Protein Kinase C Phosphorylates P-glycoprotein in Multidrug Resistant Human KB Carcinoma Cells*

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Studies were undertaken to identify the protein kinase(s) responsible for P-glycoprotein phosphorylation in multidrug-resistant (KB-V1) human carcinoma cells and to elucidate the functional role of phosphorylation. P-glycoprotein migrated on sodium dodecyl sulfate gels with apparent M, 150,000 and is termed P150. When KB-V1 membrane vesicles were incubated with [γ-32P]ATP, P150 was phosphorylated by an endogenous kinase that exhibited properties of membrane-inserted protein kinase C (PKC). Both membrane-bound P150 and purified P150 served as effective substrates for highly purified rat brain PKC which incorporated approximately 0.6 mol of phosphate/mol of P150. Enzyme assays showed that KB-V1 cells exhibit 4-fold higher PKC activity compared with the drug-sensitive KB-3 cell line. The basal phosphorylation of P150 observed in 32P-labeled cells was increased 2-fold by phorbol ester (PMA) treatment and reduced 30% by treatment with the isoquinolinesulfonamide H-7. Phosphopeptide maps of partially digested P150, phosphorylated either in vitro with PKC or in intact 32P-labeled control or PMA-stimulated cells, were indistinguishable from one another. Drug accumulation assays revealed that PMA treatment of KB-V1 cells significantly reduced [3H]vinblastine accumulation induced by verapamil or by tetrabrandine. The results suggest that PKC is primarily responsible for P150 phosphorylation in KB-V1 cells and that phosphorylation may play a modulatory role in the drug transport process.

The acquisition by tumor cells of resistance to cytotoxic drugs is recognized as a major obstacle to successful clinical chemotherapy. Cultured mammary tumor cells selected for resistance to a single drug display cross-resistance to the effects of other structurally unrelated drugs. This phenomenon of multidrug resistance is characterized by reduced cellular drug accumulation and overexpression of a plasma membrane glycoprotein of 150–170 kDa, termed P-glycoprotein or P170 (for reviews see Refs. 1–4). Based on the deduced amino acid sequence, models of P-glycoprotein predict a highly complex structure containing 12 membrane spanning domains (5, 6). Several lines of evidence suggest that P-glycoprotein acts as an energy-dependent drug efflux pump responsible for maintaining low levels of drug in multidrug-resistant cell lines. This evidence includes (i) amino acid sequence homology exists between P-glycoprotein and a number of bacterial transport proteins, and regions of homology include putative nucleotide-binding sites (5, 6); (ii) P-glycoprotein binds vinblastine, vincristine, and daunomycin (7) and is specifically labeled by radioactive photolabile analogs of vinblastine (8); (iii) certain agents which reverse drug resistance, such as verapamil, inhibit vinblastine binding to P-glycoprotein (9); (iv) sensitive cells acquire the multidrug-resistant phenotype when transfected with the gene encoding P-glycoprotein (10); and (v) treatment of drug-resistant cells with antibodies directed against P-glycoprotein can partially restore drug sensitivity (11).

Although the mechanisms which govern P-glycoprotein function are not known, one potential candidate is regulation by phosphorylation. This post-translational modification is a common and apparently universal feature of P-glycoproteins and has been observed in intact drug-resistant Chinese hamster lung (12), murine macrophage-like (13), human myelogenous leukemia (14), and human KB carcinoma (15) cells. Despite the fact that P-glycoproteins are invariably found as phosphoproteins, little is known of the functional role of phosphorylation or the enzymes involved. Since phosphorylation may modulate or be required for P-glycoprotein function, it is necessary to assess the nature and regulation of the protein kinases responsible. However, few studies have addressed these issues and the currently available information is largely indirect. Enhanced phosphorylation of P-glycoprotein by phorbol ester treatment of K562/ADM cells has been reported (14). This implies that P-glycoprotein can be phosphorylated following protein kinase C activation. Modulation of drug resistance by phorbol ester treatment of human breast cancer cell lines has been demonstrated (16). These changes correlate with increased phosphorylation of a 20-kDa protein(s), but P-glycoprotein phosphorylation was not examined. A possible involvement of protein kinase C in multidrug resistance is also suggested by experiments which show increased enzyme activity in resistant versus sensitive cells (16, 17).

