Analysis of P-glycoprotein Phosphorylation in HL60 Cells Isolated for Resistance to Vincristine*

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In the present study we have analyzed the involvement of phosphorylation in the function of P-glycoprotein and have also examined sites of phosphorylation along the P-glycoprotein polypeptide chain. The results show that in HL60 cells isolated for resistance to vincristine the protein kinase inhibitor staurosporine induces a major inhibition in the phosphorylation of P-glycoprotein. Further studies show that under the same conditions in which staurosporine inhibits P-glycoprotein phosphorylation there is a concomitant increase in cellular drug accumulation and a major inhibition in drug efflux. Additional studies using pulse-chase experiments show that the P-glycoprotein phosphate groups are metabolically active and that the protein undergoes rapid cycles of phosphorylation and dephosphorylation in the cell.

Structural analyses demonstrate that cleavage of \( ^{32}P \)-labeled P-glycoprotein at Asp-Pro linkages with formic acid results in the formation of a major phosphorylated peptide of 35 kDa and a minor peptide of 42 kDa. Western blot analysis using site-specific antiserum against P-glycoprotein suggests that P35 represents a phosphorylated fragment containing P-glycoprotein amino acids 446–744. Analysis of tryptic peptides using site-specific antiserum identifies a second major phosphorylated region of P-glycoprotein which contains amino acids 745–1088.

These studies thus suggest that phosphorylation plays an important role in the biological activity of P-glycoprotein. The results also indicate that two adjacent internal regions are highly phosphorylated in the P-glycoprotein molecule.

Evidence accumulated over the past several years suggests that in many experimental isolates multidrug resistance is related to overexpression of a 150–180-kilodalton surface membrane phosphoglycoprotein which has been referred to as P-glycoprotein (1–4). Crucial support for this has been provided by the finding that transfection of sensitive cells with an expression vector containing a full length cDNA of the P-glycoprotein gene results in the appearance of P-glycoprotein and the conversion of sensitive cells to those exhibiting a drug-resistant phenotype (5–9). Cells containing P-glycoprotein appear to be resistant as a result of reduced cellular accumulation of drug which is related to enhanced levels of an energy-dependent drug efflux system (10–12). Previous studies have shown that P-glycoprotein contains an ATPase activity (13) and is capable of binding photoactive analogs of certain drugs (14, 15). Based on these findings it has been proposed that P-glycoprotein functions as a transporter to remove drug from the resistant cell in a reaction that utilizes energy released from ATP hydrolysis. At the present time the exact mechanism by which P-glycoprotein accomplishes the efflux of drug from the resistant cell is not known. Previous studies have shown that P-glycoprotein is phosphorylated (16–18) thus raising the possibility that specific sites of phosphorylation play an important role in regulating its biological function. In the present study we have addressed this question and have examined the effect of certain protein kinase inhibitors on P-glycoprotein phosphorylation and drug accumulation. In addition we have examined sites of phosphorylation in the P-glycoprotein molecule.

EXPERIMENTAL PROCEDURES

Materials—\(^{32}P\), (8500–9100 Ci/mmol) and \([\text{H}]\)daunomycin (4.2 Ci/mmol) were purchased from Du Pont-New England Nuclear. Stausporine was from Behring Diagnostics and \(1-(5\text{-isoquinolinyI-sulfonyl})\)-2-methylpipеразине (H-7) from Sigma.

Cells—HL60 cells were isolated for resistance to vincristine as previously described (19). The HL60/Vinc isolated exhibits a 140-fold increase in resistance to vincristine and is cross-resistant to a number of other chemotherapeutic agents.

Antiserum to Synthetic Peptides of P-glycoprotein—Synthetic peptides corresponding to a deduced sequence of P-glycoprotein (20) were prepared by the Marglin and Merrifield solid state method (21). Antiserum against these peptides were prepared as previously described (22). Table I lists the anti-peptide sera used in the present experiments.

Labeling Cells with \(^{32}P\), and Immunoprecipitation of P-glycoprotein—HL60/Vinc cells growing in RPMI containing 10% fetal bovine serum were centrifuged and resuspended in TC-1 medium containing 0.05 M Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM KCl, 0.5 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 × minimal essential medium amino acids and vitamins, 5.5 mM D-glucose, and 2% fetal bovine serum. \(^{32}P\), was added to a concentration of 200 \(\mu\)Ci/ml and the cells were incubated for various time periods in a CO\(_2\) incubator at 37°C. After the labeling period the cells were centrifuged and P-glycoprotein was immunoprecipitated from cell extracts according to the procedure described by Hamada et al. (17). Immunoprecipitation of P-glycoprotein in these experiments was carried out with an antipeptide serum (ASP45, Table I) against the P-glycoprotein sequence containing amino acids 1166–1181. Radioactively labeled proteins contained in the immunoprecipitates were analyzed after electrophoresis in a 7% SDS-polycryl- amide gel (23). Labeled proteins were detected by autoradiography of the dried gel.

