 5'-TCAGGAATTCATCTGTGACATGGAGCTT-3' (antisense), and contained a 5' terminal BglII site and a 3' terminal EcoRI 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 BglII and EcoRI, purified of low-molecular-mass fragments using a Qiagen PCR purification kit, and cloned into pGEX-2T at the BamHI and EcoRI 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 DH5α) using a standard Ca²⁺-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 μg) 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 PKCo and monitoring the transfer of [³²P] from [γ-³²P]ATP to the PKC substrate peptide [Ser²⁵]PKCa 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 μl) contained 2 μM [Ser²⁵]PKCa 19–31 (or 2 μM protamine), 164 μM [γ-³²P]ATP (0.5 μCi), 18 mM MgCl₂, 2 mM CaCl₂, 46.4 μg/ml phosphatidyserine, 2.5 μM PMA, 20 mM Tris/HCl (pH 7.5) and 5 ng of purified rat brain PKCo enzyme. The phosphatidyserine 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 μl of sample on to 6.25 cm² 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.

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

Various amounts of GST-Rα[13–270], GST-Rα[90–177], a GST negative-control or an antibody raised against phosphorylated Ser[31]PKCα[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 Ser[31]PKCα[19–31] (in either the phosphorylated or non-phosphorylated forms) was then added to a final volume of 100 µl 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 standard 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 standard fluorescein read at 25 °C using a Beacon 2000® Fluorescence Polarization Instrument (Panvera Corporation, Madison, WI, U.S.A.).

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 PKCα holoenzyme (pYECNα) or a truncated form of the enzyme lacking the first 84 or 278 N-terminal amino acids (pYN284 and pYN278) were kindly provided by Dr Heimo Riedel (Wayne State University, Detroit, MI, U.S.A.). To obtain a yeast expression vector coding for bovine PKCα lacking the first 32 N-terminal amino acids, the bovine PKCα coding sequence within pYECNα 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'-CCGGCTCGAGA-AAAAACCATTGGAGGTGAAGAACCACCGGCT-3') provided a start codon, a sequence upstream of the start codon (AAAAAACCC) to maximize translation frequency in yeast [27a] and an XhoI site to facilitate cloning. The reverse primer (5'-GAAG-ATCTTCTACCACCGCCTCTGCA-3') also included a restriction site for the endonuclease BglII. The amplified PCR product was cleaved with XhoI and BglII and then inserted into the SacI and BamHI sites of the yeast expression vector YEp73 under the control of the GAL10 promoter. The vector contains a Leu2 gene for selection in leucine-deficient medium. After ligation, all plasmids were used to transform E. coli cells (strain DH5α) using standard CaCl₂-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 School). The plasmids were then introduced into Saccharomyces cerevisiae strain 334 [MATα; pep4-3; prb1-1122; ura3-52; leu2-3,112; reg1-501; gal11] 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. PKCα cDNA expression was induced in some cultures by adding 0.01% galactose in the absence or presence of 1 µM 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 PKCα-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-Rα fusion proteins was generated by PCR-mediated mutagenesis [29]. The mutant proteins consisted of the wild-type human PKCα-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-Rα[33–38] [a GST fusion protein with the regulatory domain of human PKCα 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-alanine-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 pMT2α (containing a full-length human PKCα holoenzyme cDNA) as substrate. The first PCR utilized a sense 5′-primer (5'-GGAAAGATCTGAGGTGAAGAACCACCGGCT-3') and an antisense 3′-primer (5'-CCGGCTCGAGA-AAAAACCATTGGAGGTGAAGAACCACCGGCT-3') that coded for a BglII 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'-CCGGAAATTCCTCAACTGCCCAGCATCTTCAT-3') were used to amplify another fragment of the human PKCα-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 PKCα-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 BglII and EcoRI, and ligated into the BamHI and EcoRI 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 School. The constructs were then transformed into E. coli cells (strain DH5α) using a standard CaCl₂-mediated technique. The fusion proteins coded by each construct...
RESULTS

Isolation and characterization of GST-\(\alpha^{39-177}\)

As described previously [26], we have isolated, purified, and characterized GST fusion proteins from bacterial cells, corresponding to specific regions of the human PKC\(\alpha\)-regulatory domain. One such protein contained amino acids 33–270 of the PKC\(\alpha\)-regulatory domain sequence and was referred to as GST-\(\alpha^{31-32}\) [26]. To remain consistent with the nomenclature of other GST fusion proteins described in the present study, we have renamed this protein as GST-\(\alpha^{33-270}\). Since GST-\(\alpha^{33-270}\) 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-\(\alpha^{39-177}\)). This protein (Figure 1) lacks both the N-terminal pseudosubstrate region within V1 and the C2 region containing Ca\(^{2+}\)- and phosphatidylserine-binding sites. As shown in Figure 6(B), glutathione–Sepharose-purified preparations of GST-\(\alpha^{39-177}\) 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-\(\alpha^{33-270}\) and GST-\(\alpha^{39-177}\) are potent inhibitors of PKC\(\alpha\) catalytic activity

