GTP-Stimulated Phosphorylation of P-Glycoprotein in Transporting Vesicles from KB-V1 Multidrug Resistant Cells

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ABSTRACT: We have previously shown that GTP can replace ATP as an energy source to support vinblastine transport by the multidrug transporter P-glycoprotein (Pgp) in plasma membrane vesicles isolated from the multidrug resistant cell line KB-V1 [Lelong *et al.* (1992) *FEBS Lett. 304,* 256–260]. Like [γ-^{32}P]ATP, [γ-^{32}P]GTP was also able to phosphorylate Pgp *in vitro*. Unlabeled GTP enhanced the phosphorylation of the transporter by [γ-^{32}P]ATP, whereas unlabeled ATP inhibited incorporation of label. While phosphorylation by [γ-^{32}P]ATP was Mg^{2+}-dependent, the enhanced phosphorylation of Pgp by GTP was supported by Mg^{2+} or Mn^{2+} and to a lesser extent, Ca^{2+}. Specific inhibitors of cAMP-dependent protein kinase, protein kinase C and cGMP-dependent protein kinase, did not affect phosphorylation. The phosphoprotein phosphatase inhibitor okadaic acid slightly enhanced phosphorylation, and vanadate more dramatically increased phosphorylation of the transporter. Tryptic maps of Pgp phosphorylated peptides indicate that addition of GTP altered the relative labeling of phosphopeptides. These results suggest that the overall phosphorylation of Pgp *in vitro* is determined by several different protein kinases and phosphatases, at least one of which may be GTP-regulated.

The acquisition by mammalian cells of cross-resistance to structurally unrelated chemotherapeutic drugs is often achieved by overexpression at the cell surface of a 150–170-kDa membrane phosphoglycoprotein referred to as P-glycoprotein (Pgp), P170, or the multidrug transporter [see Gottesman and Pastan (1988) and Endicott and Ling (1989)]. The transfection of drug-sensitive cells with a full-length cDNA encoding Pgp confers pleiotropic resistance toward hydrophobic drugs such as *Vinca* alkaloids, anthracyclines, and other natural product cytotoxic drugs. Cells expressing this multidrug resistance (MDR) phenotype are characterized by decreased accumulation of the chemotherapeutic agents due to energy-dependent drug efflux driven by the ATPase activity of Pgp.

Since the demonstration of the incorporation of phosphate in the multidrug transporter in many different cell lines of human and of murine origin (Carlsen *et al.*, 1977; Center, 1983, 1985; Garman *et al.*, 1983; Hamada *et al.*, 1987), several authors have asked whether the transport capacity of Pgp is modulated by phosphorylation. Previous studies suggested that increased phosphorylation of the transporter correlated with increased drug efflux from MDR cells (Center, 1983, 1985). Mellado and Horwitz have shown that phosphorylation occurring *in vitro* was partly due to protein kinase A (PKA) (Mellado & Horwitz, 1987). However, a growing body of evidence implicates protein kinase C (PKC) in phosphorylation of Pgp since several groups have reported increased PKC activity in MDR cells compared to their sensitive counterparts (Fine *et al.*, 1988; Chambers *et al.*, 1990; Hayes & Wolf, 1990). In addition, in MCF-7 cells and other cell types, both multidrug resistance and phosphorylation were increased in response to phorbol ester treatment (Hamada *et al.*, 1987; Fine *et al.*, 1988; Chambers *et al.*, 1990; Ido *et al.*, 1986; Ferguson & Chen, 1987). Furthermore, protein kinase inhibitors such as H7 and staurosporine, more or less specific for PKC, also chemosensitize MDR cells (Dong *et al.*, 1991). Chambers *et al.* (1990) showed that Pgp undergoes PKC-stimulated phosphorylation in a cell-free system consisting of isolated KB-V1 plasma membrane vesicles. In response to phorbol ester treatment, the translocation of PKC to a membrane-bound form *in vivo* was correlated with an increase in Pgp phosphorylation and a decrease in drug accumulation (Chambers *et al.*, 1990). Recently it was demonstrated that the transfection of cDNAs for PKCα or PKCβ can confer increases in multidrug resistance (Yu *et al.*, 1991; Fan *et al.*, 1992). Other phospholipid-interacting drugs with chemosensitizing effects, such as phenothiazines and cyclosporin A, have an inhibitory effect on PKC activity (Mori *et al.*, 1980; Schatzman *et al.*, 1981; Ford *et al.*, 1990; Aftab *et al.*, 1991). However, these effects are likely due to competitive inhibition of the transporter itself. Furthermore, increases in phosphorylation of Pgp can be stimulated not only by phorbol esters but also by some agents which reverse MDR (Hamada *et al.*, 1987).

When considering these studies, it should be kept in mind that phosphorylation-mediated regulatory phenomena are complex processes involving a cascade of events which could affect the activity of the transporter directly or indirectly. Furthermore, as noted, many of the phosphorylation inhibitors used in these studies may themselves be substrates for transport by Pgp, and could inhibit Pgp activity directly. To begin to address these concerns, we have studied phosphorylation of Pgp in a plasma membrane vesicle system which is known to be active in energy-dependent drug transport (Horio *et al.*, 1988; Lelong *et al.*, 1992). Both ATP and GTP stimulate transport in such a system (Lelong *et al.*, 1992). This result led us to screen for differential phosphorylation of Pgp in the presence of ATP or GTP or of a combination of both. The

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results reported here show that GTP supports the phosphorylation of Pgp, that GTP stimulates phosphorylation by ATP, and that the requirements for phosphorylation and the patterns of phosphorylation observed with ATP and GTP are different.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (~6000 Ci/mmol), [γ-^{32}P]GTP (~5000 Ci/mmol), [γ-^{35}S]ATP (>1000 Ci/mmol), and [^{3}H]-azidopine were purchased from Amersham. Staurosporine, Calphostin C (PKC inhibitor, IC_{50} = 0.05 μM), KT5720 (PKA inhibitor, K_i = 0.056 μM), and KT5823 (cGMP-dependent protein kinase inhibitor, K_i = 0.234 μM) were purchased from Kamiya Biomedical Co. Okadaic acid, protein kinase C inhibitor peptide [PKC(19–36), K_i = 0.15 μM], and protein kinase A inhibitor peptide [PKI(6–22)amide, K_i = 1.7 nM] were from GIBCO BRL. Another cAMP-dependent protein kinase inhibitor (synthetic peptide, rabbit sequence: P3294), phosphoserine, phosphothreonine, phosphotyrosine, ninhydrin spray, constant-boiling HCl, aprotinin, and trypsin-TPCK were obtained from Sigma. Thin-layer PEI–cellulose and cellulose chromatography plates were from Merck. Protein A–Sepharose was purchased from Pharmacia LKB Biotechnology.

^{14}C-Radiolabeled molecular weight standards (200 000–14 300) were purchased from GIBCO BRL; cold prestained standards were either from Amersham (Rainbow TM 200 000–14 300) or from Bio-Rad (205 000–49 500). Monoclonal antibody C219 was a generous gift from Centocor, and polyclonal rabbit antibody 4007 was prepared as described (Tanaka *et al.*, 1990). Both antibodies react with Pgp. Polyclonal rabbit IgG anti-phosphotyrosine was from UBI. Polyclonal rabbit anti-EGF receptor antiserum 2313 was generously provided by Dr. A. Johnson, NCI. Nonradioactive vinblastine and nucleotides were purchased from Sigma.

Cell Culture. The isolation and properties of the parental and the multidrug resistant human KB carcinoma cell lines used in this study have been described in detail (Akiyama *et al.*, 1985; Shen *et al.*, 1986). The parental drug-sensitive cell line KB-3-1 was maintained in Dulbecco's modified Eagle's medium (4.5 g of glucose/L) supplemented with 10% fetal bovine serum. The drug-resistant KB-V1 and KB-C1 cell lines were maintained in the same medium containing 1 μg/mL vinblastine or colchicine, respectively.

