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**SHORT COMMUNICATION**

# Differential Phosphorylation of Sites in the Linker Region of P-Glycoprotein by Protein Kinase C Isozymes α, βI, βII, γ, δ, ϵ, η, and ζ

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**ABSTRACT.** To determine whether individual protein kinase C (PKC) isozymes differentially phosphorylate sites in the linker region of human P-glycoprotein (P-gp), we used a synthetic peptide substrate, PG-2, exactly corresponding to amino acid residues spanning the region 656–689 of the multidrug resistance gene (*MDR1*). All tested PKC isozymes phosphorylated PG-2. The maximum phosphate incorporation by calcium-dependent PKC isozymes α, βI, βII, and γ was 3, 2, 2, and 3 mol phosphate/mol PG-2, respectively. The maximum phosphate incorporation by calcium-independent isozymes δ, ϵ, η, and ζ was 1.5, 0.5, 1.5, and 1.5 mol phosphate/mol PG-2, respectively. Two-dimensional tryptic phosphopeptide mapping indicated differential phosphorylation of the PKC consensus sites Ser-661, Ser-667, and Ser-671 by individual isozymes, which may be functionally significant. These data suggest that differential phosphorylation by PKC isoenzymes of PKC sites within the P-gp linker region may play a role in modulating P-gp activity. BIOCHEM PHARMACOL **58**;10: 1587–1592, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** P-glycoprotein; protein kinase C isoenzymes; PG-2; phosphorylation; stoichiometry; phosphopeptides

Overexpression of P-gp has been associated mechanistically with the MDR‖ phenotype characterized by resistance to several structurally and functionally unrelated natural product chemotherapy drugs (reviewed in Ref. 1). Although other kinases have been noted to phosphorylate P-gp *in vitro*, several lines of experimental evidence suggest that phosphorylation of P-gp by PKC may play a role in regulating multidrug resistance (reviewed in Refs. 2–4).

PKC is a family of serine/threonine kinase isozymes that are dependent upon lipid for activation and can be classified in three groups: conventional PKC isozymes (α, βI, βII, γ) that are $Ca^{2+}$-dependent and phorbol ester-responsive; novel PKC isozymes (δ, ϵ, η, θ) that are $Ca^{2+}$-independent and phorbol ester-responsive; and atypical PKC isozymes (ζ, λ) that are $Ca^{2+}$-independent and phorbol ester-unresponsive (reviewed in Ref. 5). Differences in expression, substrate specificity, and activator requirements suggest that PKC isozymes may have distinct roles in different signaling pathways [5]. Increased PKC activity and enhanced phosphorylation of P-gp have been correlated with selective expression of specific PKC isozymes in many MDR cell lines [6–12].

*In vitro*, human MDR1 P-gp is phosphorylated at Ser-661, Ser-667, and Ser-671 by a rat brain PKC preparation consisting of a mixture of isozymes [13, 14]. Human P-gp also has been found to be phosphorylated in the *in situ* state at the same amino acid residues. These sites are clustered in the linker region between the two homologous halves of the molecule. However, the specificity of different PKC isozymes for these sites has not been determined. To elucidate whether different PKC isozymes preferentially phosphorylate the PKC sites in the linker region of P-gp, we used PG-2, a synthetic peptide substrate exactly corresponding to residues 656–689 of the linker region of human MDR1 P-gp [14]. The stoichiometry and the pattern of phosphorylation based on tryptic phosphopeptide mapping were determined for PG-2 phosphorylated by PKC α, βI, βII, γ, δ, ϵ, η, and ζ isozymes, and the results are presented herein.

## MATERIALS AND METHODS

### *Preparation of PKC Isozymes*

PKC isozymes were produced and expressed in baculovirus-infected insect Sf9 cells, and partial purification of these

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‖ *Abbreviations*: MDR, multidrug resistance; PKC, protein kinase C; P-gp, P-glycoprotein; PDBu, phorbol 12,13-dibutyrate; and PS, phosphatidylserine.

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isozymes was performed as described for $Ca^{2+}$-dependent PKC isozymes [15] and $Ca^{2+}$-independent PKC isozymes [16]. Immunoblotting of these preparations for specific PKC isozymes verified the identity of the isozymes and indicated no cross-contamination of PKC isozymes [9] (data not shown).

