Modulation of P-Glycoprotein by Protein Kinase Cα in a Baculovirus Expression System†

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ABSTRACT: The modulation of P-glycoprotein by protein kinase Cα (PKCα) was examined in a baculovirus expression system. PGP was phosphorylated in membrane vesicle preparations *in vitro* only when coexpressed with PKCα, and phosphorylation was Ca^{2+}-dependent and inhibited by the PKC inhibitor Ro 31-8220. PGP and PKCα were tightly associated in membrane vesicles and were coimmunoprecipitated with antibodies against either PGP or PKCα. Photoaffinity labeling of membrane vesicles with [^{3}H]azidopine indicated that drug binding to PGP was slightly increased in the presence of PKCα. In contrast, PGP ATPase activity was increased by PKCα as well as by verapamil, but only PKC-stimulated activity in the presence of verapamil was inhibited by Ro 31-8220. Mutation of serine-671 to asparagine in the linker region of PGP abolished PKCα-stimulated ATPase activity, and also inhibited to a lesser degree verapamil-stimulated ATPase activity. These results indicate that PKCα in a positive regulator of PGP ATPase activity and suggest that this mechanism may account for the increased multidrug resistance observed in *MDR*1-expressing cells when PKCα activity is elevated.

The hallmark of multidrug resistance (MDR)[1] is the reduced intracellular accumulation of structurally unrelated natural product anticancer drugs such as the anthracyclines, *Vinca* alkaloids, actinomycin D, and epipodophyllotoxins (Beck, 1987; Gottesman & Pastan, 1988). The MDR phenotype is most often associated with the elevated expression of a plasma membrane-associated ATP-dependent unidirectional drug transporter, P-glycoprotein (PGP) (Hamada & Tsuruo, 1986, 1988; Kamimoto et al., 1989; Naito et al., 1989). PGP is the product of the *MDR*1 gene, a highly conserved multigene family that includes two genes in man termed *MDR*1 and *MDR*3 (also called *MDR*2) (Croop et al., 1988; Ng et al., 1989; Chin et al., 1989). In MDR cell lines, *MDR*1 expression is increased as a result of gene amplification or increased transcription (Fairchild et al., 1987; Fojo et al., 1985; Riordan et al., 1985; Roninson et al., 1986; Scotto et al., 1986), and overexpression of the *MDR*1 gene, but not the *MDR*3 gene, confers MDR (Schinkel et al., 1991). Several studies have shown that expression of either the genomic or the cDNA sequence of *MDR*1 confers the MDR phenotype to the recipient cells (Ueda et al., 1987; Croop et al., 1987; Debenham et al., 1982; Gros et al., 1986; Shen et al., 1986; Sugimoto & Tsuruo, 1987), although the degree of resistance is generally less when compared to drug-selected MDR cells.

Another phenotypic characteristic of all human MDR cell lines is an increase in PKC activity (Aquino et al., 1988, 1990; Fine et al., 1988; Lee et al., 1992; Melloni et al., 1989; O'Brian et al., 1989; Palayoor et al., 1987; Posada et al., 1989a,b). This attribute has been found for PKCα (Lee et al., 1992; Posada et al., 1989a; Blobe et al., 1993; Yu et al., 1991; O'Brian et al., 1991), PKCβ (Fan et al., 1992), and PKCγ (Aquino et al., 1990). Since PGP is phosphorylated in several MDR cell lines (Carlsen et al., 1977; Center, 1983, 1985; Mellado et al., 1987; Myers et al., 1989; Schurr et al., 1989) and phorbol esters stimulate PGP phosphorylation in intact cells (Yu et al., 1991; Bates et al., 1992; Hamada et al., 1987; Chambers et al., 1990a,b) and reduce drug accumulation (Chambers et al., 1990a, 1992), it may be inferred that PKC is involved in this process. PGP serves as a substrate for PKC *in vitro* (Chambers et al., 1992), and overexpression of PKCα (Yu et al., 1991), but not PKCγ (Ahmad et al., 1992), increases MDR, decreases drug accumulation, and increases phorbol ester-dependent phosphorylation of PGP. In contrast, antisense expression of PKCα in MDR MCF-7/ADR cells reduces MDR (Ahmad & Glazer, 1993). Therefore, a substantial body of data supports the thesis that PKCα is involved in modulating PGP, but the mechanism for this effect has not been determined. To investigate the possible modulatory role of PKCα on PGP function, a baculovirus expression system was developed in which we could examine the interaction of these proteins in the absence of other pleiotropic changes which occur in MDR cells.

MATERIALS AND METHODS

Baculovirus Expression of PGP and PKCα in Sf9 Cells. The human *MDR*1 cDNA was generously provided by Dr. Merrill Goldsmith, National Cancer Institute, as plasmid pMTAdr (Fairchild et al., 1987), and consists of the 3.84 kb open reading frame and 424 bp of 5′-untranslated and 382 bp of 3′-untranslated sequences. The *MDR*1 cDNA was isolated from plasmid pMTAdr by digestion with *Bam*HI followed by gel purification. Baculovirus transfer vector pVL1392 (kindly provided by Dr. Max Summers, Texas A&M University) was digested with *Bam*HI and the *MDR*1 insert ligated into the vector with T4 ligase. The orientation of the *MDR*1 insert downstream from the polyhedrin promoter was confirmed by

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[1] Abbreviations: MDR, multidrug-resistant or multidrug resistance; PKC, protein kinase C; PGP, P-glycoprotein; SDS, sodium dodecyl sulfate.