Isolation of a Purified Fraction of Plasma Membrane Vesicles. Plasma membrane vesicles were isolated from KB-V1, KB-C1, and KB-3-1 cells according to previously described protocols, with some modifications (Lever, 1977; Cornwell *et al.*, 1986). After preparation, the vesicle pellet was suspended in TSNa buffer (10 mM Tris, 250 mM sucrose, and 50 mM NaCl, pH 7.45) at a concentration of 2–4 mg/mL protein and divided into aliquots which were quick-frozen and stored at –80 °C. Typically, 12–18 mg of purified plasma membrane proteins was recovered from 60 plates of cells grown on 15 cm tissue culture dishes.

[^{3}H]Azidopine Labeling of KB-V1 Plasma Membrane Vesicles. Photoaffinity labeling was performed as described (Yang *et al.*, 1989). Competition of [^{3}H]azidopine photolabeling by drugs or by possible substrates for the transporter (including the inhibitors of kinases or phosphatases) was achieved by preincubating the vesicles with these agents (10^{-5} M range) for 10 min at room temperature, prior to labeling with azidopine. Radiolabeled vesicles were subjected to SDS–PAGE and autoradiography as described below.

In Vitro Phosphorylation of P-Glycoprotein in Plasma Membrane Vesicles. Phosphorylation was carried out at 25 °C in 10 mM Tris-HCl, 0.25 M sucrose, and 50 mM NaCl, pH 7.5, containing nucleotide/$MgCl_2$ in a 1:2 ratio (except for the low concentrations of nucleotide where the $MgCl_2$ concentration was higher, as noted in the figure legends) with 100–200 μg of plasma membrane vesicles. Unless otherwise mentioned, no vanadate was added to the incubation media. When kinase or phosphatase inhibitor studies were run, the vesicles were preincubated 10 min at room temperature with the appropriate kinase or phosphatase inhibitor prior to adding nucleotide(s)/$MgCl_2$. The phosphorylation reaction was started by the addition either of [γ-^{32}P]ATP or [γ-^{32}P]GTP or of [γ-^{35}S]ATP in the presence of either cold ATP/$MgCl_2$ or cold GTP/$MgCl_2$. For the studies on the effect of divalent cations on Pgp phosphorylation, the nucleotides were not stabilized by $MgCl_2$ but by the chloride salts of the cation to be tested. The final volume was 100 μL. All phosphorylation reactions were stopped after 10 min by the addition of EDTA (25 mM final concentration), and the samples were pelleted at maximum speed in a 5414 Eppendorf microcentrifuge followed by immediate solubilization in Laemmli's sample buffer (Laemmli, 1970), heated at 37–40 °C for 20–30 min, and either immediately run on SDS–PAGE or quick-frozen in a dry ice/methanol slurry for subsequent storage at –80 °C.

Immunoprecipitation. Cold or radiolabeled membrane vesicles (100–200 μg) from KB-V1 and KB-3-1 cells were solubilized in 0.5 mL of RIPA buffer (20 mM Tris, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate sodium salt, 0.1% SDS, 1 mM EDTA, and 1% aprotinin, pH 7.2). Extracts were mixed with 10 μL of C219 (1 mg/mL) or 10 μL of rabbit antiserum 4007 overnight at 4 °C with constant gentle mixing; then 0.1 mL of protein A–Sepharose (PAS, 25% v/v in PBS) was added, and extracts were mixed for an additional 2 h. Beads were collected and washed once with 1 mL of RIPA buffer and once with 1 mL of RIPA–2.5 M KCl followed by another wash with 1 mL of RIPA. P-Glycoprotein was dissociated from the bead-PAS-antibody complex by incubating the beads either with Laemmli sample buffer (Laemmli, 1970) or with 500 μL of elution buffer (2% SDS, 100 mM Tris, and 5% β-mercaptoethanol, pH 7.5). The samples in elution buffer were concentrated by addition of an equal volume of acetone at –20 °C, in the presence of 0.1 volume of 50% sucrose, and precipitated overnight. The samples were then centrifuged, and the pellets containing P-glycoprotein were speed-vacuum-dried (Savant centrifuge). For tryptic mapping or phosphoamino acid analysis, three to four acetone precipitates of immunoprecipitated P-glycoprotein were pooled for one lane of SDS–PAGE by solubilization in Laemmli sample buffer (Laemmli, 1970) and processed further as described below. Supernatants of beads, prior to the first wash with RIPA, were acetone-precipitated as described below, and subjected to SDS–PAGE to verify the complete immunoprecipitation of Pgp.

SDS–PAGE. Electrophoresis on 7.5% SDS–PAGE gels was performed as described by Laemmli (1970), except that the samples were not boiled but heated for 30 min at 40 °C. After electrophoresis, the gels were either fixed in 2-propanol/acetic acid/water (25:10:65) and dried at 80 °C on Whatman 3M paper under vacuum suction or stained with Coomassie blue R-250 prior to drying. The gels for phosphopeptide or phosphoamino acid analysis were immediately dried, without fixing. For the detection of ^{35}S-labeled proteins, the gels were additionally soaked for 15 min in Amplify (Amersham) after fixing and immediately dried. The [^{14}C]protein molecular weight standards and the ^{32}P- or ^{35}S-labeled phosphoproteins were detected by exposure of the dried gels to Kodak X-ray

RP2 films at −80 °C. In some experiments, intensifying screens (Dupont Cronex Lightening Plus) were used.

Electrotransfer. Western blotting was performed as described (Towbin *et al.*, 1979) on PVDF membranes (0.22 μm, Bio-Rad) with a methanol concentration in the transfer buffer of 15%. Immunodetection of P-glycoprotein was done either with monoclonal antibody C219 or with polyclonal 4007 antiserum using a Vectastain ABC kit with the biotinylated horseradish peroxidase/avidin system (Vector Laboratories) followed by diaminobenzidine/$NiCl_2$/H_2O_2 visualization. In some analyses, the ECL detection system (Amersham) was used.

Acidic SDS–PAGE. The detection of acyl phosphate as a phosphorylation intermediate was performed according to Sarkadi *et al.* (1986) with some modifications. After phosphorylation was performed at 4 °C for 2 min, the reaction was stopped by the addition of 9 volumes of ice-cold Tris–phosphate buffer containing 25 mM EDTA and centrifuged at maximum speed (~12000*g*) in a 5414 Eppendorf microcentrifuge at 4 °C. The pellet was resuspended in the following buffer: 0.15 M Tris, 5 mM EDTA, 30% sucrose, 2% SDS, and 5% 2-mercaptoethanol buffered to pH 5.5 with phosphoric acid, incubated at 37 °C for 10 min, and chilled in an ice/water slurry before loading at 4 °C into the wells of the stacking gel containing 4% acrylamide (Protogel: 40:1), 65 mM Tris–phosphate, pH 5.5, and 0.1% SDS (gels equilibrated in the cold). The running gel was a 6.5% gel [40:1 acrylamide:bis-(acrylamide)ratio] containing 65 mM Tris–phosphate/0.1% SDS, pH 6.5. The reservoir buffer contained 0.17 M MOPS adjusted to pH 5.5 with phosphoric acid, and 0.1% SDS. The samples were run for 3 h at 4 °C with a starting current of 40 mA. For acyl phosphate analysis, the gels were fixed as described above, covered with cellophane, clamped on a glass plate, and allowed to dry for 2 days at room temperature. The molecular weights of the membrane proteins were estimated by comparison with the mobilities of standard proteins run on the same gel. Autoradiography of the gels was performed at room temperature with Kodak X-Omat X-ray films.