In the present report we provide direct evidence for an involvement of protein kinase C in multidrug resistance by showing that P-glycoprotein, from drug-resistant human KB carcinoma cells, is an effective substrate for this kinase in vitro. We also show that protein kinase C appears to be primarily responsible for P-glycoprotein phosphorylation in intact cells. Preliminary studies suggest that the drug transport properties of P-glycoprotein are stimulated by protein kinase C-mediated phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (3000 Ci/mmol), [3H]azidopine (53 Ci/mmol), [32P]orthophosphate (8 mCi/ml), [3H]vinblastine (23 Ci/mmol), and nonradioactive azidopine were obtained from Amersham

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Coro. Histone III-S, PMA,1 Protein A-Sepharose, cAMP-dependent protein kinase, the inhibitor peptide of cAMP-dependent protein kinase and Staphylococcus aureus V8 protease, were obtained from Sigma. H-7 was purchased from Seikakzu, rat brain protein kinase C from Lipex, wheat germ lectin-agarose from Pharmacia LKB Biotechnology Inc., and Extract-Gel-D from Pierce Chemical Co. Molecular weight standards and the silver nitrate staining kit were from Bio-Rad and prestained molecular weight standards from Bethesda Research Laboratories. Monoclonal antibody C219, developed as described (18), was purchased from Centocore. The heat stable inhibitor protein of cAMP-dependent protein kinase was purified from Staphylococcus aureus and described as previously (19). Tetrandrine, a bis-benzisoxazolino alkaloid calcium entry blocker, was prepared from Stephania tetrandra as described (20).

Cell Culture—The isolation and properties of the multidrug-resistant human KB carcinoma cell lines used in this study have been described in detail (21). The drug-sensitive parental cell line, designated KB-3, was maintained in Dulbecco's modified Eagle's medium (4.5 g/liter glucose) containing 10% fetal bovine serum and 10 mM Hepes, pH 7.3. Drug-resistant KB-V1 cells were maintained in the same medium containing 1 mg/ml of vinblastine.

[c]2-5P]Phosphatase Labeling of Cells—Cells in 150-cm2 culture flasks were incubated for 4 h in 8 ml of phosphate-free medium containing 0.4 mg/ml of [c-32P]phosphate.

Membrane Vesicle Preparation—This was done using the nitrogen cavitation technique essentially as described (27), except that 1 mM PMSF and 0.9 mg/ml aprotinin were added to all the solutions. A typical preparation was from 20 x 24 x 24-cm dishes of confluent cells; cells were suspended in vesicle buffer at 108 cells/ml. After preparation, the vesicle pellet was suspended by 10 strokes in a loose fitting Dounce homogenizer, in 1 ml of 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10 mg/ml protein, and divided into 0.1-ml aliquots which were quick-frozen in dry ice/ethanol and stored at -70°C. For the preparation of membrane vesicles from [c-32P]labeled cells, the same procedure was employed except that cells were suspended at 107 cells/ml in vesicle buffer, and all solutions contained 5 mM dithiothreitol and 20 mM sodium vanadate to inhibit phosphatase activity.

Cell Extraction for Protein Kinase C Activity Determination—Cells which were 80-90% confluent in 150-cm2 culture flasks were used to determine protein kinase C activity. Cells were scraped into ice-cold phosphate-buffered saline and sedimented (1,000 x g, 5 min). For the determination of total cellular protein kinase C, the cell pellet was suspended in 2 ml of buffer A (10 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.2 mM CaCl2, and 1 mM PMSF) and sonicated with a Braun microprobe for 2 x 15 s at half-maximum high setting. EDTA and Triton X-100 (1 mM and 1% (w/v)) final concentrations, respectively) were added, and after gentle mixing for 1 h the suspension was centrifuged (100,000 X g, 60 min). The supernatant was divided into two parts, one for immunoprecipitation and membrane fractions, the cell pellet was suspended in 2 ml of buffer A and disrupted by nitrogen cavitation (100 psi, 15 min). EDTA was added to 1 mM final concentration and nuclei and unbroken cells removed by centrifugation (1,000 x g, 10 min). The supernatant was centrifuged at 100,000 X g for 60 min to obtain a cytosolic fraction (supernatant) and a crude membrane pellet. The pellet was suspended with 10 strokes in a loose fitting Dounce homogenizer, in 1 ml of 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, and 1% (w/v) Triton X-100. After 15 min on ice, insoluble material was removed by centrifugation (100,000 X g, 60 min).

Total cellular extracts, cytosolic, or solubilized membrane fractions were loaded onto 0.3-m columns of DEAE-Sepharocel equilibrated with buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM PMSF). After washing with 5 ml of buffer B, the protein kinase C activity was completely eluted with 2 ml of buffer B containing 100 mM NaCl. Aliquots of 20 ml of the column eluants were assayed for protein kinase C activity as described above (27).

Protein Kinase C Assay—This was performed essentially as described (22). The standard assay mixture (0.1 ml) contained 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.2 mg/ml histone III-S, 50 mM [γ-32P]ATP (specific activity 200 cpm/pmol), 0.2 mM CaCl2, 50 μg/ml phosphatidylserine, and 10 mM phospho-diglyceride (PS) and 10 μg/ml diode. Blank assays contained 0.5 mM EGTA instead of CaCl2, PS, and diode. The reaction was initiated by the addition of [γ-32P]ATP. After 5 min at 30°C, an aliquot of the mixture was applied to a disc of P-81 phosphocellulose paper and after washing in 75 mM H2PO4, radioactivity was determined by scintillation counting.