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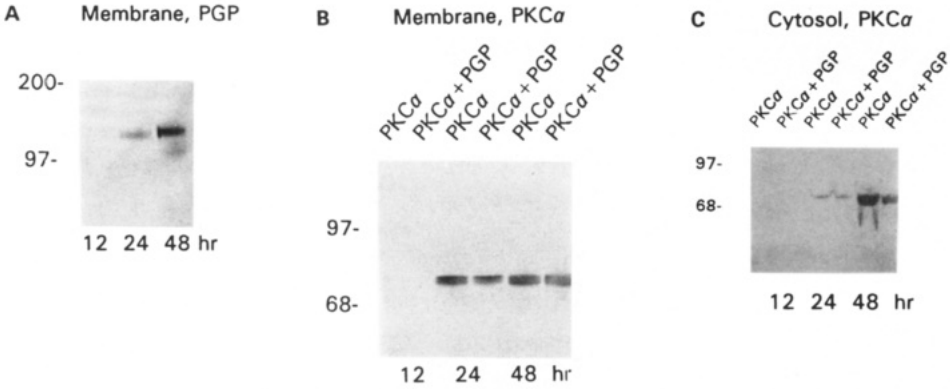

FIGURE 1: Time course of the expression of PGP and PKCα in Sf9 cells. At 12–48 h after infection, Sf9 cells were disrupted by nitrogen cavitation, and membrane vesicle and cytosol fractions were prepared by differential centrifugation. Membrane vesicle (20 μg) and cytosol (20 μg) fractions were separated by SDS–PAGE, and immunoblotting was carried out as described under Materials and Methods. (A) PGP levels in the membrane fraction; (B) PKCα levels in the membrane fraction; (C) PKC levels in the cytosol fraction. PGP and PKCα were visualized with monoclonal antibody C219 or with a polyclonal antibody against PKCα, respectively.

restriction enzyme analysis. The resulting plasmid, pVL-MDR1, and linearized mutated proviral DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (BaculoGold, Pharmingen) were used to cotransfect Sf9 cells by lipofection (Lipofectin, BRL). Recombinant plaques expressing PGP were identified by their occlusion-negative morphology and by immunoblotting with monoclonal antibody C219 (Centocor). Immunoreactive bands were visualized with the alkaline phosphatase/BCIP/NBT detection system. A recombinant baculovirus expressing PKCα was prepared as described previously (Goswami & Glazer, 1991).

Site-Directed Mutagenesis. Plasmid pVL-MDR1 was mutated by the procedure of Deng and Nickoloff (1992) using the protocol supplied with the Transformer size-directed mutagenesis kit (Clontech). Codon 671, AGT, was mutated to AAT to produce a Ser→Asn mutation using the oligonucleotide 5′-TCC-ACG-GAC-AAT-CCT-ACG-AGT-3′. Plasmid selection was based on mutation of the unique *Not*I site, GCGGCCGC to GCTGCCGC, with oligonucleotide 5′-TGG-AGC-GGC-AGC-TGC-AGA-TCT-3′. Mutations were confirmed by double-stranded sequencing using Sequenase (USB).

Membrane Vesicle Preparation. Cells (2×10^7) were infected with each recombinant virus at 2.5 pfu/cell in 10 mL of SF900 medium (Gibco/BRL), and after 1 h, an additional 10 mL of medium was added. After 48 h, cells were suspended in 5 mL of hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, and 2 mM PMSF) and incubated on ice for 30 min. Cells were pressurized at 800 psi under nitrogen in a Parr cell disruption bomb for 10 min and after decompression were centrifuged for 10 min at 500*g* at 4 °C. The supernatant was centrifuged at 100000*g* for 1 h, and the cell pellet containing the membrane vesicle fraction was suspended in the same buffer containing 0.25 M sucrose, divided into aliquots, and kept at −80 °C.

Phosphorylation of PGP. Membrane vesicles were prepared from Sf9 cells infected with *MDR*1 or *MDR*1 and PKCα. Phosphorylation assays were carried out *in vitro* as described by Yu et al. (1991), except that exogenous substrate was omitted. In some experiments, the staurosporine analog Ro 31-8220 (kindly provided by Dr. Geoffrey Lawton, Roche Products, Ltd., England) was used to inhibit PKC activity (Davis et al., 1992).

Immunoprecipitation of PGP and PKC. After *in vitro* phosphorylation, the reaction was terminated by the addition of 500 μL of immunoprecipitation buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% Triton X-100]. Immunoprecipitation was carried out with the following antibodies: 5 μg of PGP rabbit polyclonal antibody 4007 (kindly provided by Dr. Michael Gottesman, National Cancer Institute), 1 μg of PGP monoclonal antibody MRK16 (kindly provided by Dr. Takashi Tsuruo, Japanese Foundation for Cancer Research, Japan), 5 μg of PKCα/β rabbit polyclonal antibody C4 (Aquino et al., 1987) and 1.25 μg of PKCα rabbit polyclonal antibody (BRL). Immunoconjugates were adsorbed with 20 μL of Protein G-Plus/Protein A–agarose (Oncogene Science) at 4 °C for 1 h on a rocking platform, centrifuged at 12000*g* for 2 min, and washed 3 times with immunoprecipitation buffer. Adsorbed immunocomplexes were suspended in Laemmli sample buffer without boiling and separated by SDS–PAGE in an 8% minigel (Novex). Samples were transferred to nitrocellulose by electroblotting, and antigens were visualized by autoradiography.