Phosphopeptide Analysis (Tryptic Map). The methods described by Boyle *et al.* (1991) were used with some modifications. Samples of immunoprecipitated radiophosphorylated P-glycoprotein (labeled in the presence of 10 μM vanadate) were pooled (3–4 immunoprecipitates deriving each from 200 μg of purified plasma membrane/lane), and P-170 was resolved from other coprecipitated peptides by electrophoresis on SDS–PAGE. The P-glycoprotein in the unfixed dried gel was located by autoradiography at −80 °C, and the radioactive bands containing the radiolabeled protein were excised from the gel. Every gel slice was allowed to swell in fresh 50 mM NH_4HCO_3 buffer, pH 8, and the paper backing was peeled off. Gel slices were equilibrated in 1 mL of 50 mM NH_4CO_3 (pH 8 with NH_4OH) at room temperature and crushed to a slurry, and 5 μL of 10 mg/mL L-1-(tosylamino)-2-phenylethyl chloromethyl ketone treated (TPCK)-trypsin was added and incubated at 37 °C overnight. The digestion was repeated with four more additions of the same amount of TPCK-trypsin every 12 h. After incubation, the supernatants from several gel pieces were combined and dried in a speed-vacuum centrifuge (Savant). Dried pellets were suspended in twice-distilled water and were cleared by ultracentrifugation on Millipore ultra-free-MC low binding 5 μm filters. The digested Pgp was redried and dissolved several times in decreasing volumes of twice-distilled water. Radioactivity was estimated by Cerenkov counting. The final pellet was dissolved in 1% ammonium bicarbonate, pH 8.9, and spotted on a cellulose thin-layer plate without fluorescence indicator (20 × 20 cm) 3 cm from one edge of the plate, halfway between the adjacent edges. Thin-layer electrophoresis (TLE) was performed in the horizontal dimension on an LKB–Pharmacia electrophoresis tank at 1000 V (80 mA at start) for 1 h under cooling (8 °C), in 1% ammonium bicarbonate, pH 8.9. The plates were allowed to dry at room temperature overnight. Electrophoresed peptides were further resolved by chromatography in the second dimension (14 cm from the application spot) in butanol/pyridine/acetic acid/water (15:10:3:12, v/v). Labeled peptides were identified by autoradiography. Typically, trypsinized pellets derived from 12–20 pooled/processed immunoprecipitates of *de novo* phosphorylated Pgp are necessary to produce a tryptic map.

Phosphoamino Acid Analysis. Phosphopeptides of radiolabeled immunoprecipitated Pgp extracted from SDS–PAGE gels according to the protocol described above were hydrolyzed in constant-boiling HCl for 2 h at 110 °C in clamped Eppendorf tubes in a heating block (Cooper *et al.*, 1982). The hydrolysate was speed-vacuum-evaporated to dryness, and the phosphoamino acids were adsorbed on Dowex AG 1X8 resin and eluted with 0.1 N HCl as previously described (Cooper *et al.*, 1982). The eluted sample was freeze-lyophilized and further redissolved in twice-distilled water several times. Radioactivity was estimated by Cerenkov counting. Finally, the sample was taken up in 20 μL of electrophoresis buffer [pyridine/acetic acid/water (1:10:189, v/v), pH 3.5] containing phosphoserine, phosphothreonine, and phosphotyrosine as internal standards. Phosphoamino acids were spotted 3 cm from the edge of a cellulose thin-layer plate without fluorescence indicator (20 × 20 cm) and separated by monodirectional electrophoresis at 1000 V (~20 mA) for 1 h. Markers were visualized with ninhydrin spray, and ^{32}P-labeled phosphoamino acids were visualized by autoradiography.

Nucleotide Analysis by Thin-Layer Chromatography. Nucleotide analysis was as described by Cashel *et al.* (1969). Because several samples were processed in parallel, a one-dimensional chromatography system was used. The radiophosphorylated KB plasma membrane vesicles were extracted in 400 μL of chloroform/methanol 2:1 (mixture kept in dry ice); 100 μL of the aqueous upper phase containing the radiolabeled and cold nucleotides was quick-frozen in a slurry of methanol/dry ice. Each sample was thawed immediately prior to spotting 5 μL on a poly(ethylenimine) (PEI)–cellulose thin-layer plate which was prewashed by ascending development with twice-distilled water, dried, and stored at 4 °C. One-dimensional chromatography was in 0.85 M KH_2PO_4, pH 3.4. When a higher resolution of the pyrimidine triphosphates was required, an additional formate step was employed as described (Cashel *et al.*, 1969).

Protein Concentration. Protein was measured by the method of Bradford (1976) (Bio-Rad protein kit) with bovine serum albumin as standard.

RESULTS

Pgp in Transporting Vesicles Is Phosphorylated in Vitro by [γ-^{32}P]ATP. Plasma membrane vesicles capable of transporting [^3H]vinblastine (see below) were prepared from drug-resistant KB-V1 cells. Nontransporting vesicles from the drug-sensitive KB-3-1 cells were used as a control. Plasma membranes from both cell lines were phosphorylated with [γ-^{32}P]ATP, as described under Experimental Procedures, and either analyzed directly on SDS–PAGE gels or immunoprecipitated before electrophoresis, followed in most cases by Western blot analysis. The pattern of radiophosphorylated proteins obtained from KB-V1 and KB-3-1 vesicles after immunoprecipitation with anti-Pgp antisera shows that the

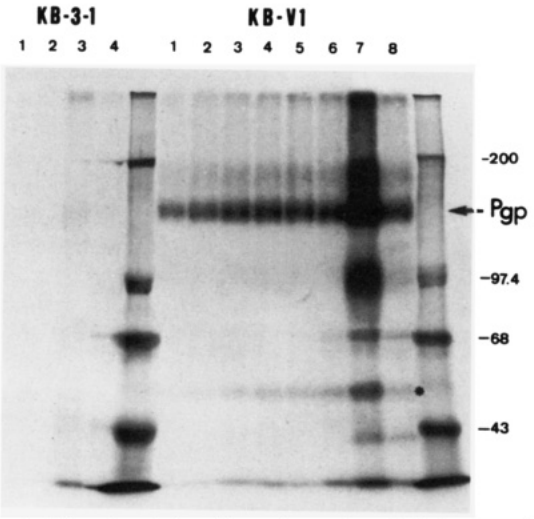

FIGURE 1: Phosphorylation of membrane-bound P-glycoprotein by [γ-^{32}P]ATP *in vitro*. Plasma membranes (100 μg) of KB-3-1 and KB-V1 cells were incubated for 10 min at room temperature in the presence of 10 μCi of [γ-^{32}P]ATP, in a final volume of 100 μL. Reactions were performed in the presence of cold nucleotides as follows: KB-3-1: GTP, 9 μM (lane 1) and 3.0 mM (lane 2); ATP, 9 μM (lane 3) and 3.0 mM (lane 4). KB-V1: ATP, 3.0 mM (lane 1), 1.5 mM (lane 2), 0.30 mM (lane 3), 0.15 mM (lane 4), 30 μM (lane 5), and 9 μM (lane 6); GTP, 3.0 mM (lane 7) and 9 μM (lane 8). Pgp was processed as described under Experimental Procedures. The molecular masses of ^{14}C-labeled standard proteins (in kilodaltons) are indicated on the right side. The results shown are representative of at least three separate experiments. The star indicates the 55-kDa proteolytic fragment of Pgp.

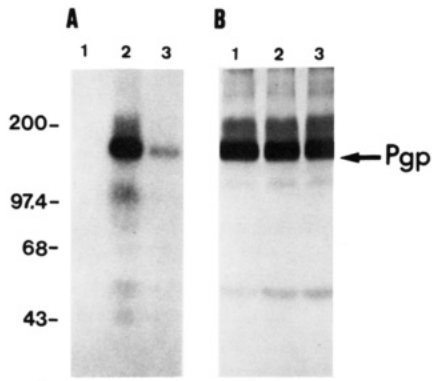

FIGURE 2: Effect of staurosporine and vanadate on phosphorylation of Pgp by [γ-^{32}P]ATP *in vitro*. Plasma membrane vesicles from KB-V1 cells (100 μg) were labeled in the presence of 30 μCi of [γ-^{32}P]ATP as described under Experimental Procedures, in the absence (control, lanes 3) or in the presence of either 10 μM staurosporine (lanes 1) or 10 μM vanadate (lanes 2) followed by immunoprecipitation (antiserum 4007) and Western blotting. Panel A shows autoradiography of the C219-immunostained blot which is shown in panel B. Results shown are representative of at least four separate experiments. The arrow shows the position of Pgp.