### *Phorbol Binding*

To equalize the PKC isozyme activities for study, [$^3$H]PDBu binding to PKC isozymes was measured in a rapid filtration assay. Aliquots of PKC isozymes (containing up to 200 μg protein) were incubated for 1 hr at 4° in the presence of buffer containing 20 mM Tris–HCl, pH 7.5, phosphatidylserine (PS) (40 μg/mL), 50 nM [$^3$H]PDBu (specific activity 18.6 Ci/mmol), and 2 mM $CaCl_2$ ($Ca^{2+}$-dependent isozymes) or 10 mM EGTA ($Ca^{2+}$-independent isozymes) in a final volume of 100 μL. The incubations were terminated by spotting 80 μL of sample onto DE-81 filters and washing twice with 20 mM Tris–HCl, pH 7.5, 20% methanol, and either 0.2 mM $CaCl_2$ or 1 mM EGTA. Total binding and non-specific binding were determined in triplicate in the presence of vehicle (1% DMSO) or 100 μM unlabeled PDBu, respectively. Specific binding was calculated from the difference of the total and non-specific binding.

### *Peptide Synthesis*

The synthetic peptide PG-2, corresponding to residues 656–689 of human MDR1 P-gp, was synthesized as previously described [14]. Concentration and specificity of PG-2 stock solutions were determined by amino acid compositional analysis [14].

### *Substrate Phosphorylation*

The phosphorylation reaction mixtures contained: 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 500 μM [$\gamma$-$^{32}$P]ATP (specific activity 50–150 cpm/pmol), 40 μg/mL of PS, 1 μg/mL of dioleoylglycerol, 0.2 μM calyculin A and either 0.2 mg/mL of histone H1 or 30 μM PG-2. Calcium-dependent and -independent PKC activities were evaluated in the presence of 100 μM $CaCl_2$, or 10 mM EGTA, respectively. Phosphorylation was initiated by the addition of PKC isozymes or buffer alone (in which the isozymes were stored) and was performed at 37° in a total volume of 100 μL. The reactions were terminated either by adding an equal volume of 2X SDS sample buffer or by trichloroacetic acid precipitation. Incorporation of $^{32}$P from [$\gamma$-$^{32}$P]ATP into PG-2 was measured at timed intervals by spotting 5 μL of the reaction mixture on P-81 paper and placing the paper in 75 mM phosphoric acid. The papers were washed three times in fresh phosphoric acid and dried, and radioactivity was determined by scintillation counting.

**TABLE 1. Specific binding of [$^3$H]PDBu to PKC isozymes**

| Isozyme | Protein (mg/ml) | [$^3$H]PDBu Specific binding (pmol/mg) |
|---|---|---|
| PKC α | 2.25 | 105.9 ± 7.5 |
| PKC βI | 0.94 | 34.7 ± 2.1 |
| PKC βII | 2.83 | 24.3 ± 1.2 |
| PKC γ | 1.04 | 30.3 ± 5.1 |
| PKC δ | 2.47 | 73.0 ± 3.5 |
| PKC ε | 2.56 | 51.9 ± 3.8 |
| PKC η | 3.10 | 4.2 ± 0.5 |
| PKC ζ | 5.40 | 0.0 ± 0.0 |

Specific binding of [$^3$H]PDBu to partially purified PKC isozymes was measured in a rapid filtration assay as described under Materials and Methods. Data are means ± SEM (N = 3).

### *Polyacrylamide Electrophoresis of PG-2 and Tryptic Peptide Mapping*

Phosphorylated PG-2 was resolved on Tricine-SDS polyacrylamide gels (16.5% acrylamide) [17], and the labeled 4-kDa band was identified by autoradiography. PG-2 was excised from the gel and soaked in 0.2 M $NH_4HCO_3$ for subsequent tryptic digestion. In some experiments, the phosphorylated peptide was precipitated by the addition of trichloroacetic acid. The precipitates were washed twice, acetone precipitated, and dried. Proteolytic digestion of the samples using *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin was performed at 37° for 16 hr as described [14]. Two-dimensional separation of phosphopeptides was accomplished using the Hunter Thin Layer Electrophoresis System. Electrophoresis was performed toward the cathode in formic acid:glacial acetic acid:water (1:3:36, by vol.), pH 1.9, for 2.5 hr at 450 V. Ascending chromatography in the second dimension was performed in *n*-butanol:pyridine:glacial acetic acid:water (50:33:1:4, by vol.). The dried plates were autoradiographed with Kodak XAR film.