Affinity Fractionation and Determination of P-glycoprotein by Lectin Chromatography—Solubilization with the detergent CHAPS (29) and conditions of lectin chromatography (24) were based on previously described methods. Briefly, membrane vesicles (3 mg of protein) from KB V1 cells were suspended at 4°C in 0.6 ml of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM PMSF, and 1% (w/v) CHAPS (buffer C). After mixing with a microprobe for 2 x 15 s at half-maximum high setting, the supernatant was centrifuged (100,000 X g, 60 min) and the supernatant applied to a wheat germ lectin agarose column (0.5 ml) equilibrated in buffer C. After 60 min at 4°C, to allow binding of P-glycoprotein to occur, the column was washed with 10 ml of buffer C. Bound material was eluted with buffer C containing 0.5 mM n-acetylglucosamine; 0.4 ml fractions were collected. P-glycoprotein was recovered mainly in the first three fractions which were pooled. Detergent was removed by applying the pooled material to a column (0.2 ml) of Extracti-Gel D equilibrated in 50 mM Tris-HCl, pH 8, 100 mM NaCl, and 1 mM PMSF, and collecting fractions which contained protein.

In Vitro Phosphorylation of P-glycoprotein in Membranes and after Purification—Membrane vesicles from KB-V1 cells (0.1-0.2 mg of protein) were incubated at 30°C in 0.1 ml containing 0.1 mM EGTA, 5 mM MgCl2, and 10 μM [γ-32P]ATP (specific activity, 5000 cpm/pmol). Reactions designed to stimulate protein kinase C included 0.2 mM CaCl2 and 50 μg/ml PS, and those designed to inhibit included H-7 (20 or 100 μM) in addition to CaCl2 and PS. Reactions designed to activate cAMP-dependent protein kinase included 100 μM cyclic AMP, and those designed to inhibit included 5 μg/ml of the heat-stable inhibitor protein or inhibitor peptide of the kinase, in addition to 100 μM cyclic AMP. For studies of the phosphorylation of P-glycoprotein by rat brain protein kinase C, membrane vesicles from rat brain neurons (0.1 mg) and P-glycoprotein (10 μg) purified by lectin chromatography were incubated as described above with or without 50-100 ng or pure rat brain protein kinase C in the absence or presence of 0.2 mM CaCl2 and 50 μg/ml PS. All phosphorylation reactions were terminated by adding EDTA to 10 mM. Analysis of phosphorylated proteins was performed by SDS-gel electrophoresis and autoradiography.

Immunoprecipitation—Extracts were prepared from cell pellets by incubation for 30 min in 10 mM Tris-HCl, pH 7.2, 0.15 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 100 μM cyclic AMP, and those designed to inhibit included H-7 (20 or 100 μM) in addition to CaCl2 and PS. Reactions designed to activate cAMP-dependent protein kinase included 100 μM cyclic AMP, and those designed to inhibit included 5 μg/ml of the heat-stable inhibitor protein or inhibitor peptide of the kinase, in addition to 100 μM cyclic AMP. For studies of the phosphorylation of P-glycoprotein by rat brain protein kinase C, membrane vesicles from rat brain neurons (0.1 mg) and P-glycoprotein (10 μg) purified by lectin chromatography were incubated as described above with or without 50-100 ng or pure rat brain protein kinase C in the absence or presence of 0.2 mM CaCl2 and 50 μg/ml PS. All phosphorylation reactions were terminated by adding EDTA to 10 mM. Analysis of phosphorylated proteins was performed by SDS-gel electrophoresis and autoradiography.

 Electrophoresis—Electrophoresis was performed as described (25). The gels were stained or not with Coomassie Brilliant Blue or silver nitrate. Molecular mass standards were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (92 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). c22P-Labeled proteins were detected by exposure of dried gels to Kodak X-Omat film at -70°C. In some experiments, an intensifying screen was used. For the detection of tritium-labeled proteins, the gel was fixed for 1 h with 8% methanol, dried for 1 h with Enlightening dried, and exposed to x-ray film as above. Prestained protein molecular mass standards were used with the following apparent molecular masses: myosin (194 kDa), phosphorylase b (91 kDa), bovine serum albumin (66 kDa), and ovalbumin (35 kDa).
Protein Kinase C Phosphorylates P-glycoprotein