170-kDa Pgp and its 55-kDa fragment were present in membranes isolated from KB-V1 cells (Figure 1, panel KB-V1, lanes 1–6), but not in membranes from KB-3-1 (Figure 1, panel KB-3-1, lanes 3 and 4). This was further confirmed by Western blot analysis with antibody C219 of the immunoprecipitated radiophosphorylated 150- and 55-kDa peptides (Figure 2, panels A and B) which shows that Pgp and its 55-kDa fragment are the major phosphorylated polypeptides in these immunoprecipitates. The converse experiment, e.g., immunoprecipitation with C219 followed by immunoblotting with 4007, gave the same results, with the exception that 4007 was more efficient for immunoprecipitation (data not shown), and has therefore been chosen for processing the samples in most of the experiments. When KB-V1 membrane vesicles were incubated in the presence of [γ-^{32}P]ATP and increasing concentrations of cold ATP, a decrease in Pgp phosphorylation was observed (Figure 1). Interestingly, strong Pgp phosphorylation was observed when KB-V1 membrane vesicles were incubated in the presence of [γ-^{32}P]ATP and cold GTP (Figure 1, lane 7). Under similar conditions, no Pgp labeling was observed with KB-3-1 membrane vesicles.

Figure 2 also shows that in the presence of staurosporine, a broad-spectrum protein kinase inhibitor, no phosphorylation of the 150- and 55-kDa peptides was observed (Figure 2, panel A, lane 1). In contrast, vanadate, a metabolic inhibitor with pleiotropic effects including an inhibitory effect on phosphatases and on the ATPase activity of the transporter itself (Ambudkar *et al.*, 1992), strongly increased the phosphorylation of the transporter. Supplementation of the incubation medium with cAMP (100 μM) did not change the phosphorylation of Pgp in the presence of either radiolabeled ATP alone or in combination with 30 μM cold ATP, suggesting that PKA is not involved in this phosphorylation event. These results suggest the involvement of kinases and/or phosphatases in the phosphorylation of Pgp *in vitro*, but do not identify the responsible enzymes.

These results identify the radiophosphorylated protein of apparent molecular mass 150 kDa as the multidrug transporter (Pgp) and the 55-kDa peptide as its fragment (Bruggemann *et al.*, 1989). A 10 min incubation time corresponding to the plateau of Pgp phosphorylation *in vitro*, in the presence of [γ-^{32}P]ATP or of [γ-^{32}P]GTP, was chosen in order to be able to validly compare the results obtained with the different batches of vesicles.

De Novo Phosphorylation of the Transporter by [γ-^{32}P]-GTP in KB-V1 Plasma Membrane Vesicles. In a previous study, we reported that GTP could, to some extent, substitute for ATP to drive vinblastine transport but had neither a synergistic nor an additive effect on drug transport by KB-V1 plasma membrane vesicles (Lelong *et al.*, 1992). We speculated that GTP might act on the transport process both as a replacement source for ATP and as an allosteric effector. GTP has also been tested for its ability to phosphorylate the transporter *in vitro*. KB-V1 plasma membrane vesicles were incubated in the presence of [γ-^{32}P]GTP, and Pgp was immunoprecipitated and electrophoresed as described under Experimental Procedures. The results shown in Figure 3 (lanes 1–7) show that Pgp is also phosphorylated by GTP alone. Under our experimental conditions, the time course of phosphorylation in the presence of [γ-^{32}P]GTP showed an increase of Pgp radiolabeling with a maximum reached at 5–10 min (data not shown). Figure 3 also shows that when the concentration of cold GTP was increased (lanes 1–6, with [GTP] lowest in lane 6 and highest in lane 1), an increase in the radiolabeling of Pgp by [γ-^{32}P]GTP was observed. However, when the incubation was performed in the presence of increasing concentrations of cold ATP, radiolabeling of Pgp decreased (Figure 3, lanes 8–13, with [ATP] lowest in lane 13 and highest in lane 8). These results suggest either a dramatically different K_m for GTP vs ATP stimulation of Pgp or a complex interaction of ATP and GTP with kinases and phosphatases present in the vesicle preparation. However, the fact that Pgp is phosphorylated by [γ-^{32}P]GTP suggests the involvement of a kinase either specifically driven by this nucleotide or capable of using it as an alternative for ATP.

GTP Increases the Phosphorylation of Pgp by [γ-^{32}P]ATP. The radioactivity of Pgp and its 55-kDa fragment, immunoprecipitated from vesicles incubated in the presence of a combination of radiolabeled ATP and 3.0 mM cold GTP, was considerably higher than with cold ATP (30 μM) alone (Figure 1, panel KB-V1, lanes 7 and 5, respectively). No phos-

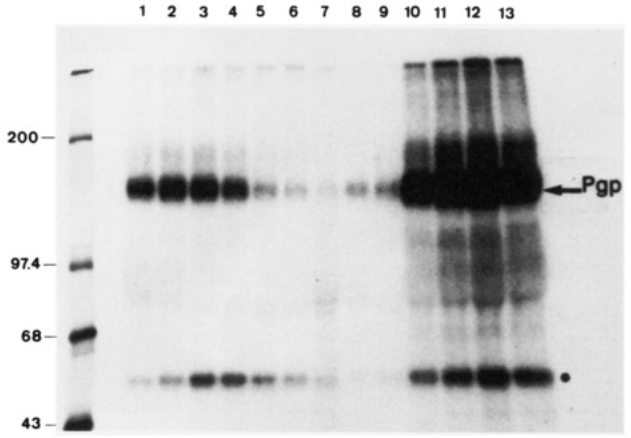

FIGURE 3: Phosphorylation of membrane-bound P-glycoprotein by [γ-^{32}P]GTP *in vitro*. Plasma membranes (100 μg) of KB-V1 cells were incubated for 10 min at room temperature in the presence of 10 μCi of [γ-^{32}P]GTP, in a final volume of 100 μL. Reactions were performed in the presence of decreasing concentrations of cold GTP (3.0 mM, 1.5 mM, 0.30 mM, 0.15 mM, 30 μM and 15 μM) (lanes 1–6, respectively) or in the presence of decreasing concentrations of cold ATP (lanes 8–13, respectively). Lane 7, no cold nucleotide added (control). The autoradiography shows SDS–PAGE of the 4007-immunoprecipitated [γ-^{32}P]Pgp. Molecular masses of ^{14}C-labeled standard proteins (in kilodaltons) are indicated on the left side. The star indicates the 55-kDa proteolytic fragment of Pgp.