## RESULTS

### *Standardization of PKC Isozymes*

The phorbol ester-responsive PKC isozymes used in these studies were standardized on the basis of PDBu binding equivalences. Table 1 summarizes the PDBu binding activity of the isozymes. As expected, no specific binding of [$^3$H]PDBu was observed for the atypical PKC isozyme PKC ζ, which lacks a regulatory diacylglycerol/phorbol ester binding site [16, 18]. Therefore, standardization of the PKC ζ isozyme used in PG-2 phosphorylation experiments was based on H1 histone kinase activity and was adjusted to be equal to that of PKC η H1 histone kinase activity.

### *Stoichiometry of PG-2 Phosphorylation by PKC Isozymes*

Figure 1 shows the amino acid sequence of PG-2, the major PKC sites, and the sequences of tryptic phosphopeptides, as deduced from a previous study [14]. The time course and


**FIG. 1. Sequence of PG-2. The amino acid sequence of PG-2 corresponds to residues 656–689 of human MDR1. Sequences shown in the shaded boxes correspond to tryptic peptides containing the previously described *in vitro* and *in vivo* major PKC sites: Ser-661, Ser-667, and Ser-671.**

stoichiometry of PG-2 phosphorylation by individual PKC isozymes were determined. Figure 2A shows that PG-2 was phosphorylated to a maximum stoichiometry of approximately 3 mol phosphate/mol PG-2 by a rat brain mixture of PKC, as well as PKC α and PKC γ. PKC βI and βII phosphorylated PG-2 to a maximum stoichiometry of 2.3 and 2 mol phosphate/mol PG-2, respectively. Figure 2B shows that the maximum stoichiometry of PG-2 phosphorylation at 120 min by PKC ε was 0.5 mol phosphate/mol PG-2; for PKC δ and PKC η it was approximately 1.1 mol phosphate/mol PG-2; and for PKC ζ it was 1.4 mol phosphate/mol PG-2. These results indicated that PG-2 was phosphorylated less extensively by the $Ca^{2+}$-independent PKC isozymes than by the $Ca^{2+}$-dependent PKC isozymes. In all cases, only serine residues were phosphorylated by PKC isozymes, as determined by phosphoamino acid analyses (data not shown).

### *Two-Dimensional Analysis of PG-2 Tryptic Phosphopeptides*

Previous studies have shown that rat brain PKC, comprising a mixture of PKC isozymes, phosphorylated PG-2 to a maximum stoichiometry of 3 mol phosphate/mol PG-2, and Ser-661, Ser-667, and Ser-671 were the three sites of phosphorylation [14]. The tryptic phosphopeptides containing these sites (Fig. 1) had a similar net charge at low pH. However, they differed significantly in their hydrophobicity (from high to low: 661-SSLIR > 671-SVR > 667-STR) to be separated during cellulose thin-layer chromatography in the second dimension. We subjected PG-2, maximally phosphorylated by individual PKC isozymes, to two-dimensional tryptic phosphopeptide mapping analysis.

As shown in Fig. 3, phosphorylation of PG-2 by PKC α, PKC βI, PKC βII, PKC γ, PKC η, and PKC ζ resulted in the appearance of phosphate in all three consensus sites for PKC phosphorylation. However, quantitative differences were noted. In particular, Ser-661 was phosphorylated less extensively by PKC βI and βII, consistent with the phosphate stoichiometry of 2 mol phosphate/mol PG-2. In addition, some clear differences were noted for PKC δ and PKC ε. PKC δ mainly phosphorylated Ser-667 and Ser-671 and poorly phosphorylated Ser-661. PKC ε mainly phosphorylated Ser-667, poorly phosphorylated Ser-671, and did not phosphorylate Ser-661. Generally, there was good correspondence between the number of phosphopeptides observed and the phosphorylation stoichiometry, confirming that each phosphopeptide contained a single site. However, exceptions were PKC η and PKC ζ, where three phosphopeptides were observed, but the stoichiometry was much less than 3 mol phosphate/mol PG-2, indicating submaximal phosphorylation. Nonetheless, these results clearly indicate differential *in vitro* phosphorylation of the PKC sites in the linker region of P-gp by different PKC isozymes.