ATPase Activity. ATPase activity was measured by the procedure of Sarkadi et al. (1992) in the presence and absence of 10 μM verapamil. In order to determine only PGP-associated ATPase activity, the assay mix contained 2 mM EGTA (an inhibitor of Ca^{2+}-dependent ATPase), 5 mM sodium azide (an inhibitor of F_1,F_2-ATPase), and 1 mM ouabain (an inhibitor of Na^+,K^+-ATPase).

Photoaffinity Labeling with [^{3}H]Azidopine. Membrane vesicles were incubated in a buffer containing 10 mM Tris-HCl (pH 7.4), 10% PBS, 0.3 mM $MgCl_2$, and 3 mM ATP containing 4% DMSO and 0.25 μM [^{3}H]azidopine (specific activity 44 Ci/mmol; Amersham Corp.) in a final volume of 0.05 mL as described previously (Safa et al., 1990). The reaction mixture was preincubated for 30 min at 25 °C in the absence or presence of 50 μM nonradioactive competing ligand and then irradiated for 20 min with a UV lamp equipped with two 15-W self-filtering 302-nm lamps. Photolabeled membranes (75 μg of protein/lane) were analyzed directly by SDS–PAGE on a 5–15% gradient gel containing 4.5 M urea, followed by fluorography.

RESULTS

Time Course of Expression and Translocation of PGP and PKCα. PGP was distributed exclusively in the membrane fraction at 12–48 h after infection (Figure 1A). PKCα was also distributed in the membrane fraction 24 h after infection when expressed either alone or with PGP (Figure 1B), but PKCα was present mainly in the cytosol at 48 h after infection (Figure 1C). PKC activity 48 h after infection was 99.3%

Table 1: Protein Kinase Cα Activity and Distribution in Insect Cells Expressing P-Glycoprotein[a]

	cytosol			membrane		
virus	protein (mg of protein)	total act. (nmol/min)	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]	protein (mg of protein)	total act. (nmol/min)	sp act. [nmol min^{-1} (mg of protein)$^{-1}$]
PKCα	5.2 (81.3%)	615 (99.7%)	118 (100%)	1.2 (18.7%)	4.3 (0.7%)	3.6 (100%)
PKCα + PGP	3.8 (82.6%)	189 (97.4%)	50 (42%)	0.8 (17.4%)	5.0 (2.6%)	6.3 (175%)

[a] Baculovirus infection of Sf9 cells and PKC assays are described under Materials and Methods.

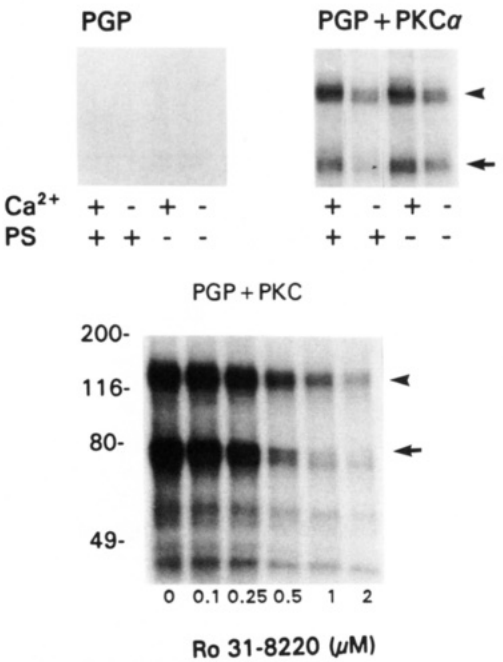

FIGURE 2: Effect of PKC cofactors and Ro 31-8220 on PGP and PKCα phosphorylation *in vitro* in membrane vesicles. (A) Phosphorylation assays were carried out with 20 μg of protein in the presence (+) or absence (–) of either PKC cofactor. An autoradiogram of the dried gel is shown. The *arrowhead* and *arrow* represent the 140-kDa phosphorylated PGP and the 80-kDa autophosphorylated PKCα, respectively. (B) Inhibition of PGP phosphorylation by inhibitor Ro 31-8220. Membrane vesicles were prepared from cells infected with PGP and PKCα, and phosphorylation *in vitro* was determined in the presence of Ca^{2+} and varying concentrations of Ro 31-8220. The *arrowhead* and *arrow* represent the 140-kDa phosphorylated PGP and the 80-kDa autophosphorylated PKCα, respectively.