Identification of P-glycoprotein in Membrane Vesicle Preparations—Membrane vesicles were prepared from drug-resistant KB-V1 and from drug-sensitive KB-3 cells and 100 μg of each preparation analyzed by 6% acrylamide-SDS-gel electrophoresis. A broad band of molecular mass 150 kDa, present in KB-V1 membranes but absent in KB-3 membranes, is readily detected by Coomassie Blue staining of the gel (Fig. 1, panel A). Evidence that the 150-kDa protein detected by staining represents the multidrug resistance P-glycoprotein was provided by analyzing immunoprecipitates of cell extracts treated with the C219 monoclonal antibody, which recognizes a cytoplasmic COOH-terminal domain of P-glycoprotein (18). The antibody recognizes a protein of 150 kDa present in KB-V1 but absent in KB-3 cells (Fig. 1, panel B). Other protein bands evident in both lanes of the gel presumably represent immunoglobulin and nonspecific absorption of cellular proteins to Protein A-Sepharose. Further identification of KB-V1 P-glycoprotein was obtained by photolabeling of membrane preparations with [3H]azidopine. This dihydropyridine calcium channel blocker has been shown to bind with high specificity to P-glycoproteins from several drug-resistant cell lines (29, 31). Analysis by SDS-gel electrophoresis and fluorography (Fig. 1, panel C) revealed in KB-V1 membranes a prominent radiolabeled band of 150 kDa (lane 3). Non-radioactive azidopine competed with [3H]azidopine binding (lane 4). No radiolabeling was observed in proteins from KB-3 membranes (lanes 1 and 2). On the basis of these analyses, the protein we describe which migrates with an apparent molecular mass of 150 kDa (P150) is clearly the same as the 150–170 kDa (P170) multidrug resistance P-glycoprotein from KB-V1 cells described previously (8, 15, 32).

Endogenous Phosphorylation of P150 in Membrane Vesicle Preparations—As a first step toward identifying the type of protein kinases which phosphorylate P150, membrane vesicle preparations from KB-V1 cells were incubated with 10 μM [γ-32P]ATP and 5 mM MgCl₂. In the presence of 1 mM EGTA, P150 was found to be phosphorylated by a membrane-associated protein kinase(s) (Fig. 2, lane 1). In order to identify this endogenous kinase, various protein kinase activators and inhibitors were tested. The phosphorylation of P150 was unaffected by the inclusion of the reaction mixture of 0.2 mM free Ca²⁺ either alone (lane 2) or with 50 μM...
added phosphatidylserine (lane 3). At a concentration of 100 mM, the isouquinolin sulfonamide H-7, an inhibitor of both protein kinase C and cAMP-dependent protein kinase (33), blocked the endogenous phosphorylation of P150 (lane 4). H-7 was also effective at a concentration of 20 µM (not shown). No significant effect on the level of P150 phosphorylation was observed when 100 µM cyclic AMP (lane 5), or specific protein or peptide (lanes 6 and 7) inhibitors of the cAMP-dependent protein kinase were tested. The specificities of the inhibitors were confirmed in assays of purified preparations of cAMP-dependent protein kinase and protein kinase C, using H1 histone as substrate (not shown). The results of Fig. 2 suggest that protein kinase C or a related kinase, and not the cAMP-dependent protein kinase, phosphorylates P150 in isolated membranes.

Phosphorylation of Membrane-bound P150 by Rat Brain Protein Kinase C—We next tested the ability of the membrane-bound form of P150 to serve as a substrate for exogenous protein kinase C. The rat brain preparation we utilized was homogeneous as judged by SDS-gel electrophoresis, which showed a single 80-kDa species (not shown). KB-V1 membrane vesicles were incubated with the rat brain protein kinase C preparation under phosphorylation conditions and products analyzed by SDS-gel electrophoresis. As shown in Fig. 3 (panels A and B), a membrane protein of Mr 150,000 is phosphorylated in a Ca2+/phospholipid-dependent manner. Immunoprecipitation demonstrated that the Mr 150,000 protein is indeed P-glycoprotein (Fig. 3, panel C). It is evident that P150 is the major protein kinase C substrate present. Another substrate of 60 kDa was noted (Fig. 3, panel B, lanes 1–4). Additional, less prominent substrates were revealed by exposing the gel to film for longer periods and by analysis of low molecular weight proteins using 12% acrylamide gels (not shown). Longer exposure times also revealed the same pattern of endogenous protein phosphorylation seen earlier (Fig. 2).

Phosphorylation of Purified P150 by Rat Brain Protein Kinase C—To further characterize the phosphorylation of P150 by protein kinase C and to estimate the stoichiometry of phosphorylation, phosphorylation of the purified protein was examined. Purification of P150 to near homogeneity was achieved by solubilization from KB-V1 membranes using the detergent CHAPS, followed by lectin affinity chromatography (Fig. 4, panel A). Fractions containing purified P150 were pooled and CHAPS removed by Extracti-Gel D chromatography. A time course of phosphorylation of the purified detergent-free preparation with rat brain protein kinase C was performed. The results of one representative experiment (Fig. 4, panel B) show that phosphorylation proceeds rapidly. Most of the 32P is incorporated in the first 15 min of incubation, the remainder over the next 45 min, with no additional incorporation after another 60 min. A stoichiometry of phosphorylation of 0.6 mol of phosphate/mol of P150 was calculated from the 60-min time point of this experiment. Similar values have been obtained in other experiments (n = 3). These substoichiometric values may reflect the fact that P150 is highly phosphorylated when isolated (see below), thus limiting further phosphate incorporation in vitro. Calculations were based on the specific activities of the [γ-32P]ATP, the 32P content of the P150 band excised from gels, and the amount of P150 applied to the gels. The latter value was obtained from the protein concentration and assuming the preparation to be homogeneous.