The conventional PKC isozymes and the atypical PKC ζ gave a pattern more similar to mixed rat brain PKC, and a distinctly different pattern was observed for the novel PKC isozymes δ and ε. It should be noted that the phosphopeptides containing Ser-667 and Ser-671 are closely adjacent and, in some of the patterns, appear to merge, but they are, nonetheless, distinct. Map patterns were highly reproducible and performed in triplicate. For example, Fig. 3 shows that two independent analyses of PKC γ phosphorylation of PG-2 gave the same pattern of phosphopeptides.

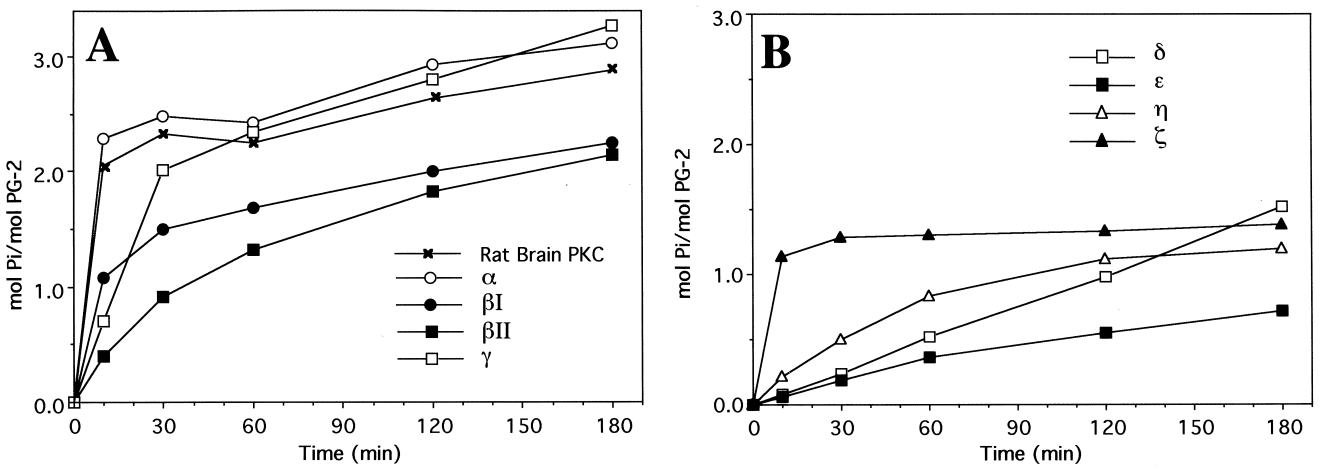


**FIG. 2. Time course and stoichiometry of PG-2 phosphorylation by PKC isozymes. Phosphorylation of PG-2 was performed as described under Materials and Methods. At the indicated times, aliquots of the reaction mixture were removed, and phosphate incorporation was determined. (A) $Ca^{2+}$-dependent PKC isozymes; (B) $Ca^{2+}$-independent PKC isozymes. Data shown are representative of three independent experiments, and replicates differed by ≤ 15%.**