Effect of Various Agents on Drug Accumulation and Efflux—Sensitive or resistant HL60 cells were seeded at a density of 3 × 10\(^5\)/ml in 1 ml of RPMI/10% fetal calf serum. Staurosporine or H-7 was added and this was followed by the addition of \([\text{H}]\)daunomycin. The cells were thereafter incubated in a CO\(_2\) incubator at 37°C and after

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.
**P-glycoprotein Phosphorylation in HL60/Vinc Cells**

### Table 1

| Peptide No. | Peptide sequence→ | P-glycoprotein amino acids |
|-------------|-------------------|---------------------------|
| 23          | SDNQDGGFMNLIV     | 96–110                    |
| 54          | FDTRVGERAGQLSGQKQRI | 519–538                  |
| 11          | VSKFDPKNTGTGALT   | 801–815                   |
| 21          | NVTDFEVNYPTPR     | 1034–1048                 |
| 45          | TKVKGRTGQLSGQQKQRI | 1166–1181               |
| 12          | YFMSMVSVQAGTRKQ    | 1266–1280                |

*Peptide number refers to original laboratory designation.

**Effect of Staurosporine on P-glycoprotein Phosphorylation**

HL60/Vinc cells were incubated in the absence or presence of 1 μM staurosporine and in the presence of 32P, for time periods of 15 and 30 min. At the end of the labeling period cell extracts were prepared and P-glycoprotein was immunoprecipitated as described under “Experimental Procedures.” Under the conditions used, labeling of P-glycoprotein is rapid and the protein becomes highly phosphorylated during a 15-min incubation period (Fig. 1, *left panel, lane A*). Increasing the incubation time to 30 min results in a parallel increase in the 32P labeling of P-glycoprotein (Fig. 1, *left panel, lane C*). Two protein kinase inhibitors, staurosporine (24) and H-7 (25), have been examined for their effect on P-glycoprotein phosphorylation. Both inhibitors have previously been shown to inhibit protein kinase C and cyclic AMP-dependent protein kinase (24, 25). In the presence of 1 μM staurosporine there is a major inhibition in P-glycoprotein phosphorylation during the 15- or 30-min incubation periods (Fig. 1, *left panel, lanes B and D*). Cells treated with staurosporine under these conditions and those to be described are completely viable as determined by trypan blue exclusion. Additional studies show that, during a 1-h incubation of cells with 32P, P-glycoprotein is highly labeled in resistant cells (Fig. 1, *right panel, lane A*) but is not detectable in cells sensitive to drug (Fig. 1, *right panel, lane E*). Incubation of resistant cells under these conditions various time periods were centrifuged. The cell pellets were suspended in 0.2% SDS buffer and the radioactivity was determined. HL60 cells isolated for resistance to vincristine are cross-resistant to daunomycin. 1H]Daunomycin (600 cpn/pg, final concentration 0.4 μg/ml) was used in drug uptake and efflux studies since it can be obtained at a high specific activity and the levels of this material which accumulate in cells is considerably greater than that for vincristine. For fluorescence microscopy, cells were centrifuged and suspended in drug efflux media (EM) containing 0.05 M Tris-HCl (pH 7.6), 0.14 M NaCl, 5.0 mM CaCl2, 0.5 mM MgCl2, 1.8 mM NaH2PO4, 5.6 mM NaHPO4, 2 mM glutamine, 1 × minimal essential medium amino acids and vitamins, and 2% fetal calf serum. Sodium azide was added to 10 mM and the cells were incubated with 3H]daunomycin for 40 min at 37°C. After this time period the cells were centrifuged and divided into two portions in EM containing 15 mM glucose. To one portion staurosporine was added to 1 μM. The cells were incubated at 37°C and after various time periods aliquots were centrifuged and radioactivity contained in the cell pellet was determined.

**Western Blots** Western blot analysis using antisera against synthetic peptides which correspond to the deduced sequence of P-glycoprotein was carried out as described previously (22).