Protein Kinase C Activity and Distribution in Unstimulated and PMA-stimulated Drug-resistant and Drug-sensitive KB Cells—Since P-glycoprotein serves as a substrate in vitro for protein kinase C, it was of interest to determine and compare the activities of the kinase in drug-resistant versus drug-sensitive KB-V1 cells. The enzyme activity was determined by measuring 32P incorporation into P150 by rat brain protein kinase C. Panel A, purification of P150 from KB-V1 membrane vesicles by CHAPS solubilization and wheat germ lectin chromatography was performed as described under “Experimental Procedures.” Purification was monitored by 6% acrylamide SDS-gel electrophoresis and Coomassie Blue staining. Lane 1, membrane vesicles (50 µg); lane 2, CHAPS extract (50 µg); lane 3, column flow-through and wash (20 µg); lane(s) 4, first four fractions eluted with 0.5 M n-acetyl glucosamine. In order of elution, 5, 14, 6, and 3 µg of protein were loaded in adjacent lanes of the gel. Panel B, time course of phosphorylation of P150. Purified detergent-free P150 (10 µg) was incubated at 30 °C in a reaction mixture of 0.2 ml containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.2 mM CaCl2, 50 µg/ml PS, 100 ng of rat brain protein kinase C, and 0.25 mM [γ-32P]ATP (108 cpm/pmol). Aliquots of 30 µl were removed at intervals from 1 to 120 min and subjected to 6% acrylamide SDS-gel electrophoresis. The stained, dried gel was autoradiographed and the bands corresponding to P150 located and excised and 32P content determined by scintillation counting. The graph shows incorporation of 32P versus time of incubation. The inset shows the autoradiograph of the P150 region of the gel, with time of incubation (min) shown above the corresponding gel lane.

![Fig. 3. Phosphorylation of membrane-bound P150 by rat brain protein kinase C.](image)

![Fig. 4. Phosphorylation of purified P150 by rat brain protein kinase C. Panel A, purification of P150 from KB-V1 membrane vesicles by CHAPS solubilization and wheat germ lectin chromatography was performed as described under "Experimental Procedures." Purification was monitored by 6% acrylamide SDS-gel electrophoresis and Coomassie Blue staining. Lane 1, membrane vesicles (50 µg); lane 2, CHAPS extract (50 µg); lane 3, column flow-through and wash (20 µg); lane(s) 4, first four fractions eluted with 0.5 M n-acetyl glucosamine. In order of elution, 5, 14, 6, and 3 µg of protein were loaded in adjacent lanes of the gel. Panel B, time course of phosphorylation of P150. Purified detergent-free P150 (10 µg) was incubated at 30 °C in a reaction mixture of 0.2 ml containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.2 mM CaCl2, 50 µg/ml PS, 100 ng of rat brain protein kinase C, and 0.25 mM [γ-32P]ATP (108 cpm/pmol). Aliquots of 30 µl were removed at intervals from 1 to 120 min and subjected to 6% acrylamide SDS-gel electrophoresis. The stained, dried gel was autoradiographed and the bands corresponding to P150 located and excised and 32P content determined by scintillation counting. The graph shows incorporation of 32P versus time of incubation. The inset shows the autoradiograph of the P150 region of the gel, with time of incubation (min) shown above the corresponding gel lane.)
sensitive cells. Basal protein kinase C activity was therefore measured in DEAE-Sephadex purified total cellular extracts of KB-V1 and KB-3 cells. The chromatographic step was necessary to remove inhibitors of the enzyme and to separate protein kinase C from other histone III-S utilizing kinases. When normalized to the same number of cells, protein kinase C activity was approximately 4-fold higher in KB-V1 compared with KB-3 cells (Table I). Subcellular fractionation studies revealed that the enzyme activity was elevated both in the cytosolic and in the membrane fractions (not shown). The distribution of protein kinase C activity is similar for both cell lines; in KB-V1, 76% of the enzyme is cytosolic and the remainder membrane-bound, and in KB-3, 70% of the enzyme is cytosolic and the remainder membrane-bound. Translocation of protein kinase C from the cytosol to the membrane in response to phorbol ester treatment occurs in many cell types (34). KB-V1 and KB-3 cells respond similarly; treatment with PMA (200 nM, 10 min) results in rapid translocation of protein kinase C in both cell lines (Table I).