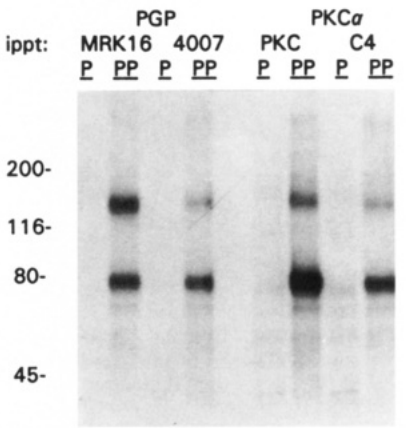

FIGURE 3: Immunoprecipitation of PGP or PKCα after phosphorylation *in vitro*. Membrane vesicles from Sf9 cells infected with either PGP (*P*) or PGP and PKCα (*PP*) were incubated *in vitro* with [γ-^{32}P]ATP and calcium and immunoprecipitated with either monoclonal (*MRK16*) or polyclonal (*4007*) antibodies against PGP or with polyclonal antibodies against PKCα (*PKC*) or the catalytic domain of PKC (*C4*). Samples were separated by SDS–PAGE in 8% gels and detected by autoradiography. The *upper* and *lower* bands represent PGP and PKCα, respectively.

cytosolic and 0.7% membrane-bound (Table 1); however, when PKCα was coexpressed with PGP, PKC activity was 97.4% cytosolic and 2.6% membrane-bound. Whether this change in PKC distribution resulted from PGP expression or the lower expression of PKCα when coexpressed with PGP is uncertain.

Cofactor Dependence of Phosphorylation and Inhibition by PKC Inhibitor Ro 31-8220 in Membrane Vesicles Containing PGP and PKCα. The requirements for PGP phosphorylation *in vitro* were examined in membrane vesicles containing PKCα and PGP or PGP alone (Figure 2A). No phosphorylation of PGP was observed in membranes in the absence of PKCα, but PGP phosphorylation as well as PKCα autophosphorylation was readily observed in membranes containing both proteins. Autophosphorylation of PKC or PGP phosphorylation was dependent only on calcium, and may reflect membrane integration of PKC and PGP and the elimination of a requirement for exogenous phospholipid. In order to substantiate that PGP phosphorylation is mediated through PKC, *in vitro* phosphorylation was carried out in the presence of the selective PKC inhibitor Ro 31-8220 (Figure 2B). PGP phosphorylation as well as PKC autophosphorylation was inhibited in a dose-dependent fashion. Metabolic labeling of Sf9 cells expressing PGP with $H_3{}^{32}PO_4$ for 3 h produced low levels of PGP phosphorylation which was greatly enhanced in cells expressing both PGP and PKCα (results not shown).

To determine whether PGP and PKCα were tightly associated in membrane vesicles, membrane vesicles containing PGP and PKCα and phosphorylated *in vitro* were immunoprecipitated either with PGP antibodies MRK16 or 4007 or with two polyclonal PKC antibodies (Figure 3). MRK16 and the PKCα antibody ("PKC") were more effective than antibody 4007 and PKC antibody C4 for immunoprecipitating PGP–PKC complexes, and may be due, in part, to the different epitopes recognized by each antibody. Neither MRK16 nor the PKCα antibody nonspecifically immunoprecipitated PKCα and PGP in membranes expressing either PGP or PKCα, respectively (results not shown). These results suggest that baculovirus-expressed PGP is a native protein that is tightly associated with PKC in membrane vesicles.

Photoaffinity Labeling of Membrane Vesicles with Azidopine. To determine whether PKC affected drug binding to PGP, PGP was photoaffinity-labeled with [^{3}H]azidopine (Figure 4). Membrane vesicles containing both PGP and PKC were photolabeled to a slightly greater extent than membrane vesicles containing PGP alone (Figure 4). Photolabeling in both preparations was completely inhibited by unlabeled azidopine, and less effectively by vinblastine, doxorubicin, and colchicine. It was also noted that PKC was labeled by [^{3}H]azidopine, but this effect could not be completely eliminated with unlabeled azidopine, suggesting nonspecific binding. Immunoblotting of membrane vesicles containing either PGP or both PGP and PKCα indicated that the levels of PGP were similar in both instances (results not shown).

ATPase Activity in Membrane Vesicles Containing PGP. ATPase activity was measured in membrane vesicles contain-

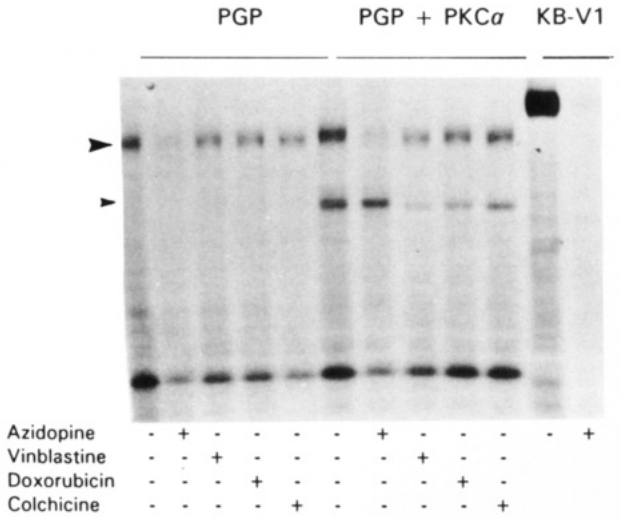

FIGURE 4: Photoaffinity labeling of membrane vesicles containing PGP and PKCα with [^{3}H]azidopine. Membrane vesicles were preincubated for 30 min at 25 °C in the absence (–) or presence (+) of 50 μM competing ligand before photoaffinity labeling with [^{3}H]-azidopine as described under Materials and Methods. Photolabeled membranes (75 μg of protein/lane) were analyzed directly by SDS–PAGE on a 5–15% gradient gel containing 4.5 M urea, followed by autoradiography. Membrane vesicles contained PGP (*PGP*) or PGP and PKCα (*PGP+PKCα*) or were from KB-V1 cells (*KB-V1*). *Large arrowhead*, PGP; *small arrowhead*, PKCα.