**Results**

**Analysis of Tryptic Peptides** Immunoprecipitated from 32P-labeled cell extracts and proteins were resolved by SDS-polyacrylamide gel electrophoresis. 32P-Labeled P-glycoprotein was detected by autoradiography of the fixed gel. The gel slice containing P-glycoprotein was removed and thereafter incubated overnight with 0.05 M NH4HCO3 containing 25 μg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. The gel slice was removed by centrifugation and the supernatant was evaporated to dryness twice in water. The sample was electrophoresed in an SDS/10% polyacrylamide gel and the labeled cleavage products were detected after autoradiography. For drug efflux studies cells were centrifuged and suspended in TCG medium and the cells were incubated at 37°C in a CO2 incubator. After 15 and 30 min aliquots were taken of cells grown in the absence or presence of staurosporine. Cell extracts were prepared and immunoprecipitation was carried out as described under “Experimental Procedures.” The radioactively labeled proteins contained in the immunoprecipitates were analyzed after SDS-polyacrylamide gel electrophoresis and autoradiography. The results are shown in the left panel (lanes A–D). Lanes A and C, 15- and 30-min labeling, respectively, in the absence of staurosporine; lanes B and D, 15- and 30-min labeling, respectively, in the presence of staurosporine. In a separate experiment HL60/Vinc cells were suspended in TCG medium and the cells were incubated in the absence or presence of 50, 100, or 300 nM staurosporine for 10 min. 32P (200 μCi/ml) was added to these cells and also to sensitive cells suspended in the same medium. Incubation at 37°C was continued for 1 h after which time cell extracts were prepared and immunoprecipitation was carried out as described under “Experimental Procedures.” Radioactively labeled proteins were analyzed after electrophoresis in an SDS/7% polyacrylamide gel. The results are shown in the right panel (lanes A–E). Lanes A–D are resistant cells containing 0, 50, 100, and 300 nM, respectively, of staurosporine. Lane E, immunoprecipitation of an extract from sensitive cells. P-glycoprotein is detected as a 180-kilodalton protein.

**FIG. 1. Effect of staurosporine on P-glycoprotein phosphorylation.** HL60/Vinc cells growing in RPMI/10% fetal calf serum were centrifuged and suspended in TCG medium. The cells were divided into two portions with one containing 1 μM staurosporine. 32P was added to a final concentration of 200 μCi/ml and the cells were incubated at 37°C in a CO2 incubator. After 15 and 30 min aliquots were taken of cells grown in the absence or presence of staurosporine. Cell extracts were prepared and immunoprecipitation was carried out as described under “Experimental Procedures.” The radioactively labeled proteins contained in the immunoprecipitates were analyzed after SDS-polyacrylamide gel electrophoresis and autoradiography. The results are shown in the left panel (lanes A–D). Lanes A and C, 15- and 30-min labeling, respectively, in the absence of staurosporine; lanes B and D, 15- and 30-min labeling, respectively, in the presence of staurosporine. In a separate experiment HL60/Vinc cells were suspended in TCG medium and the cells were incubated in the absence or presence of 50, 100, or 300 nM staurosporine for 10 min. 32P (200 μCi/ml) was added to these cells and also to sensitive cells suspended in the same medium. Incubation at 37°C was continued for 1 h after which time cell extracts were prepared and immunoprecipitation was carried out as described under “Experimental Procedures.” Radioactively labeled proteins were analyzed after electrophoresis in an SDS/7% polyacrylamide gel. The results are shown in the right panel (lanes A–E). Lanes A–D are resistant cells containing 0, 50, 100, and 300 nM, respectively, of staurosporine. Lane E, immunoprecipitation of an extract from sensitive cells. P-glycoprotein is detected as a 180-kilodalton protein.
ditions with 50, 100, or 300 nM staurosporine also results in a progressive increase in the inhibition of P-glycoprotein phosphorylation (Fig. 1, right panel, lanes B–D). Further studies using H-7 show that at concentrations of 100 μM this agent does not inhibit P-glycoprotein phosphorylation during a 1-h incubation period (not shown).