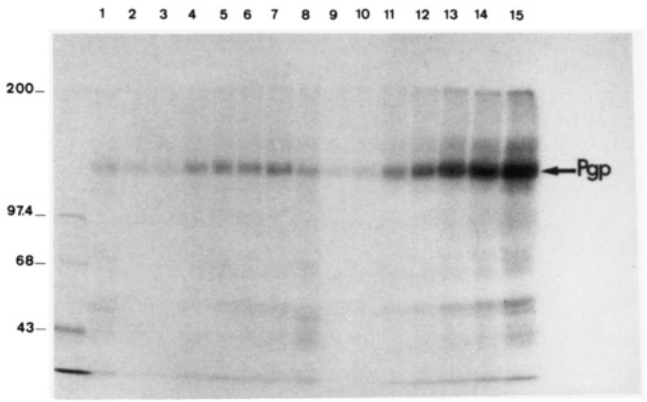

FIGURE 4: Phosphorylation of membrane-bound P-glycoprotein by [γ-^{32}P]ATP in the presence of low and high concentrations of GTP *in vitro*. Membranes from KB-V1 cells were radiolabeled with [γ-^{32}P]-ATP (30 μCi) in the presence of various concentrations of cold ATP in combination with a low concentration of cold GTP (30 μM GTP, lanes 2–8) or with a high concentration of cold GTP (3.0 mM GTP, lanes 9–15). Lane 1 represents the control where neither cold ATP nor cold GTP was added. Lanes 2–6: 30 μM GTP in combination with 3.0 mM, 1.5 mM, 0.30 mM, 0.15 mM, 30 μM, and 15 μM ATP, respectively. Lane 8: no cold ATP was added, but 30 μM GTP was present. Lanes 9–14: 3.0 mM GTP in combination with 3.0 mM, 1.5 mM, 0.30 mM, 0.15 mM, 30 μM, and 15 μM ATP, respectively. Lane 15: no cold ATP was added, but 3 mM GTP was present. The autoradiography shows the SDS–PAGE of 4007-immunoprecipitated samples. Molecular masses of ^{14}C-labeled standard proteins (in kilodaltons) are indicated on the left side.

phorylation of a 150-kDa band was seen in KB-3-1 plasma membrane with [γ-^{32}P]ATP+GTP (Figure 1, panel KB-3-1, lanes 1 and 2). Phosphorylation in the presence of several concentrations of GTP and varying amounts of cold ATP shows that the greatest phosphorylation was obtained with a striking excess of GTP (3.0 mM) in combination with 0.3 mM ATP or less (Figures 3 and 4). We have tried to determine the K_m for phosphorylation of Pgp with [γ-^{32}P]ATP and [γ-^{32}P]GTP. Though variability was found with different preparations of plasma membrane vesicles, the K_m for [γ-^{32}P]-ATP+ATP-driven phosphorylation was always lower (0.2–1.4 mM) than for [γ-^{32}P]GTP+GTP (4–7.5 mM). An accurate determination of the kinetic parameters will require

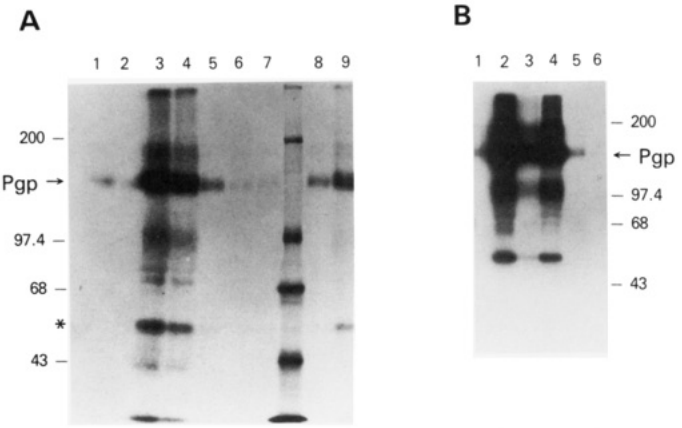

FIGURE 5: Effect of nonhydrolyzable analogs of nucleotides on phosphorylation of membrane-bound Pgp *in vitro*. (Panel A) Membranes of KB-V1 vesicles were subjected to phosphorylation with [γ-^{32}P]ATP (10 μCi/sample) in the presence of 3.0 or 1.5 mM ATP (lanes 1, 2) or GTP (lanes 3, 4) or AMP-PNP (lanes 6, 7). The following combinations were tested: 1.5 mM each of ATP+GTP (lane 5); 1.5 mM each of ATP+AMP-PNP (lane 8); 1.5 mM each of GTP+AMP-PNP (lane 9). The concentration ranges were chosen to mimic conditions enabling drug transport by Pgp *in vitro* (Lelong *et al.*, 1992). (Panel B) Effect of nonhydrolyzable analogs of GTP on Pgp phosphorylation by [γ-^{32}P]ATP *in vitro*. In this experiment, 30 μCi of [γ-^{32}P]ATP was used to label samples of KB-V1 plasma membranes (100 μg of protein, final volume 100 μL, 10 min incubation time) in the absence of cold "carrier" (control, lane 1: no cold ATP, no other cold nucleotide) or in the presence of 3.3 mM either cold GTP, GMP-PNP, GTPγS, cGMP, or cAMP (lanes 2–6, respectively). All the incubations were stopped by EDTA, and samples were immunoprecipitated with 4007 and processed as described under Experimental Procedures. The molecular masses of ^{14}C-labeled standards are indicated in kilodaltons. Exposure of autoradiograms was the same for all panels presented.

purification and reconstitution of Pgp and each of the kinases and phosphatases contributing to phosphorylation in this system.

Two possibilities to explain why GTP simulates [γ-^{32}P]-ATP-dependent phosphorylation are that GTP is regenerating ATP via a nucleotide diphosphate (NDP) kinase system (Kimura et al., 1990) or that GTP helps to prevent ATP hydrolysis. To exclude the possible interconversion of GTP and ATP by an NDP kinase, the radiolabeled nucleotides present in the incubation medium at the end of the experiment were analyzed by thin-layer chromatography. Our results with [γ-^{32}P]ATP in combination either with cold ATP or with cold GTP showed that no interconversion occurred (data not shown).

To determine whether the effect of GTP and ATP on phosphorylation required hydrolysis of these nucleotides, various nonhydrolyzable analogs were tested. In Figure 5A, substitution of ATP (1.5 and 3.0 mM) with the same concentrations of its nonhydrolyzable analog AMP-PNP also diminished the phosphorylation on the transporter by [γ-^{32}P]-ATP, as would be expected if the nonhydrolyzable analogs compete for the ATP binding site of the transporter (Figure 5A, lanes 1, 2 and 6, 7, respectively). Equimolar concentrations of ATP+AMP–PNP did not alter the phosphorylation (Figure 5A, lane 8). When AMP-PNP was used in combination with an equimolar concentration of GTP, the enhancer effect on Pgp phosphorylation by GTP (Figure 5A, lanes 3, 4) was diminished (Figure 5A, lane 9). Similar studies have been performed with nonhydrolyzable analogs of GTP and with purine cyclic nucleotides. In Figure 5B, GMP-PNP at 3.3 mM produced a slight increase in phosphorylation by [γ-^{32}P]-ATP, but this effect was notably lower than that observed with 3.3 mM GTP (Figure 5B, lanes 2, 3); 3.3 mM GTPγS, another analog of GTP, was able to stimulate phosphorylation

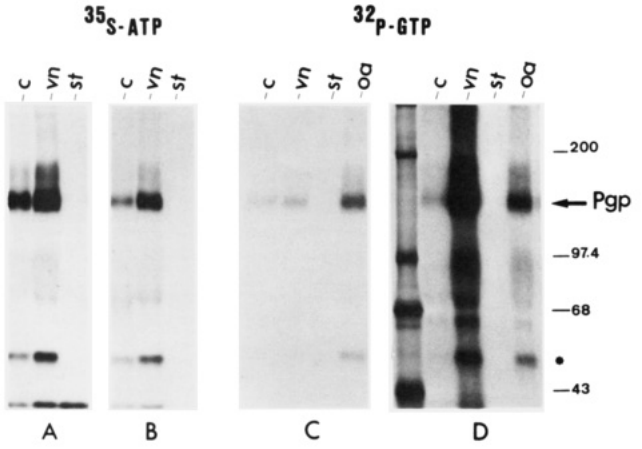

FIGURE 6: Effect of vanadate, staurosporine, and okadaic acid on phosphorylation of membrane-bound Pgp by [γ-^{35}S]ATP or by [γ-^{32}P]-GTP *in vitro*. Membranes of KB-V1 vesicles (100 μg, 100 μL final volume) were subjected to phosphorylation with [γ-^{35}S]ATP (10 μCi/sample) in the presence of either 3.0 mM GTP (panel A) or 30 μM ATP (panel B). Phosphorylation of Pgp by [γ-^{32}P]GTP (10 μCi/sample) is shown in the presence of either 3.0 mM GTP (panel C) or 30 μM ATP (panel D). Phosphorylation reactions were performed either in the absence (c, control) or in the presence of 10 μM vanadate (vn) or 10 μM staurosporine (st) or 10 μM okadaic acid (oa). After radiophosphorylation, the samples were immunoprecipitated with antiserum 4007 and processed as described under Experimental Procedures. The molecular masses of ^{14}C-labeled protein standards are indicated in kilodaltons.