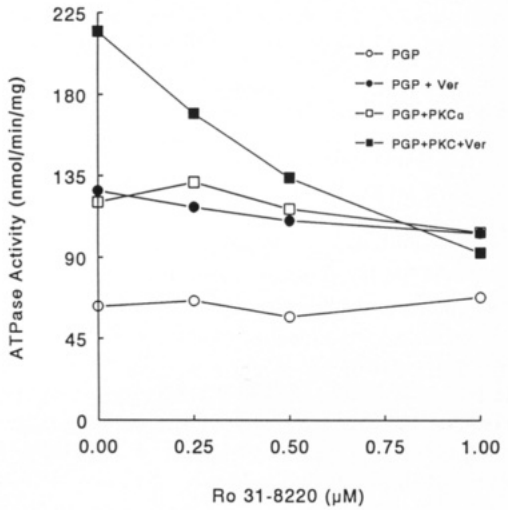

FIGURE 5: Effect of verapamil and Ro 31-8220 on ATPase activity in membrane vesicles from Sf9 cells expressing either PGP or PGP and PKCα. Membrane vesicles were prepared by nitrogen cavitation and assayed for ATPase activity as described by Sarkadi et al. (1992). Assays were carried out under linear kinetics for 20 min at 37 °C with 10 μg of membrane vesicle protein in the presence or absence of 10 μM verapamil and varying concentrations of Ro 31-8220.

ing PGP alone or both PGP and PKCα (Figure 5) in the presence of sodium azide, ouabain, and EGTA, inhibitors of F_1,F_o-ATPase, Na^+,K^+-ATPase, and Ca^{2+}-dependent ATPase, respectively. PGP-associated ATPase activity was increased approximately 2-fold when coexpressed with PKCα compared to membrane preparations containing PGP alone. Moreover, Ro 31-8220 inhibited only verapamil- and PKC-stimulated ATPase activity in a concentration-dependent manner, but did not affect ATPase activity when stimulated by either verapamil or PKC alone.

To directly determine the dependence of PGP ATPase activity on PKC-mediated phosphorylation, Ser671 was mutated to Asn within a PKC consensus phosphorylation sequence in the linker region of PGP (Figure 6). The

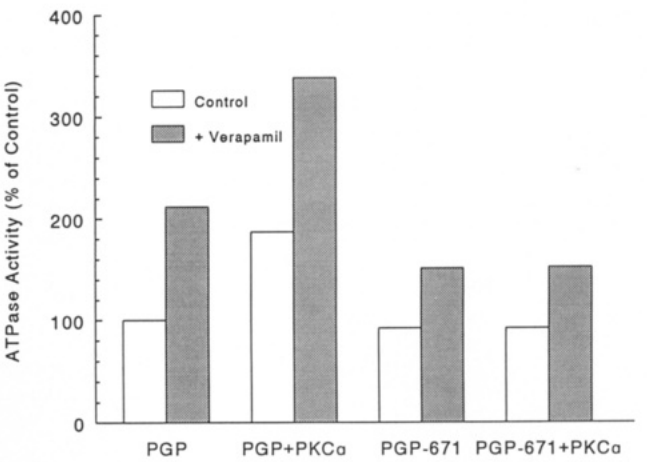

FIGURE 6: Effect of mutating Ser671 in PGP on PKCα- and verapamil-stimulated ATPase activity. Membrane vesicles were prepared from Sf9 cells expressing either PGP or PGP and PKCα, and assayed for ATPase activity as described in Figure 5. Assays were carried out for 30 min in the presence and absence of 10 μM verapamil. ATPase activity [nmol min^{-1} (mg of protein)$^{-1}$] was 108 for wild-type PGP and 99 for the PGP Ser671 mutant, and is expressed as a percentage of the activity of wild-type PGP.

Ser671Asn mutant exhibited a similar level of ATPase activity as wild-type PGP; however, mutant PGP was resistant to the stimulatory effect of PKCα on ATPase activity, and exhibited an attenuated response to verapamil compared to wild-type PGP.

DISCUSSION

In the present investigation, a recombinant baculovirus expression system was developed in which the effect of PKC on two functional properties of PGP could be measured, viz., ATPase activity and drug binding. Sf9 insect cells contain no endogenous PKC activity or other proteins associated with pleiotropic drug resistance and, therefore, represent an ideal system in which to evaluate the effects of protein phosphorylation in the absence of other interfering factors. Membrane vesicles containing PGP alone exhibited verapamil-stimulated ATPase activity that was comparable to the activity reported by Sarkadi et al. (1992). However, in addition, we have found that ATPase activity is stimulated by PKCα in the presence or absence of verapamil and that the effect of PKC is abolished by mutation of Ser671 in PGP. The internucleotide linker region of PGP contains several consensus PKC phosphorylation sites (Chen et al., 1986). Ser671 is the site identified in human PGP that is phosphorylated by PKC *in vitro* (Chambers et al., 1992), and is equivalent to Ser669 identified as the major phosphorylation site in murine mdr1b (Orr et al., 1993). Our results indicate that Ser671 appears to be crucial for both drug-stimulated and PKC-stimulated ATPase activity since mutation of Ser671 completely negated the stimulatory effect of PKCα and partially inhibited the effect of verapamil. In addition, the PKC inhibitor Ro 31-8220 abolished PKC stimulation of ATPase activity in the presence of verapamil, suggesting that the pathways involved in ATPase activation by PKC and verapamil are not mutually exclusive despite the appearance of additivity. This possibility is further supported by the study of Hamada et al. (1987), who found that verapamil stimulated phosphorylation of PGP as much as phorbol esters. Since drug transport by PGP is an energy-dependent process (Skovsgaard & Nissen, 1982), it is likely that activation of ATPase is coupled to drug transport. These results provide an explanation for the increased multidrug resistance that is accompanied by increased PGP phosphorylation and decreased