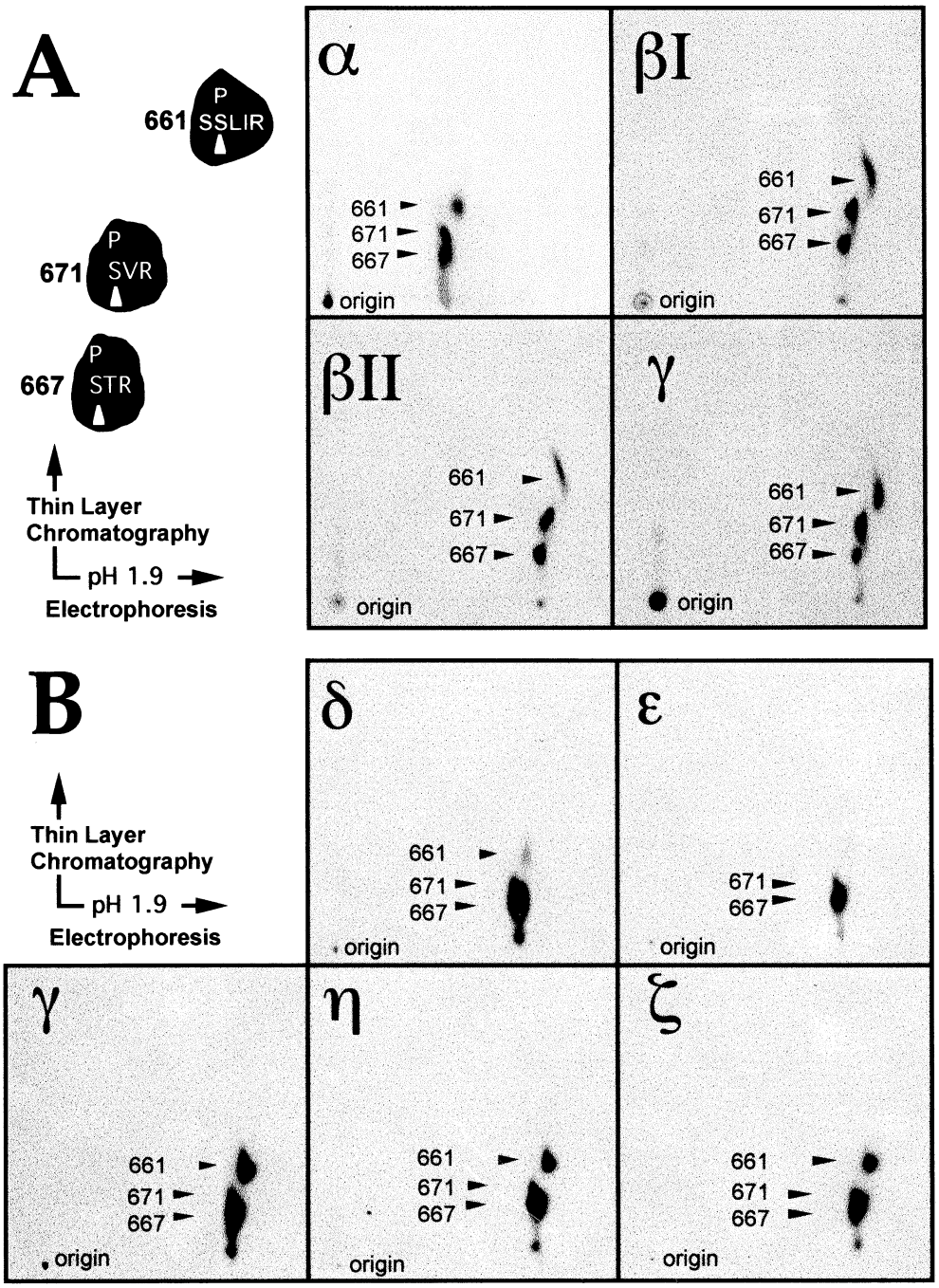


**FIG. 3. Autoradiograms of phosphopeptide maps of PG-2 phosphorylated by PKC isozymes. Two-dimensional tryptic phosphopeptide maps of PG-2 phosphorylated by PKC isozymes were generated as described under Materials and Methods. The sequences of these phosphopeptides in order of migration from the bottom to top are: STR (Ser-667), SVR (Ser-671), and SSLIR (Ser-661). Electrophoresis was performed from left to right, and ascending chromatography was performed from bottom to top as indicated. (A) $Ca^{2+}$-dependent PKC isozymes; (B) $Ca^{2+}$-independent PKC isozymes. Results with PKC γ are shown in both panels for comparison.**