Effect of Staurosporine on Drug Accumulation and Efflux

Studies have been carried out to correlate the staurosporine inhibition of P-glycoprotein phosphorylation with the effect of this agent on cellular drug levels. Thus in these experiments HL60-sensitive and -resistant cells were incubated in the absence or presence of staurosporine and the accumulation of [3H]daunomycin was determined. The results clearly demonstrate that under conditions in which staurosporine inhibits the phosphorylation of P-glycoprotein there is a concomitant increase in the accumulation of [3H]daunomycin (Fig. 2A). The effect of staurosporine is rapid and within 10 min of incubation drug accumulation increases severalfold (Fig. 2A). Of interest is the finding that in contrast to HL60/Vinc cells staurosporine has essentially no effect on [3H]daunomycin accumulation in drug-sensitive cells (Fig. 2B). As described above P-glycoprotein phosphorylation is inhibited in the presence of 50, 100, or 300 nM staurosporine. With increasing concentrations of this agent there is parallel increase in the inhibition of phosphorylation (Fig. 1). Drug uptake studies under these same conditions demonstrates that in the presence of 50, 100, or 300 nM staurosporine there is a 1.2-, 2.6-, and 4.0-fold increase respectively in daunomycin accumulation during a 1-h incubation period. Experiments have also been carried out to examine the effect of H-7 on cellular accumulation of [3H]daunomycin. Thus we find that incubation for 1 h in H-7 at concentrations up to 100 μM results in no detectable increase in drug accumulation in resistant cells (not shown). We have also examined the effect of staurosporine on drug efflux from resistant cells. Thus cells which have been loaded with [3H]daunomycin and incubated in the absence of staurosporine lose about 75% of the drug during a 15-min incubation period (Fig. 3). In contrast in the presence of 1 μM staurosporine there is a major inhibition of drug efflux from the resistant cell (Fig. 3). This inhibition in efflux is rapid and can be observed after a 5-min incubation with staurosporine (Fig. 3).

Analysis of P-glycoprotein Phosphorylation during a Pulse-chase

In these experiments drug-resistant cells were incubated in 32P, and thereafter suspended in high phosphate medium. After various incubation periods cell extracts were prepared and P-glycoprotein was immunoprecipitated and analyzed after polyacrylamide gel electrophoresis. The results clearly demonstrate that during the 40-min chase period the levels of P-glycoprotein phosphorylation are greatly reduced (Fig. 4C). Densitometric scans of the autoradiogram show that during the 20-, 40-, 60-, and 120-min chase periods there is a 15, 60, 73, and 97% loss, respectively, in labeled phosphate from P180 protein was measured. Lanes A–E are times 0, 20, 40, 60, and 120 min, respectively, after the chase period.
Phosphorylation Sites in P-glycoprotein

Analysis of Tryptic Peptides and Phosphorylated Amino Acids—In these studies 32P-labeled P-glycoprotein was immunoprecipitated and electrophoresed in a polyacrylamide gel. The gel slice containing P-glycoprotein was treated with ammonium bicarbonate and trypsin, and tryptic peptides were thereafter analyzed as described under "Experimental Procedures." The results of these studies show that major phosphorylation is confined to only three tryptic peptides (Fig. 5A). Six additional minor peptides not visible in the figure can also be detected after prolonged exposure of the autoradiogram. Additional studies demonstrate that serine is the single amino acid phosphorylated in P-glycoprotein contained in HL60/Vinc cells (Fig. 5B).

Analysis of 32P-Labeled Tryptic Peptide Fragments of P-glycoprotein—In these experiments HL60/Vinc or sensitive cells were labeled with 32P and membranes were prepared and thereafter treated with low levels of trypsin. The membranes were solubilized and immunoprecipitation was carried out in the presence of a site-specific antiserum (ASP45) against P-glycoprotein (Table I). The results of these studies demonstrate that a 55-kDa 32P-labeled fragment is selectively immunoprecipitated from membranes of resistant cells (Fig. 6). Other experiments have shown that P55 is not immunoprecipitated from untreated resistant membranes (not shown). The ASP45 antiserum (Table I) which was prepared against a COOH-terminal synthetic peptide containing amino acids 1167–1182 of P-glycoprotein should also react with a highly homologous sequence in the NH2-terminal region of P-glycoprotein containing amino acids 521–536 (20). Based on this specificity of ASP45 it would be predicted that P55 is derived from the COOH terminus of P-glycoprotein. This is based on the finding that the amino-terminal end of P-glycoprotein is highly glycosylated (20) and it would thus be predicted that a sequence from this end of the molecule could not extend into the ASP45 binding site and migrate in a polyacrylamide gel as a 55-kilodalton component. To confirm this, experiments were carried out in which membranes were incubated in the absence or presence of trypsin and the peptide fragments were analyzed by Western blot analysis using additional site-specific antisera (Table I). Antiserum against peptide 23 (Table I) which is directed to COOH-terminal sequences of P-glycoprotein (Table I). The binding sites for these antisera have only slight homology with P-glycoprotein sequences contained in the amino-terminal region of the molecule (20). Antiserum against peptide 23 (Table I) which is directed against an amino-terminal sequence and which has no homologous part in P-glycoprotein does not react with P55 (Fig. 7, Lane C). In the absence of trypsin treatment P55 is not detected in the isolated membranes (not shown). Further studies have also shown that the antiserum ASP12 immunoprecipitates 32P-labeled P55. P55 was not, however, immunoprecipitated by ASP23 (not shown). The results, taken