As part of our objective to localize and characterize potentially novel regions within the human PKC\(\alpha\)-regulatory domain involved in the inhibition of catalytic domain activity, we compared the relative abilities of GST-\(\alpha^{33-270}\) and GST-\(\alpha^{39-177}\) to inhibit the activity of purified rat brain PKC\(\alpha\). 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-\(\alpha^{33-270}\) and GST-\(\alpha^{39-177}\) were capable of inhibiting strongly the ability of PKC\(\alpha\) to phosphorylate a PKC substrate peptide, under lipidd

 activated conditions (IC\(_{50}\) ≈ 0.5 \(\mu\)M). Complete inhibition of PKC catalytic activity occurred typically at a concentration of fusion protein exceeding 10 \(\mu\)M, with GST-\(\alpha^{39-177}\) exhibiting less PKC-inhibitory capacity than GST-\(\alpha^{33-270}\) (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 protamine was used as substrate (results not shown).

Inhibition of PKC\(\alpha\) catalytic activity by pseudosubstrate-less GST-\(\alpha^{33-270}\) and GST-\(\alpha^{39-177}\) fusion proteins is not through sequestration of Ca\(^{2+}\), phosphatidylserine or phorbol ester

Since GST-\(\alpha^{39-177}\) 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\(\alpha\)-regulatory domain sequence (within either the C1 and/or V2 region of the molecule). Moreover, since Ca\(^{2+}\)- and phosphatidylserine-binding sites in PKC\(\alpha\) lie within the C2 region [30], GST-\(\alpha^{39-177}\) appeared capable of inhibiting PKC catalytic activity despite lacking binding sites for Ca\(^{2+}\) and phosphatidylserine, suggesting that inhibition of PKC by GST-\(\alpha^{39-177}\) (and probably GST-\(\alpha^{33-270}\)) was not by sequestration of these PKC activators in our reactions. GST-\(\alpha^{39-177}\), however, possesses both the C1a and C1b phorbol-binding sites [31]. Thus PKC inhibition by GST-\(\alpha^{39-177}\) 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-\(\alpha^{39-177}\) and GST-\(\alpha^{33-270}\) to inhibit PKC in the presence of phosphatidylserine and Ca\(^{2+}\), but under varying concentrations of phorbol ester. As shown in Figure 2(B), both GST-\(\alpha^{39-177}\) and GST-\(\alpha^{33-270}\) were capable of inhibiting PKC activity in the absence of phorbol ester; moreover, addition of phorbol ester (at concentrations up to 20 \(\mu\)M) had no effect on the ability of the fusion protein to inhibit PKC. Taken together, these findings suggest that both GST-\(\alpha^{33-270}\) and GST-\(\alpha^{39-177}\) inhibit PKC by a mechanism not involving the sequestration of Ca\(^{2+}\), phosphatidylserine or phorbol ester. This conclusion is further supported by our recent observation that GST-\(\alpha^{33-270}\)
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-Rα¹³⁻²⁷⁰ or GST-Rα¹⁷⁷⁻¹⁹⁻³¹, even at concentrations as high as 10 μM. 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 PKCα-regulatory domain lying outside the pseudosubstrate sequence**

Yeast cells expressing wild-type bovine PKCα 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 PKCα 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 PKCβ₁-holoenzyme blocked the ability of PKCβ₁ 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 PKCα-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 (pND32, pND84 and pND278 respectively) were prepared as described in the Experimental section. These plasmids were introduced into yeast cells by lithium acetate
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 PKCα. 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 PKCα holoenzyme lacking either amino acids 1–84 or 1–278 from the N-terminus of the protein (αND84 and αND278 respectively). These proteins were expressed at one-third of the expression level of PKCα-expressing cells. The strain of yeast cells expressing a bovine PKCα holoenzyme lacking amino acids 1–32 from the N-terminus of the protein (αND32) had expression levels approx. 5–10% of that for PKCα-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 PKCα-expressing yeast cells also stimulated growth (Figure 5B). However, in contrast with control cells, PKCα-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 PKCα lacking the pseudosubstrate sequence (αND32; Figure 5C) grew at a slower rate than wild-type PKCα-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 αND32 construct compared with PKCα (Figure 4).] Moreover, we also observed that growth inhibition of cells expressing the αND32 protein could be further augmented by the addition of 1 μM PMA. These findings suggest that the αND32 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 PKCα 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 (αND84) also grew more slowly than control cells in the absence of PMA (Figure 5D), consistent with the activation of PKCα by removal of the pseudosubstrate region. Moreover, addition of 1 μM 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 αND84 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 αND84 protein (Figure 5D). Since expression of the αND278 protein was similar to that of the αND84 protein (Figure 4), the enhanced growth inhibition for the αND278 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 αND278 construct compared with cells expressing the αND84 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-Rαα−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-Rαα−270 proteins was constructed by PCR-mediated mutagenesis [29], as described in the Experimental section. These proteins contain the full PKCα-regulatory domain but lack the N-terminal pseudosubstrate region (amino acids 1–32). In addition, tandem 12-amino-acid stretches within GST-Rαα−270 have been replaced with alanine residues using PCR-mediated mutagenesis. One exception was the protein GST-Rαc33–38, 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

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

Yeast cells were transfected with constructs coding for the wild-type bovine PKCα holoenzyme, or N-terminally truncated forms of the enzyme lacking 32, 84 or 278 amino acids (strains PKCα, αND32, αND84 or αND278 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 PKCα (M6). The molecular masses of reference proteins are indicated on the left-hand side of the immunoblot.