## DISCUSSION

PG-2 is an excellent model substrate to investigate the phosphorylation of human MDR1 P-gp because the *in vitro* and *in vivo* sites have been shown to be confined to the region of the molecule represented by this peptide. Specifically, Ser-661, Ser-667, and Ser-671 are the major *in vitro* and *in vivo* sites, and they all occur within classic PKC consensus motifs [13, 19, 20]. However, despite these results, the number and identity of the kinases that phosphorylate P-gp in MDR cells are not certain. It seems most likely that P-gp is phosphorylated by one or more PKC isozymes and probably also by other related kinases [2–4]. We therefore undertook the present study to determine whether PG-2 was phosphorylated by individual PKC

isozymes. Our results demonstrated that PG-2 is an effective substrate for all of the PKC isozymes tested, but the extent and pattern of phosphorylation vary with different PKC isozymes. This could be important because cell- and tissue-specific expression of PKC isozymes have been found previously.

A critical role for the linker region serine-671 in the positive regulation of P-gp ATPase activity by PKC α was suggested by the demonstration that mutation of this serine to asparagine abolished the enhancement of drug-stimulated P-gp ATPase activity by PKC α in a baculovirus expression system [21]. Previously, transfection of MCF-7 cells expressing MDR1 with PKC α was shown to increase drug resistance and enhance phosphorylation of P-gp [22], while expression of PKC α antisense cDNA in the same MDR MCF7 cells was shown to decrease PKC activity, P-gp phosphorylation, and P-gp function [23].

Simultaneous mutation of all linker region consensus PKC phosphorylation sites either to nonphosphorylatable residues or negatively charged residues, and subsequent expression of these mutant P-gps, did not reveal phenotypic differences in drug resistance patterns between mutated and wild-type P-gps [24, 25]. However, the selective and enhanced phosphorylation of individual sites by specific PKC isozymes, as suggested by the present work, may be functionally important. PKC isozyme patterns of protein expression are known to be tissue- and cell type-specific from mammalian studies. It is possible that this tissue-specific expression of PKC isozymes could enhance or diminish activation of P-gp activity depending upon which PKC isozyme(s) is (or are) expressed and phosphorylate(s) P-gp. In addition, several PKC isozymes have been shown by co-immunoprecipitation studies to be physically associated with P-gp in MDR cells [26]. The ability of different PKC isozymes to phosphorylate selective PKC consensus sites singularly or in combinations within the linker region may modulate drug transport and ATPase activities of P-gp above or below basal activity levels. Our work and its implications may compliment conclusions made from previous studies in which all PKC phosphorylation sites in the linker region were mutated to a non-phosphorylatable state [24, 25]. Phosphorylation of individual sites may have opposing effects on MDR function, not revealed in the mutagenesis studies where all sites were altered simultaneously. Studies of single site mutants will help clarify this point and may implicate specific PKC isozymes in the differential modulation of P-gp function. This needs to be examined fully to understand the effects of PKC isoenzyme phosphorylation upon P-gp function.