drug retention in MCF-7 breast carcinoma cells overexpressing *MDR*1 and PKCα (Yu et al., 1991), and for the partial reversal of resistance by antisense PKCα (Ahmad & Glazer, 1993).

Several studies have used PKC inhibitors to reverse MDR (O'Brian et al., 1989; Posada et al., 1989a; Sampson et al., 1993; Dong et al., 1991), but these effects have been difficult to interpret since all of these inhibitors are known to interact with PGP and prevent drug binding (Miyamoto et al., 1992; Sato et al., 1990; Wakusawa et al., 1992). In addition, inhibitors such as staurosporine and H-7 lack PKC selectivity (Meyer et al., 1989) and produce cytotoxicity that may be unrelated to inhibition of protein kinase activity (Smith et al., 1988). Despite the fact that inhibitors such as Ro 31-8220 show greater PKC isoform selectivity (Dieter & Fitzke, 1991), their ability to serve as substrates for PGP may pose an insurmountable limitation to studies trying to dissect out the role of PKC in regulating PGP function. Therefore, the major advantage of the baculovirus system is that it permits the assessment of the drug binding and ATPase functions of PGP independently of its transport function and the influence of other pleiotropic factors associated with MDR.

PGP phosphorylation by PKCα in membrane vesicles was Ca^{2+}- but not phospholipid-dependent. This is not an unexpected finding since other studies have shown that the cofactor requirements of PKC for catalytic activity and substrate binding vary considerably depending on the substrate (Bazzi & Nelsestuen, 1987; Hyatt et al., 1994) and that membrane-associated PKC exhibits changes in its cofactor requirements (Bazzi & Nelsestuen, 1988, 1990). Our data also indicate that PGP strongly associates with PKCα, and although this has not been demonstrated previously in MDR cells, PGP was found to cofractionate with an uncharacterized phospholipid-dependent protein kinase with the characteristics of Ca^{2+}-independent PKC isoforms (Staats et al., 1990). Whether this occurs in MDR cells remains to be established.

Our data suggest that PKC has a slight stimulatory effect on substrate binding as measured by photoaffinity labeling with azidopine. Germann et al. (1990) reported that Sf9 cell membranes containing PGP exhibited less azidopine binding than KB-V1 cells, and that vinblastine and vincristine were less effective competitors of binding in insect cell membrane vesicles compared to KB-V1 cells. We did not observe differences in the ability of vinblastine, doxorubicin, or colchicine to compete with azidopine in Sf9 cell membranes containing either PGP or PGP and PKCα, although they were less effective competitors than azidopine. The relative order of competition of these drugs for azidopine binding to membranes containing PGP and PKCα was azidopine > vinblastine > doxorubicin > colchicine, a result that is similar to what was observed previously (Safa et al., 1990). Although azidopine binding was lower in Sf9 membrane vesicles than in membranes from KB-V1 cells, this effect may be a result of either membrane lipid composition or hypoglycosylation of PGP in insect cells. A recent study by Schinkel et al. (1993) indicated that hypoglycosylation of PGP did not affect its ability to confer MDR in mammalian cells, and therefore the lipid composition of insect cell vs mammalian cell membranes is the most likely cause of the differences observed in drug binding (Higgins & Gottesman, 1992).

Thus, the additive effects of verapamil and PKC suggest that PGP can be activated by two, but not necessarily, mutually exclusive processes: substrate binding and PGP phosphorylation. Although the mechanism for these effects has not been determined, it is likely that PGP phosphorylation results in a conformational change in PGP in the linker region which results in enhanced ATP hydrolysis and energy-dependent drug transport. We have, in fact, observed increased vinblastine accumulation *in vitro* using membrane vesicles from Sf9 cells phosphorylated by PKC (unpublished results). There is precedent for this model for another member of the ATPase binding cassette (ABC) protein family, the cystic fibrosis transmembrane regulator (Ames & Lecar, 1992), where PKC phosphorylation of the R domain (analogous to the linker region in PGP) activates its Cl^- conductance activity (Hwang et al., 1989; Picciotto et al., 1992). Thus, PGP becomes the second ABC protein to be modulated by PKC.

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REFERENCES

Ahmad, S., & Glazer, R. I. (1993) *Mol. Pharmacol. 43*, 858–862.

Ahmad, S., Trepel, J. B., Ohno, S., Suzuki, K., Tsuruo, T., & Glazer, R. I. (1992) *Mol. Pharmacol. 42*, 1004–1009.

Ames, G. F.-L., & Lecar, H. (1992) *FASEB J. 6*, 2660–2666.