Effects of PMA and H-7 on Phosphorylation of P150 in Intact KB-V1 Cells—We next examined P150 phosphorylation in intact KB-V1 cells metabolically labeled with [32P]orthophosphate and the effects on phosphorylation of protein kinase C modulators. Membrane vesicles were prepared from 32P-labeled cells and subjected to SDS-gel electrophoresis and autoradiography. A prominent 32P-labeled membrane phosphoprotein of 150 kDa present in KB-V1 (Fig. 5, lane 2) but absent in KB-3 (lane 1) was observed, as reported previously (15). The 150-kDa phosphoprotein was identified as P-glycoprotein by its reactivity with the C219 monoclonal antibody (Fig. 5, lane 3) and by its chromatographic properties on wheat germ lectin-agarose (lane 4). The results of one experiment with protein kinase C modulators are shown in Fig. 6. Treatment of 32P-labeled KB-V1 cells with the protein kinase C activator PMA (200 nM, 30 min) resulted in a significant increase in the 32P content of P150 (Fig. 6, lanes 1 and 2). Excision of the P150 bands from the gel and quantitation of 32P by scintillation counting showed that under these conditions PMA stimulates P150 phosphorylation 2-fold. Similar results were obtained when 32P quantitation of immunoprecipitated phosphorylated P150 was performed (see Fig. 8). The PMA-induced augmentation of P150 phosphorylation coincided with translocation of cytosolic protein kinase C to the membrane (Table I). Treatment of 32P-labeled cells with the protein kinase C inhibitor H-7 (20 μM, 16 h) led to a decrease of approximately 30% of basal P150 phosphorylation (Fig. 6, lane 3). When H-7-treated cells were stimulated with PMA, P150 phosphorylation was increased 2-fold relative to H-7-treated unstimulated cells (Fig. 6, lane 4). Thus, despite the presence of H-7, a pool of protein kinase C is apparently available for activation by PMA resulting in renewed phosphorylation of P150. This result also shows that the inhibitory effects of H-7 are not due to depletion of the cellular ATP pools. Essentially the same results as those illustrated in Fig. 6 have been obtained in two additional experiments of this type.

Phosphopeptide Analysis of Phosphorylated P150—To determine whether P150 was phosphorylated at the same site(s) in vivo by protein kinase C and in intact cells, the protein was isolated from in vitro phosphorylation reactions and from control and PMA-stimulated 32P-labeled KB-V1 cells. The phosphorylated P150 preparations were subjected to partial digestion with S. aureus V8 protease and phosphopeptide analysis. Fig. 7 shows that the patterns of phosphopeptides generated were identical in each case. We conclude that protein kinase C appears to be primarily responsible for P150 phosphorylation in intact cells.

Phorbol Ester Treatment Causes Reduced Drug Accumulation—To elucidate the effect of protein kinase C-mediated phosphorylation on P150 function, we examined [3H]vinblastine accumulation in PMA-treated KB-V1 cells. As expected, after incubation with [3H]vinblastine for 30 min, only a low level of drug accumulated in KB-V1 cells (0.6 pmol vinblas-tine/mg cellular protein) (Table II), compared with KB-3 cells (6.5 pmol/mg). Preincubation of KB-V1 cells with PMA (200 nM, 10 min), with continued exposure of cells to PMA during the 30-min incubation with [3H]vinblastine, had no observable effect on base-line drug accumulation. Incubation of cells with 20 μM verapamil led to increased [3H]vinblastine accumulation, to 1.6 pmol/mg, as expected for this compound (28). If cells were exposed to both PMA and verapamil, however, [3H]vinblastine accumulation was reduced to 1.16 pmol/mg.

**Table I**

<table>
<thead>
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<th>Cell line</th>
<th>Protein kinase C activitya</th>
<th>Protein kinase C distributionb</th>
<th>PMA</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>% cytosol/membrane</td>
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<tr>
<td>KB-3</td>
<td>63 ± 14</td>
<td>70/30</td>
<td>17/83</td>
<td></td>
</tr>
<tr>
<td>KB-V1</td>
<td>261 ± 38</td>
<td>96/24</td>
<td>19/81</td>
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a Expressed as pmol phosphate transferred to histone III-S/min/10⁶ cells. Values are mean ± S.E. of triplicate plates of cells.

b Results shown are from a representative experiment where cells were either unstimulated (-PMA) or stimulated with 200 nM PMA for 10 min (+PMA).
Thus, verapamil-induced vinblastine accumulation is reduced 28% by PMA treatment. A very similar result was obtained when we used tetraindrine instead of verapamil. As shown in Table II, this bis-benzylisoquinoline alkaloid Ca\({^2}\) entry blocker potently reverses drug resistance and at a concentration of 5 \(\mu\)M induces a relatively high level of \([^{32}\text{P}]\)vinblastine accumulation (3.53 pmol/mg). When cells were treated with both PMA and tetraindrine, \([^{32}\text{P}]\)vinblastine accumulation was reduced to 2.43 pmol/mg, a 31% decrease compared with the value obtained with tetraindrine alone. The inactive analog phorbol 12,13-diacetate had no effect on verapamil- or tetraindrine-induced \([^{32}\text{P}]\)vinblastine accumulation (not shown).