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## References

1. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62**: 385–427, 1993.
2. Germann UA, Chambers TC, Ambudkar SV, Pastan I and Gottesman MM, Effects of phosphorylation of P-glycoprotein on multidrug resistance. *J Bioenerg Biomembr* **27**: 53–61, 1995.
3. Chambers TC, Phosphorylation of proteins involved in multidrug resistance. In: *Multidrug Resistance in Cancer Cells: Molecular, Biochemical, Physiological, and Biological Aspects* (Eds. Gupta S and Tsuruo T), pp. 303–318. John Wiley & Sons, Chichester, 1996.
4. Fine RL, Chambers TC and Sachs CW, P-glycoprotein, multidrug resistance and protein kinase C. *Stem Cells* **14**: 47–55, 1996.
5. Asaoka Y, Nakamura S, Yoshida K and Nishizuka Y, Protein kinase C, calcium and phospholipid degradation. *Trends Biochem Sci* **17**: 414–417, 1992.
6. Fine RL, Patel J and Chabner BA, Phorbol esters induce MDR in human breast cancer cells. *Proc Natl Acad Sci USA* **85**: 582–586, 1988.
7. O'Brian CA, Fan D, Ward NE, Seid C and Fidler IJ, Level of protein kinase C activity correlates directly with resistance to adriamycin in murine fibrosarcoma cells. *FEBS Lett* **246**: 78–82, 1989.
8. Posada J, McKeegan E, Worthington K, Morin M, Jaken S and Tritton T, Human multidrug resistant KB cells overexpress PKC: Involvement in drug resistance. *Cancer Commun* **1**: 285–292, 1989.
9. Blobe GC, Sachs CW, Khan WA, Fabbro D, Stabel S, Wetsel WC, Obeid LM, Fine RL and Hannun YA, Selective regulation of expression of protein kinase C (PKC) isoenzymes in multidrug-resistant MCF-7 cells. *J Biol Chem* **268**: 658–664, 1993.
10. Gollapudi S, Patel K, Jain V and Gupta S, Protein kinase C isoforms in multidrug resistant P388/ADR cells: A possible role in daunorubicin transport. *Cancer Lett* **62**: 69–75, 1992.
11. Aquino A, Warren BS, Omichinski J, Hartman KD and Glazer RI, Protein kinase C-γ is present in adriamycin resistant HL-60 leukemia cells. *Biochem Biophys Res Commun* **166**: 723–728, 1990.
12. O'Brian CA, Fan D, Ward NE, Dong Z, Iwamoto L, Gupta KP, Earnsest LE and Fidler IJ, Transient enhancement of multidrug resistance by the bile acid deoxycholate in murine fibrosarcoma cells *in vitro*. *Biochem Pharmacol* **41**: 797–806, 1991.
13. Chambers TC, Pohl J, Raynor RL and Kuo JF, Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *J Biol Chem* **268**: 4592–4595, 1993.
14. Chambers TC, Pohl J, Glass DB and Kuo JF, Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. *Biochem J* **299**: 309–315, 1994.
15. Stabel S, Schaap D and Parker PJ, Expression of protein kinase C isotypes using baculovirus vectors. *Methods Enzymol* **200**: 670–673, 1991.
16. Liyanage M, Frith D, Livneh E and Stabel S, Protein kinase C group B members PKC-δ, -ε, -η and ζ: Comparison of properties of recombinant proteins *in vitro* and *in vivo*. *Biochem J* **283**: 781–787, 1992.
17. Schagger H and von Jagow G, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 1000 kDa. *Anal Biochem* **166**: 368–379, 1987.
18. Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K and Nishizuka Y, Protein kinase C ζ subspecies from rat brain: Its structure,

expression and properties. *Proc Natl Acad Sci USA* **86**: 3099–3103, 1989.
19. Chambers TC, Zheng B and Kuo JF, Regulation by phorbol ester and protein kinase C inhibitors, and by a protein phosphatase inhibitor (okadaic acid), of P-glycoprotein phosphorylation and relationship to drug accumulation in multidrug-resistant human KB cells. *Mol Pharmacol* **41**: 1008–1015, 1993.
20. Kennelly PJ and Krebs EG, Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem* **261**: 15555–15558, 1991.
21. Ahmad S, Safa AR and Glazer RI, Modulation of P-glycoprotein by protein kinase C α in a baculovirus expression system. *Biochemistry* **33**: 10313–10318, 1994.
22. Yu G, Ahmad S, Aquino A, Fairchild CR, Trepel JB, Ohno S, Suzuki K, Tsuruo T, Cowan KH and Glazer RI, Transfection with protein kinase C α confers increased multidrug resistance to MCF-7 cells overexpressing P-glycoprotein. *Cancer Commun* **3**: 181–189, 1991.
23. Ahmad S and Glazer RI, Expression of the antisense cDNA for protein kinase C α attenuates resistance in doxorubicin-resistant MCF-7 breast carcinoma cells. *Mol Pharmacol* **43**: 858–862, 1993.
24. Germann UA, Chambers TC, Ambudkar SV, Licht T, Cardarelli CO, Pastan I and Gottesman MM, Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. *J Biol Chem* **271**: 1708–1716, 1996.
25. Goodfellow HR, Sardini A, Ruetz S, Callaghan R, Gros P, McNaughton PA and Higgins CF, Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. *J Biol Chem* **271**: 13668–13674, 1996.
26. Yang J-M, Chin K-V and Hait WN, Interaction of P-glycoprotein with protein kinase C in human multidrug resistant carcinoma cells. *Cancer Res* **56**: 3490–3494, 1996.