Aquino, A., Hartman, K. D., Knode, M. C., Huang, K.-P., Niu, C.-H., & Glazer, R. I. (1988) *Cancer Res. 48*, 3324–3329.

Aquino, A., Warren, B., Omichinski, J., Hartman, K. D., & Glazer, R. I. (1990) *Biochem. Biophys. Res. Commun. 166*, 723–728.

Bates, S. E., Currier, S. J., Alvarez, M., & Fojo, A. T. (1992) *Biochemistry 31*, 6366–6372.

Bazzi, M. D., & Nelsestuen, G. L. (1987) *Biochemistry 26*, 1974–1982.

Bazzi, M. D., & Nelsestuen, G. L. (1988) *Biochem. Biophys. Res. Commun. 152*, 336–343.

Bazzi, M. D., & Nelsestuen, G. L. (1990) *Biochemistry 29*, 7624–7630.

Beck, W. T. (1987) *Biochem. Pharmacol. 36*, 2879–2887.

Blobe, G. C., Sachs, C. W., Khan, W. A., Fabbro, D., Stabel, S., Wetsel, W. C., Obeid, L. M., Fine, R. L., & Hannun, Y. A. (1993) *J. Biol. Chem. 268*, 658–664.

Carlsen, S. A., Till, J. E., & Ling, V. (1977) *Biochim. Biophys. Acta 467*, 238–250.

Center, M. S. (1983) *Biochem. Biophys. Res. Commun. 115*, 159–166.

Center, M. S. (1985) *Biochem. Pharmacol. 34*, 1471–1476.

Chambers, T., Chalikonda, I., & Eilon, G. (1990a) *Biochem. Biophys. Res. Commun. 169*, 253–259.

Chambers, T. C., McAvoy, E. M., Jacobs, J. W., & Eilon, G. (1990b) *J. Biol. Chem. 265*, 7679–7686.

Chambers, T., Zheng, B., & Kuo, J. F. (1992) *Mol. Pharmacol. 41*, 1008–1015.

Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., & Roninson, I. B. (1986) *Cell 47*, 381–389.

Chin, J. E., Soffir, R., Noonan, K. E., Choi, K., & Roninson, I. B. (1989) *Mol. Cell. Biol. 9*, 3808–3820.

Croop, J. M., Guild, B. C., Gros, P., & Housman, D. E. (1987) *Cancer Res. 47*, 5982–5988.

Croop, J. M., Gros, P., & Housman, D. E. (1988) *J. Clin. Invest. 81*, 1303–1309.

Davis, P. D., Elliott, L. H., Harris, W., Hill, C. H., Hurst, S. A., Keech, E., Kumar, M. K. H., Lawton, G., Nixon, J. S., & Wilkinson, S. E. (1992) *J. Med. Chem. 35*, 994–1001.

Debenham, P. G., Kartner, N., Siminovitch, L., Riordan, J. R., & Ling, V. (1982) *Mol. Cell. Biol. 2*, 881–889.

Deng, W. P., & Nickoloff, J. A. (1992) *Anal. Biochem. 200*, 81–88.

Dieter, P., & Fitzke, E. (1991) *Biochem. Biophys. Res. Commun. 181*, 396–401.

Dong, Z., Ward, N. E., Fan, D., Gupta, K. P., & O'Brian, C. A. (1991) *Mol. Pharmacol. 39*, 563–569.