One explanation for the PMA-mediated decrease in verapamil or tetraindrine-induced vinblastine accumulation is that phosphorylation of P150 by protein kinase C stimulates active drug transport. However, this interpretation is complicated by possible effects of vinblastine, verapamil, or tetraindrine on P150 phosphorylation, particularly verapamil which has been shown to increase P-glycoprotein phosphorylation in K562/ADM cells (14). To address this issue we examined P150 phosphorylation after exposure of cells to these compounds at a dose and duration which corresponded to conditions used in the drug accumulation assays described above. First, KB-V1 cells were maintained in vinblastine-free medium overnight, labeled with \([^{32}\text{P}]\), and were either untreated or treated with PMA (200 nM, 10 min) or vinblastine (30 nM, 30 min). P150 was immunoprecipitated from cellular extracts and analyzed by SDS-gel electrophoresis and autoradiography, as described under "Experimental Procedures." An autoradiograph of the P150 region of the gel is shown. Panel A, cultures of KB-V1 cells were maintained in neither vinblastine-free medium for 16 h and labeled with \([^{32}\text{P}]\) or vinblastine (VBL) (30 nM, 30 min). Control (CONT), treated with PMA (PMA) (200 nM, 40 min) or vinblastine (VBL) (30 nM, 30 min). P150 immunoprecipitated from aliquots containing 100 \(\mu\)g of protein. Immunoprecipitated material was analyzed by SDS-gel electrophoresis and autoradiography, as described under "Experimental Procedures." An autoradiograph of the P150 region of the gel is shown. Panel B, cultures of KB-V1 cells were maintained in medium containing 30 nM vinblastine and labeled with \([^{32}\text{P}]\) (0.4 mCi/dish) for 4 h. Cultures were either untreated (CONT), treated with PMA (PMA) (200 nM, 40 min) or verapamil (VERAP) (20 \(\mu\)M, 30 min) or tetraindrine (TET) (5 \(\mu\)M, 30 min). Analysis of P150 phosphorylation was performed as described in panel A using cell extracts containing 15 \(\mu\)g of protein.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PMA</th>
<th>Verapamil</th>
<th>PMA + Verapamil</th>
<th>Tetraindrine</th>
<th>Tetraindrine + PMA</th>
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<tr>
<td>([^{32}\text{P}])Vinblastine accumulation</td>
<td>0.60 ± 0.14</td>
<td>0.64 ± 0.01</td>
<td>1.60 ± 0.08*</td>
<td>1.16 ± 0.09*</td>
<td>3.53 ± 0.37b</td>
<td>2.43 ± 0.21b</td>
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\* 0.1 > \(p\) > 0.05.

\* 0.01 > \(p\) > 0.001.

\* 0.001 > \(p\).
phosphorylation. In a second experiment, KB-V1 cells were maintained in medium containing 30 nM vinblastine, and 32P-labeled cultures were either untreated or treated with PMA (200 nM, 40 min), verapamil (20 μM, 30 min), or tetrandonine (5 μM, 30 min). Analysis of P150 phosphorylation showed that, compared with PMA, neither verapamil nor tetrandonine significantly altered basal P150 phosphorylation (Fig. 8, panel B).

**DISCUSSION**

The P-glycoproteins overexpressed in drug-resistant cell lines play a pivotal role in the multidrug resistance phenotype but little is known of the mechanisms which regulate their function as drug transporters. Since P-glycoproteins are invariably found as phosphoproteins, it seems likely that conformational modification by phosphorylation contributes to P-glycoprotein function. We therefore investigated P-glycoprotein phosphorylation using the KR-V1 drug-resistant human carcinoma cell line as a model system. In this report we present evidence that protein kinase C phosphorylates P150 in KB-V1 cells. This conclusion is based on the results of studies conducted in vitro and in intact cells.

We found that P150 in isolated membrane preparations is phosphorylated by endogenous protein kinase activity and exploited this property to characterize the kinase(s) (Fig. 2). That endogenous P150 phosphorylation was inhibited by H-7 but not by specific inhibitors of cAMP-dependent protein kinase is suggestive of protein kinase C-catalyzed phosphorylation. However, the endogenous phosphorylation was not dependent on Ca++. In a series of experiments, Bassi and Nelsestuen (35, 36) have shown that membrane-inserted protein kinase C does not require Ca++ for activity. Therefore, the lack of Ca++ dependence of P150 phosphorylation in membranes is not inconsistent with a protein kinase C-catalyzed reaction. Furthermore, solubilization of KR-V1 membranes with 1% (w/v) Triton X-100, followed by DEAE-Sephacel chromatography, allowed direct demonstration of the presence of intact protein kinase C (Table I). Collectively, these results imply that P150 can serve as a substrate for constitutively active protein kinase C in membrane vesicle preparations from KB-V1 cells. Other reports have described endogenous P-glycoprotein phosphorylation in membrane preparations from drug-resistant cells (12, 24, 37). In membranes from the J7-Cl-100 line (24), stimulation of P-glycoprotein endogenous phosphorylation by 10 μM cAMP, at concentrations of MgCl₂ up to 5 mM, was observed, suggesting an involvement of the cAMP-dependent protein kinase. This is in contrast to the results we obtained (Fig. 2, lanes 5–7), perhaps reflecting differences in the type(s) of protein kinase present in the different membrane preparations.