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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 PKCα

Control yeast cells (A), or yeast cells expressing full-length PKCα (B), or yeast cells expressing PKCα 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 PKCα enzymes in yeast was under the control of a galactose-inducible promoter. Cells were grown in the absence of any additional agent (•), in the presence of 1 μM PMA (○), in the presence of 0.01 % galactose (□) or in the presence of both galactose and PMA (▪). 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 (± S.E.M.). The depicted experiment is representative of three independent experiments with similar findings.

probed with anti-PKCα antibodies. As shown in Figure 6(B), all proteins were of similar molecular mass and were recognized on immunoblots probed with a PKCα polyclonal antibody. In contrast, only a select number of these proteins were recognized by a mAb (M9) raised against the regulatory domain of PKCα (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-Rα33–270 proteins

To localize additional PKC-inhibitory sequences within the regulatory domain of PKCα 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-Rα33–270. It was theorized that those stretches of amino acids which, when mutated, blocked or reduced the ability of GST-Rα33–270 to inhibit the activity of purified PKCα 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-Rα33–270 and ‘linker-scanning’ mutants of GST-Rα33–270 to inhibit the activity of purified rat brain PKCα 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-Rα33–270 to inhibit PKCα activity. These observations are consistent with our observation above that a PKCα 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<sup>33–270</sup>, GST-R<sub>a</sub><sup>39–177</sup> and various ‘linker-scanning’ mutant forms of GST-R<sub>a</sub><sup>33–270</sup>, 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 to nitrocellulose and probed with a mAb to the PKCa-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<sub>a</sub><sup>33–270</sup> on the ability of the protein to inhibit rat brain PKCa

Equimolar (4 μM) preparations of GST, GST-R<sub>a</sub><sup>35–275</sup> or various mutant forms of GST-R<sub>a</sub><sup>33–270</sup> (labelled ‘m’; see Figure 6) were added to rat brain PKCa 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<sub>a</sub><sup>35–275</sup> 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 (± S.E.M.) detected for PKC in three experiments in the presence of the protein indicated relative to a GST control.

The regulatory domain appears to have some impact on the PKC-inhibitory capacity of GST-R<sub>a</sub><sup>33–270</sup>, 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 PKCa-regulatory domain and to rule out possible sequestration of Ca<sup>2+</sup> or phosphatidyserine as the mechanism of inhibition, we generated a GST fusion protein (GST-R<sub>a</sub><sup>39–177</sup>) containing only the C1 and V2 regions of the enzyme (Figure 1). We found that this protein inhibited strongly lipid-activated rat brain PKCa<sub>in vitro</sub> (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 PKCa 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<sub>a</sub><sup>33–270</sup>. 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<sub>a</sub><sup>39–177</sup>, purified rat brain PKCa<sub>α</sub> and [Ser<sup>20</sup>]PKC<sub>α</sub> 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<sub>a</sub><sup>33–270</sup>. 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 PKCa holoenzyme lacking the first 84 N-terminal amino acids (ND84) exhibited significantly higher catalytic activity <em>in vitro</em> when compared with the wild-type PKCa 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 PKCa 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 Asp<sup>55</sup> to alanine (D<sup>55→A</sup>) in human PKCa<sub>α</sub> 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 PKCα 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 PKCα. 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 PKCα 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 PKCα 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 PKCα-inhibitory capacity of GST-Rα 31–270. 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 PKCα. In a study by Slater et al. [44], the authors demonstrate that the D35 → A mutant of PKCα is somewhat potentiated by phorbolester, despite the loss of C1α–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 Bitova 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 PKCγ. 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.

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 PKCα in its inactive conformation. These include multiple interactions between the C1 and C2 domains of PKCα, 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 PKCα 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 PKCα, 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 PKCα then results in the release of the pseudosubstrate sequence from the catalytic cleft and the formation of the active conformation of PKCα [44]. In the present study, we present evidence for the existence of additional regions within the human PKCα-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 PKCα 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 PKCα 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 PKCα 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.

We thank Dr Susan Jaken for her gift of an antibody to the regulatory domain of PKCα (M9). This work was supported by a grant (MOP-15037) from the Canadian Institutes of Health Research to A. M. P., and core support funds from Cancer Care, Ontario, and the Northern Cancer Research Foundation.

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Received 2 January 2003/4 April 2003; accepted 23 April 2003
Published as BJ Immediate Publication 23 April 2003, DOI 10.1042/BJ20030011