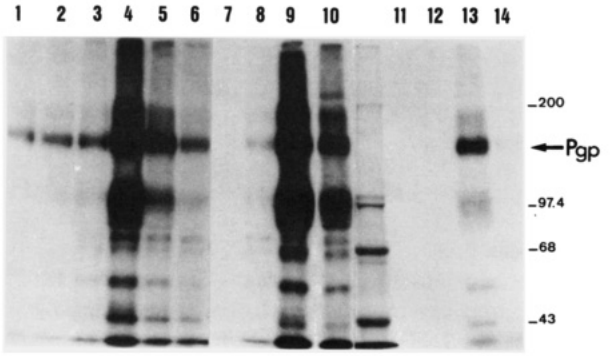

FIGURE 7: Effect of cations on phosphorylation by [γ-^{32}P]ATP of membrane-bound Pgp *in vitro*. Membranes of KB-V1 vesicles were phosphorylated with [γ-^{32}P]ATP (10 μCi/sample) in the presence of either 3.0 mM $MgCl_2$ (lanes 1–6), 3.0 mM $MnCl_2$ (lanes 7–10), or 3.0 mM $CaCl_2$ (lanes 11–14). Labeling was performed with the following nucleotide concentrations: ATP, 3.0 mM (lanes 1, 7, 11), 0.30 mM (lane 2), and 30 μM (lanes 3, 8, 12); GTP, 3.0 mM (lanes 4, 9, 13), 0.30 mM (lane 5), and 30 μM (lanes 6, 10, 14). After radiolabeling, samples were immunoprecipitated and processed as described under Experimental Procedures.

of Pgp almost as well as GTP at the same concentration (Figure 5B, lanes 2 and 4, respectively). We also found that the substitution of [γ-^{32}P]ATP by [γ-^{35}S]ATP could also support the ^{35}S thiophosphorylation of the transporter in the presence of cold ATP (Figure 6, panel B). Using [γ-^{35}S]ATP, simulation of phosphorylation by cold GTP (3.0 mM) was still observed (Figure 6, panel A, first lane, and panel B, first lane). Neither cAMP nor cGMP could mimic the GTP effect on Pgp phosphorylation (Figure 5B, lanes 5, 6).

Effect of Divalent Cations on the Modulation by GTP of Pgp Phosphorylation. As shown in Figure 7, Mn^{2+} could support phosphorylation of Pgp by [γ-^{32}P]ATP, but total phosphorylation was lower than that observed in the presence of Mg^{2+} (Figure 7, lanes 7–8 and 1–3, respectively). The same figure shows that Mg^{2+} and also Mn^{2+} fully supported the stimulation of phosphorylation seen in the presence of cold GTP (Figure 7, lanes 4–6 and 9–10, respectively). Ca^{2+} could not replace Mg^{2+} when ATP was the sole nucleotide present (Figure 7, lanes 11–12). However, in the presence of a high concentration of GTP (3.0 mM), Ca^{2+} allowed phosphorylation of Pgp (Figure 7, lane 13). These results suggest that the stimulatory effect of GTP may be via a kinase and/or a phosphatase with different cation specificity than the ATP-dependent kinase. Zn^{2+} (0.1 mM) totally inhibited phosphorylation in this system, whether ATP or GTP was present (data not shown). It has been suggested that verapamil can increase Pgp phosphorylation (Hamada *et al.*, 1987). However, under our reaction conditions, this calcium channel inhibitor (10 μM) did not alter phosphorylation when tested with [γ-^{32}P]ATP in the presence of either cold ATP or GTP (data not shown).

Lack of Evidence for Pgp Autophosphorylation. We searched for evidence of Pgp autophosphorylation with negative results. SDS–PAGE-electrotransferred immunoprecipitates of Pgp showed no in-blot kinase activity when assayed as previously described (Ferrell & Martin, 1989) (data not shown). However, the conditions generally used for protein separation (SDS–PAGE) exclude the preservation of acyl phosphate intermediates such those found for the Ca^{2+} ATPase incubated in the presence of [γ-^{32}P]ATP under specific conditions (Sarkadi *et al.*, 1986). Using conditions described under Experimental Procedures, which preserve acyl phosphates, we found a major labeled band at 116 kDa in both KB-3-1 and KB-V1 plasma membranes that was vanadate sensitive. This band probably represented plasma membrane Ca^{2+} ATPase and served as a positive control for these experiments. No radiolabeled band corresponding to the multidrug transporter was found (data not shown), indicating that Pgp has no detectable acyl phosphates under our reaction conditions.

Effect of Inhibitors of Kinases and Phosphatase on Pgp Phosphorylation. Vanadate, a metabolic inhibitor and an inhibitor of transport of vinblastine by Pgp (Horio *et al.*, 1988), as well as an inhibitor of its ATPase activity (Ambudkar *et al.*, 1992), induced a strong increase in the phosphorylation of Pgp in KB-V1 plasma membrane vesicles in the presence of either [γ-^{32}P]ATP and cold ATP or [γ-^{35}S]ATP and cold ATP (Figure 2A, lane 2; Figure 6, panels A, B, lanes vn). This "enhancing" effect of vanadate on phosphorylation of Pgp is also seen when [γ-^{32}P]GTP is used in combination with cold GTP (Figure 6C, lane vn), but is more prominent when labeled GTP and cold ATP are used (Figure 6D, lane vn).

Among the kinase(s) inhibitors tested, staurosporine is a powerful inhibitor with a rather broad spectrum (Rüegg & Burgess, 1989). With KB-V1 plasma membrane vesicles, there is nearly complete inhibition of Pgp phosphorylation at a concentration of 10 μM under all tested reaction conditions (Figure 2, lane 1; Figure 6, lanes st). Several concentrations of staurosporine were tested for its inhibitory effect on Pgp phosphorylation by [γ-^{32}P]ATP. We observed that inhibition was obtained for concentrations higher than 1 μM (data not shown). At low concentrations (<10 nM), staurosporine (K_i for PKC = 0.7 nM) had no effect on Pgp phosphorylation. Due to the high concentration required to observe total inhibition of phosphorylation and the structure of the molecule, staurosporine has been tested for its capacity to inhibit [^{3}H]-azidopine labeling. In agreement with the results of Sato *et al.* (1990), we have observed that staurosporine competed effectively for [^{3}H]azidopine labeling of KB-V1 vesicles similarly to drugs recognized by the transporter such as vinblastine or verapamil (data not shown). Staurosporine is a polycyclic hydrophobic drug containing a protonatable amine. This is a common feature of the MDR drugs. Other kinase or phosphatase inhibitors (or activators), such as