Studies with highly purified rat brain protein kinase C confirmed that P150 serves as a substrate (Figs. 3 and 4). Indeed, membrane-bound P150 was found to be a major substrate for the exogenous kinase. Phosphorylation by added protein kinase C was dependent on the presence of Ca++ and phospholipid. Purified P150 was also found to be an effective substrate for protein kinase C provided the detergent CHAPS was removed prior to phosphorylation. The presence of detergents such as CHAPS has hampered phosphorylation studies of other membrane-solubilized protein kinase substrates, such as the dihydrodipyrindine-sensitive Ca++ channel (38). Phosphorylation of purified detergent free P150 by rat brain protein kinase C was rapid with a stoichiometry of approximately 0.6 mol of phosphate/mol protein. This relatively low value was reproducibly obtained and may be due to the fact that P150 is highly phosphorylated when isolated, as indicated by the 32P-labeling experiments (Figs. 5, 6, and 8). Therefore, phosphate incorporation by protein kinase C in vitro would be limited to those P150 molecules which have available sites for phosphorylation. Experiments are in progress to determine the stoichiometry of phosphorylation of purified preparations of P150 which have been treated with alkaline and protein phosphatases.

Evidence for an involvement of protein kinase C in P150 phosphorylation in intact cells was obtained by demonstrating that the protein kinase C modulators PMA and H-7 alter the state of phosphorylation of P150 in 32P-labeled cells. Treatment of cells with 20 μM H-7 resulted in modest (30%) but reproducible inhibition of P150 basal phosphorylation. P150 phosphorylation was increased 2-fold by exposure of cells to 200 nM PMA for 30 min. This result confirms that of Hamada et al. (14) who were the first to implicate protein kinase C by reporting phorbol ester stimulated phosphorylation of P-glycoprotein in K562/ADM cells. Furthermore, phosphopeptide maps of P150 phosphorylated in vitro and in intact cells, without or with PMA treatment, were identical. The results of the phosphopeptide analysis in particular, together with the other results described above, are fully consistent with the notion that protein kinase C is primarily responsible for P-glycoprotein phosphorylation in intact KB-V1 cells.

Based on measurements of enzyme activity, protein kinase C has been shown to be elevated 7-fold in a drug-resistant MCF-7 cell line (16) and 2-fold in drug-resistant HL-60 cells (17), compared with the respective drug-sensitive cells. We found an approximately 4-fold increase in enzyme activity in KD-V1 compared with KD-3 cells. Thus, increased protein kinase C activity may be a general phenomenon associated with multidrug resistance. In view of the findings presented in this paper, it is tempting to speculate that multidrug-resistant cells require elevated protein kinase C activity to maintain suitable phosphorylation levels of the overexpressed drug transport P-glycoproteins.

The most intriguing question raised by our and other studies concerns the function of P-glycoprotein phosphorylation. Presumably, phosphorylation modulates in some way the ability of P-glycoprotein to transport drugs. We therefore studied drug accumulation in KB-V1 cells and the effect of phorbol ester, under conditions which we showed resulted in protein kinase C activation (Table I) and enhanced phosphorylation of P150 (Figs. 6 and 8). It was found that phorbol ester treatment significantly reduces the accumulation of [3H] vinblastine induced by either verapamil or tetrandonine, two structurally unrelated Ca++ channel blockers which display resistance reversal properties (Table II). Control experiments ruled out possible influences on basal P150 phosphorylation of these two compounds, as well as vinblastine (Fig. 8). The results of Table II are strikingly similar to those reported previously describing the effects of phorbol ester on vincristine and doxorubicin accumulation in multidrug-resistant human breast cancer cells (16). Phorbol esters therefore increase drug resistant in two independent multidrug-resistant cell lines. These studies provide evidence for a modulatory role of protein kinase C in the drug transport process. Whether stimulation of drug transport occurs as a direct consequence of phosphorylation remains to be established but the evidence presented here would support such a hypothesis. In this regard it is interesting to note that a number of transport and secretion processes in cells are known to be regulated by protein kinase C-catalyzed phosphorylation of the component proteins (34). Phosphorylation may also influence the ATPase activity found associated with P-glycoprotein from K562/
ADM cells (23) and KB-V1 cells. In contrast to observations made in K562/ADM cells describing verapamil-induced phosphorylation of P-glycoprotein (14), verapamil did not significantly alter levels of P150 phosphorylation in KB-V1 cells. Tetrandrine, which we discovered is a potent inhibitor of drug transport, was similarly without effect. These findings serve to emphasize two points of importance. First, the mechanism by which verapamil inhibits drug transport may differ in multidrug-resistant cell lines of divergent origin. Second, alteration in the levels of P-glycoprotein does not necessarily occur in response to agents which inhibit transport. Clearly, more needs to be learned of the functional significance of phosphorylation of P-glycoprotein and the contribution of inhibitors of transport to this pathway. On the basis of the results presented here, we anticipate that further research in these areas may result in the identification of novel inhibitors of the multidrug transporter.

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