Fairchild, C. R., Ivy, S. P., Kao-Shan, C.-S., Whang-Peng, J., Rosen, N., Israel, M. A., Melera, P. W., Cowan, K. H., & Goldsmith, M. E. (1987) *Cancer Res. 47*, 5141–5148.
Fan, D., Fidler, I. J., Ward, N. E., Seid, C., Earnest, L. E., Housey, G. M., & O'Brian, C. A. (1992) *Anticancer Res. 12*, 661–668.
Fine, R. L., Patel, J., & Chabner, B. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 582–586.
Fojo, A. T., Whang-Peng, J., Gottesman, M. M., & Pastan, I. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7661–7665.
Germann, U. A., Willingham, M. C., Pastan, I., & Gottesman, M. M. (1990) *Biochemistry 29*, 2295–2303.
Goswami, B. B., & Glazer, R. I. (1991) *BioTechniques 10*, 626–630.
Gottesman, M. M., & Pastan, I. (1988) *J. Biol. Chem. 263*, 12163–12166.
Gros, P., Neriah, Y. B., Croop, J. M., & Housman, D. E. (1986) *Nature 323*, 728–731.
Hamada, H., & Tsuruo, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7785–7789.
Hamada, H., & Tsuruo, T. (1988) *J. Biol. Chem. 263*, 1454–1458.
Hamada, H., Hagiwara, K.-I., Nakajima, T., & Tsuruo, T. (1987) *Cancer Res. 47*, 2860–2865.
Higgins, C. F., & Gottesman, M. M. (1992) *Trends Biochem. Sci. 17*, 18–21.
Horio, M., Gottesman, M. M., & Pastan, I. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3580–3584.
Hwang, T. C., Lu, L., Zeitlin, P. L., Gruenert, D. C., Huganir, R., & Guggino, W. B. (1989) *Science 244*, 1351–1353.
Hyatt, S. L., Liao, L., Chapline, C., & Jaken, S. (1994) *Biochemistry 33*, 1223–1228.
Juranka, P. F., Zastawny, R. L., & Ling, V. (1989) *FASEB J. 3*, 2583–2592.
Kamimoto, Y., Gatmaitan, Z., Hsu, J., & Arias, I. M. (1989) *J. Biol. Chem. 264*, 11693–11698.
Lee, S. A., Karaszkiewicz, J. W., & Anderson, W. B. (1992) *Cancer Res. 52*, 3750–3759.
Mellado, W., & Horwitz, S. B. (1987) *Biochemistry 26*, 6900–6904.
Melloni, E., Pontremoli, S., Viotti, P. L., Patrone, M., Marks, P. A., & Rifkind, R. A. (1989) *J. Biol. Chem. 264*, 18414–18418.
Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Rosel, J., Muller, M., Caravatti, G., & Matter, A. (1989) *Int. J. Cancer 43*, 851–856.
Miyamoto, K. I., Wakusawa, S., Inoko, K., Takagi, K., & Koyama, M. (1992) *Cancer Lett. 64*, 177–183.
Myers, M. B., Rittmann-Grauer, L., O'Brien, J. P., & Safa, A. R. (1989) *Cancer Res. 49*, 3209–3214.
Naito, M., Hamada, H., & Tsuruo, T. (1988) *J. Biol. Chem. 263*, 11887–11891.
Ng, W. F., Sarangi, F., Zastawny, R. L., Veinot-Drebot, L., & Ling, V. (1989) *Mol. Cell. Biol. 9*, 1224–1232.
O'Brian, C. A., Fan, D., Ward, N. E., Seid, C., & Fidler, I. J. (1989) *FEBS Lett. 246*, 78–82.
O'Brian, C. A., Fan, D., Ward, N. E., Dong, Z., Iwamoto, L., Gupta, K. P., Earnest, L. E., & Fidler, I. J. (1991) *Biochem. Pharmacol. 41*, 797–806.
Orr, G. A., Han, E. K. H., Browne, P. C., Nieves, E., O'Connor, B. M., Yang, C. P. H., & Horwitz, S. B. (1993) *J. Biol. Chem. 268*, 25054–25062.
Palayoor, S. T., Stein, J. M., & Hait, W. N. (1987) *Biochem. Biophys. Res. Commun. 148*, 718–725.
Picciotto, M. R., Cohn, J. A., Bertuzzi, G., Greengard, P., & Nairn, A. C. (1992) *J. Biol. Chem. 267*, 12742–12752.
Posada, J. A., McKeegan, E. M., Worthington, K. F., Morin, M. J., Jaken, S., & Tritton, T. R. (1989a) *Cancer Commun. 1*, 285–292.
Posada, J., Vichi, P., & Tritton, T. R. (1989b) *Cancer Res. 49*, 6634–6639.
Riordan, J. R., Deuchars, K., Kartner, N., Alan, N., Trent, J., & Ling, V. (1985) *Nature 16*, 817–819.
Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D.-W., Gottesman, M. M., & Pastan, I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4538–4542.
Safa, A. R., Stern, R. K., Choi, K., Agresti, M., Tamai, I., Mehta, N. D., & Roninson, I. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7225–7229.
Sampson, K. E., Wolf, C. L., & Abraham, I. (1993) *Cancer Lett. 68*, 7–14.
Sarkadi, B., Price, E. M., Boucher, R. C., Germann, U. A., & Scarborough, G. A. (1992) *J. Biol. Chem. 267*, 4854–4858.
Sato, W., Yusa, K., Naito, M., & Tsuruo, T. (1990) *Biochem. Biophys. Res. Commun. 173*, 1252–1257.
Schinkel, A. H., Roelofs, M. E. M., & Borst, P. (1991) *Cancer Res. 51*, 2628–2635.
Shinkel, A. H., Kemp, S., Dolle, M., Rudenko, G., & Wagenaar, E. (1993) *J. Biol. Chem. 268*, 7474–7481.
Schurr, E., Raymond, M., Bell, J. C., & Gros, P. (1989) *Cancer Res. 49*, 2729–2734.
Scotto, K. W., Biedler, J. L., & Melera, P. W. (1986) *Science 232*, 751–755.
Shen, D.-W., Fojo, A., Roninson, I. B., Chin, J. E., Soffir, R., Pastan, I., & Gottesman, M. M. (1986) *Mol. Cell. Biol. 6*, 4039–4045.
Skovsgaard, T., & Nissen, N. I. (1982) *Pharmacol. Ther. 18*, 293–311.
Smith, C. D., Glickman, J. F., & Chang, K.-J. (1988) *Biochem. Biophys. Res. Commun. 156*, 1250–1256.
Staats, J., Marquardt, D., & Center, M. S. (1990) *J. Biol. Chem. 265*, 4084–4090.
Sugimoto, Y., & Tsuruo, T. (1987) *Cancer Res. 47*, 2620–2625.
Ueda, K., Cardarelli, C., Gottesman, M. M., & Pastan, I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3004–3008.
Wakusawa, S., Nakamura, S., Tajima, K., Miyamoto, K. I., Hagiwara, M., & Hidaka, H. (1992) *Mol. Pharmacol. 41*, 1034–1038.
Yu, G., Ahmad, S., Aquino, A., Fairchild, C. R., Trepel, J. B., Cowan, K. H., Tsuruo, T., Ohno, S., & Glazer, R. I. (1991) *Cancer Commun. 3*, 181–189.