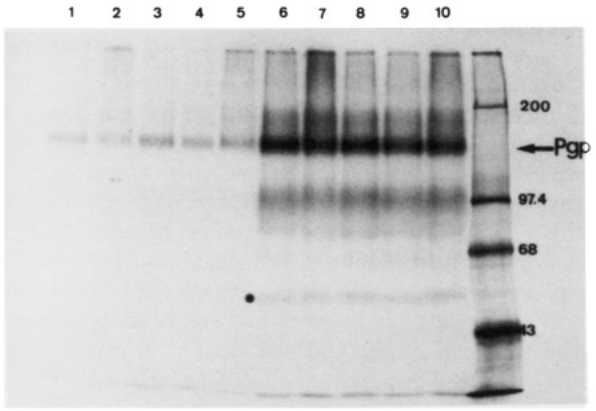

FIGURE 8: Effect of protein kinase inhibitors on phosphorylation by [γ-^{32}P]ATP of membrane-bound Pgp *in vitro*. Membranes of KB-V1 vesicles were subjected to phosphorylation with [γ-^{32}P]ATP (30 μCi/sample) in the presence of either 30 μM ATP (lanes 1–6) or 3.0 mM GTP+30 μM ATP (lanes 6–10). Labeling was performed in the absence (controls, lanes 1, 6) or in the presence of the following protein kinase inhibitors: 5 μM calphostin C (lanes 2, 7); 0.6 μM protein kinase A inhibitor KT 5720 (lanes 3, 8); 2 μM cGMP-dependent protein kinase KT 5823 (lanes 4, 9); and a combination of calphostin C (5 μM) + KT 5720 (0.6 μM) (lanes 5, 10). After radiolabeling, samples were immunoprecipitated and processed as described under Experimental Procedures. The star indicates the position of the 55-kDa proteolytic fragment of Pgp.

calphostin C or trifluoperazine, also possess a structure which could be recognized by the transporter. Further uptake experiments performed as described earlier (Lelong *et al.*, 1992) showed that 2 μM and 10 μM staurosporine diminished energy-dependent [^{3}H]vinblastine accumulation in isolated KB-V1 vesicles by 56% and 78%, respectively. This effect may be accounted for either by inhibition of Pgp phosphorylation or by the competition of staurosporine with vinblastine for transport or both. Because it may be a competitive inhibitor of Pgp, staurosporine does not seem to constitute the tool of choice either to demonstrate coupling of phosphorylation and transport or to screen for the kinase(s) involved.

A variety of different inhibitors of PKA did not alter ATP-driven phosphorylation (data shown for KT 5720; Figure 8, lane 3). Figure 8 also shows that calphostin C also failed to inhibit phosphorylation of Pgp (lane 2). Similar results were obtained with the pseudopeptide [19–36], another specific inhibitor for PKC (data not shown). The cGMP-dependent protein kinase inhibitor KT 5823 and a mixture of both PKC and PKA inhibitors also failed to alter ATP-driven phosphorylation of the transporter (calphostin C and KT 5720: Figure 8, lanes 4 and 5, respectively). These kinase inhibitors also had no effect on GTP stimulation of phosphorylation by [γ-^{32}P]ATP (Figure 8, lanes 7–10, respectively, control in lane 6).

In agreement with Chambers *et al.* (1992), we also observed that okadaic acid, a potent inhibitor of protein phosphatases, slightly increased Pgp phosphorylation in KB-V1 vesicles incubated in the presence of radiolabeled ATP (data not shown). Moreover, vinblastine uptake measured as described previously (Horio *et al.*, 1988; Lelong *et al.*, 1992) in the presence of this inhibitor (15 μM) showed only a slight decrease of about 30%, whereas vanadate (10 μM) totally abolished transport under the same conditions. Using [γ-^{32}P]GTP in combination with cold ATP or cold GTP, in the presence of okadaic acid, we observed a similar stimulation of phosphorylation (Figure 6, panels C, D, lanes oa). Okadaic acid did not show competition for [^{3}H]azidopine labeling of the transporter in KB-V1 vesicles (data not shown).

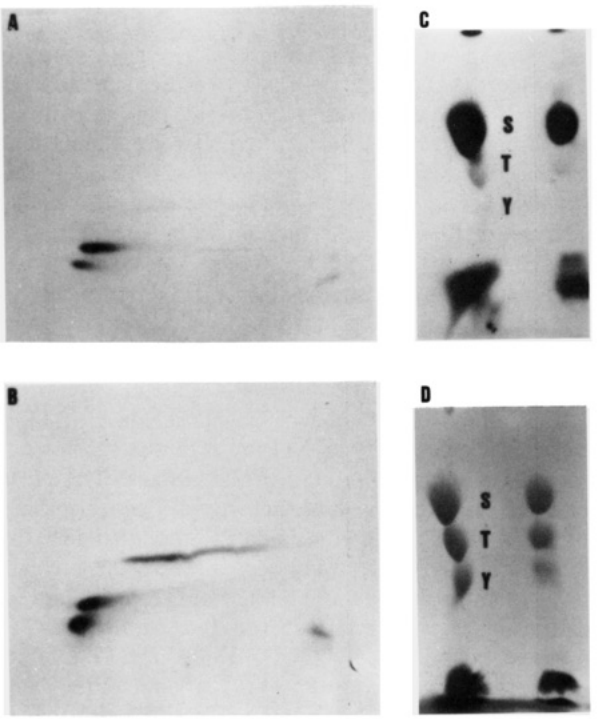

FIGURE 9: Phosphopeptide and phosphoamino acid analysis of membrane-bound Pgp radiophosphorylated *in vitro* in the presence of [γ-^{32}P]ATP in the absence or presence of cold GTP. Several samples (200 μg of protein each) of KB-V1 vesicles were phosphorylated by [γ-^{32}P]ATP (30 μCi/200 μg of protein, final volume 100 μL) in the presence of either 30 μM ATP or 3.0 mM GTP+0.2 mM ATP. Briefly, as described under Experimental Procedures, after labeling, samples were subjected to immunoprecipitation by 4007. Three to four immunoprecipitates were pooled per lane of a 7.5% SDS–PAGE gel and electrophoresed. After autoradiography of the gel, several Pgp-containing bands were excised and processed for phosphopeptide release as described under Experimental Procedures. Phosphopeptides were pooled and either subjected to two-dimensional thin-layer electrophoresis (panels A, B) or processed further for phosphoamino acid analysis (panels C, D). Autoradiography of the tryptic map of phosphorylated Pgp in the presence of either 30 μM ATP (panel A) or 3.0 mM GTP+0.2 mM ATP (panel B) is shown. Panel C shows autoradiography of the phosphoamino acids deriving from the phosphopeptides of Pgp phosphorylated in the presence of either 30 μM ATP (left side) or 3.0 mM GTP+0.2 mM ATP (right side) and separated by thin-layer electrophoresis. Panel D shows the corresponding ninhydrin-stained plate of panel C: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

Analysis of ^{32}P-Labeled Tryptic Peptide Fragments and Phosphorylated Amino Acids. In these studies, [^{32}-P]Pgp labeled either by a combination of [γ-^{32}P]ATP+cold ATP or by [γ-^{32}P]ATP+cold GTP was immunoprecipitated and resolved on SDS–PAGE, and tryptic peptides were obtained and analyzed as described under Experimental Procedures. The concentrations (0.2 mM ATP and 3.0 mM GTP as cold carrier) of the respective nucleotide combinations were chosen to optimize ^{32}P incorporation into the Pgp immunoprecipitates. Vanadate was also added to avoid loss of label. The autoradiographs show that for both types of labeling, most of the phosphorylation is found in two peptides (Figure 9, panels A, B). The addition of GTP reversed the relative ratio of phosphorylation of these two peptides (Figure 9, panels A, B). Phosphoamino analysis of the tryptic peptides reveals that serine is the main amino acid phosphorylated with a weak phosphorylation of threonine after long exposure of the autoradiographs (Figure 9, panels C, D). No effect of GTP on the overall pattern of phosphoamino acids was observed.

DISCUSSION

Pgp has been shown to be phosphorylated by ATP in numerous cell lines (Carlsen *et al.*, 1977; Center, 1983, 1985;

Garman *et al.*, 1983; Hamada *et al.*, 1987). We have previously shown that GTP can serve as an energy source for the transport of vinblastine in KB-V1 vesicles (Lelong *et al.*, 1992). Our present results show that Pgp can also be phosphorylated by [γ-^{32}P]GTP alone. Strong stimulation of radiophosphorylation of Pgp by [γ-^{32}P]ATP was obtained when phosphorylation was done in the presence of a combination of unlabeled ATP in the micromolar range together with unlabeled GTP in the millimolar range. A strong stimulation of Pgp radiophosphorylation by [γ-^{32}P]GTP is also obtained in the presence of unlabeled ATP in the micromolar range.

Why does GTP stimulate phosphorylation by ATP? There are four major possibilities: (1) GTP stimulates a Pgp kinase; (2) GTP inhibits a phosphoprotein phosphatase; (3) GTP increases the availability of ATP by acting as substrate for an NDP kinase or inhibiting an ATPase activity; and (4) GTP produces a conformational change of Pgp which favors additional phosphorylation. Our data suggest that more than one of these possibilities may contribute to the increased phosphorylation observed in presence of GTP. In addition, three lines of circumstantial evidence favor the idea of a GTP-stimulated kinase activity (and perhaps a GTP-inhibited phosphoprotein phosphatase): (1) the cation requirement for GTP stimulation of phosphorylation is less stringent than for ATP-dependent phosphorylation alone since Mg^{2+}, Mn^{2+}, and Ca^{2+} can also support the GTP-stimulated phosphorylation (Figure 7); (2) the relative phosphorylation of the two major phosphopeptides seen with GTP stimulation is different from that seen with ATP alone (Figure 9); and (3) addition of cold GTP resulted in increased phosphorylation of Pgp by [γ-^{32}P]-GTP or by [γ-^{32}P]ATP or by [γ-^{35}S]ATP, whereas addition of cold ATP decreased phosphorylation by [γ-^{32}P]GTP or by [γ-^{32}P]ATP (Figures 1, 3, and 4), suggesting that GTP may be altering the K_m and/or turnover number of a kinase or phosphatase involved in the phosphorylation of Pgp. Additional protein bands are seen on the autoradiograms when phosphorylation is done in the presence of GTP (Figures 1 and 3–8). This observation may be due to nonspecific association with the immunoprecipitated Pgp of radiolabeled peptides, whose phosphorylation is also stimulated by GTP, or to coimmunoprecipitation of phosphoproteins associated with the transporter.

Using GMP-PNP in combination with [γ-^{32}P]ATP, we observed a decrease in the "enhancing" effect on phosphorylation of Pgp (Figure 5B). In contrast, GTPγS, a hydrolyzable analog of GTP, mimicked the effect of GTP on [γ-^{32}P]ATP phosphorylation of Pgp (Figure 5B). This result suggests that GTP hydrolysis is required to observe an enhanced phosphorylation of the transporter by [γ-^{32}P]ATP. Such a result suggests that GTP may not be acting as an allosteric activator in this system, but could be a substrate for a kinase which in turn activates a Pgp kinase or inhibits a Pgp phosphatase.

Since Pgp has been shown to possess an endogenous ATPase activity (Horio *et al.*, 1988; Lelong *et al.*, 1992; Ambudkar *et al.*, 1992; Hamada & Tsuruo, 1988), one can hypothesize that while ATP hydrolysis occurs, phosphate groups are translocated as a part of the reaction process. We found no evidence of autophosphorylation of the transporter, or of an acyl phosphate intermediate. Furthermore, partially purified and reconstituted Pgp (Ambudkar *et al.*, 1992) is only very weakly phosphorylated by [γ-^{32}P]ATP, suggesting that purification/reconstitution removes one or more kinases (unpublished data). Therefore, we believe that an exogenous kinase or kinases are responsible for phosphorylation.

Numerous studies have addressed the characterization of the kinase involved in the Pgp phosphorylation and attribute this function mainly to PKC or to PKA (Hamada *et al.*, 1987; Mellado & Horwitz, 1987; Fine *et al.*, 1988; Chambers *et al.*, 1990a,b; Hayes & Wolf, 1990; Ido *et al.*, 1986; Ferguson & Chen, 1987). The results we obtained with an *in vitro* system of plasma membrane vesicles capable of drug transport, using different specific kinase inhibitors, allow us to exclude the exclusive participation of the "classical" protein kinases in Pgp phosphorylation, as none of the tested inhibitors when used at a concentration close to their known K_i significantly affected the level of Pgp phosphorylation. A similar result was reported by Chambers *et al.* (1992) with KB-V1 plasma membranes and by Staats *et al.* (1990) with HL-60 membranes. Chambers *et al.* (1990) found only a 30% decrease in the phosphorylation of Pgp in the presence of H7, a protein kinase inhibitor used at high concentrations (100 and 20 μM) where it affects both PKC and PKA [K_{iH7} for PKC, 6 μM; K_{iH7} for PKA, 3 μM (Hidaka *et al.*, 1984)]. The fact that Ca^{2+} did not support ATP-dependent phosphorylation argues against the involvement of Ca^{2+}-dependent PKC isoforms in the phosphorylation we observed. Staats *et al.* (1990) have reported the presence of a new kinase (PK1) localized in plasma membranes of HL-60 cells that phosphorylates Pgp. This new enzyme requires Mn^{2+}, can use Mg^{2+}, and is inactive in the presence of calcium. PK1 is completely inhibited by staurosporine, but not H7, and is activated by lipids. This PK1 kinase is probably similar to the Mn^{2+}/phospholipid-activated kinase described earlier by Elias and Davis (1985) and has properties similar to the kinase activity we observe in our system.

The GTP-dependent phosphorylation we observe is different from the previously reported GTP-dependent protein kinases (Friedman *et al.*, 1985; Diaz-Nido *et al.*, 1988). These later kinases can use either ATP or GTP. In contrast to our results, with these reported kinases the presence of cold GTP in the incubation media dramatically decreases the protein phosphorylation driven by radiolabeled ATP, whereas we see stimulation by ATP. The results of Amir-Saltsman and Salomon (1989), showing that GTP can stimulate phosphorylation of different plasma membrane proteins, are more parallel, but not identical, to ours. This latter phosphorylation is maximum at 20 mM GTP but is also inhibited by Ca^{2+}. Moreover, in this particular study, vanadate did not change the level of phosphorylation as occurs in our system. Thus, none of the previously described kinase activities correspond precisely to the activity we have observed in the KB-V1 vesicles.

In agreement with the results of Hamada *et al.* (1987) and Ma *et al.* (1991), we have observed that phosphoserine was the main phosphorylated amino acid with [γ-^{32}P]ATP+ATP. The same result was obtained in the presence of [γ-^{32}P]-ATP+cold GTP (Figure 9). This strengthens our hypothesis suggesting participation of another kinase.

In agreement with Chambers *et al.* (1992), we found that okadaic acid, a phosphoprotein phosphatase inhibitor, increased the phosphorylation of Pgp when [γ-^{32}P]ATP was used. A similar result was obtained in the presence of cold GTP. Increased phosphorylation was obtained when vanadate was added to the phosphorylation medium. This may be explained either by the inhibition of other ATP-consuming systems by vanadate and/or inactivation of phosphatases present in the vesicles. The addition of vanadate in incubation media has been used earlier in phosphorylation studies (Hamada *et al.*, 1987; Chambers *et al.*, 1992), but its effect on the phosphorylation of the transporter has not been reported.

In our plasma membrane vesicle system, the multidrug transporter is phosphorylated only by intrinsic plasma membrane kinases. Under the experimental conditions described in this work, our data exclude the participation of intrinsic "conventional" PKC but do not exclude the involvement of "atypical" isoform(s). Recently Chambers *et al.* (1993), using exogenously added PKC comprising a mixture of PKC isoforms, have identified specific serine phosphorylation sites in the linker region of the multidrug transporter. Our data and several reports on Pgp phosphorylation in other systems suggest the participation of several kinases other than "classical" PKC whose effect on transport function remains unknown.

To discriminate among the possible kinases and phosphatases involved in the phosphorylation of the transporter and to determine their effect on transport activity, a less complex system may prove useful. Purification of some of the kinases and phosphatases responsible for phosphorylation of P-glycoprotein in drug-transporting vesicles is in progress.

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