Fig. 5. Tryptic peptide and phosphoamino acid analysis of P-glycoprotein. HL60/Vinc cells suspended in TCG medium were labeled with 32P (200 μCi/ml) for 3 h at 37°C. At the end of the incubation period cell extracts were prepared and P-glycoprotein was immunoprecipitated and electrophoresed in an SDS/7% polyacrylamide gel. P-glycoprotein was detected in the unfixed gel by autoradiography and the gel slice containing the protein was excised. Tryptic peptide (A) and phosphoamino acid analysis (B) was carried out as under "Experimental Procedures." The letters S, T, and Y indicate the position to which serine, threonine, and tyrosine migrate, respectively. Radioactivity which migrates ahead of the amino acids represents free inorganic phosphate.

Fig. 6. Immunoprecipitation of P-glycoprotein tryptic peptide fragments. Sensitive and resistant HL60 cells suspended in TCG medium were labeled with 32P, (200 μCi/ml) for 3 h at 36°C. The cells were centrifuged and suspended in ice-cold 0.01 M Tris-HCl (pH 7.6), 1 μM MgCl2. The cells were homogenized with 25 strokes of a glass homogenizer and thereafter centrifuged at 500 × g for 5 min. The supernatant was collected and membranes were pelleted after centrifugation for 45 min at 10,000 × g. Cell membranes (50 μg/ml) were suspended in 0.1 M Tris-HCl (pH 7.6) and thereafter incubated with trypsin (5 μg/ml) for 15 and 30 min at 37°C. At the end of the incubation period trypsin inhibitor (20 μg/ml) was added and the membranes were solubilized in a solution containing 50 mM Tris-HCl (pH 8.0), 0.14 M NaCl, 2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, 0.5% deoxycholate. Membranes were incubated for 30 min on ice and thereafter centrifuged for 20 min at 10,000 × g. The supernatant was collected and immunoprecipitation was carried out as described using antiserum ASP45 (Table I). Proteins contained in the immunoprecipitates were analyzed after electrophoresis in an SDS/7% polyacrylamide gel. Lanes B and D, sensitive membranes, 15 and 30 min trypsin treatment, respectively. Lanes A and C, resistant membranes, 15 and 30 min trypsin treatment, respectively.

Fig. 7. Western blot analysis of a P-glycoprotein tryptic fragment. Unlabeled membranes were prepared from resistant cells and thereafter incubated in the absence or presence of trypsin as described in the legend to Fig. 6. At the end of the incubation period Western blot analysis was carried out as described using P-glycoprotein site-specific antisera (Table I). Antiserum used in these experiments were ASP11 (Lane A), ASP12 (Lane B), and ASP23 (Lane C).
together, strongly suggest that phosphorylated P55 is derived from the COOH terminus of P-glycoprotein.

Formic Acid Cleavage Products of \(^{32}\)P-Labeled P-Glycoprotein—Previous studies have shown that formic acid selectively cleaves proteins at Asp-Pro linkages (26). Based on the deduced sequence of human P-glycoprotein (20) it would be predicted that formic acid cleavage of this protein would result in five distinct fragments having the molecular weights indicated in Table II. In polyacrylamide gels the amino-terminal fragment 1 would migrate as higher molecular mass material (70–80 kDa) due to the presence of extensive glycosylation (20). To determine if the predicted fragments could be detected we carried out a Western blot analysis of formic acid cleaved P-glycoprotein. Site-specific antisera (Tables I and II) were used which should allow the detection of all fragments except fragment 3 (Table II). The results demonstrate that two of the four predicted formic acid cleavage products can be identified. Thus the antiserum ASP54 (Tables I and II) reacts with a 35-kilodalton fragment which would correspond to the predicted fragment 2 containing amino acids 446–744 (Table II) (Fig. 8A). ASP54 also reacts with a 20-kilodalton formic acid cleavage product (Fig. 8A). P20 would therefore correspond to the predicted fragment 5 which would contain an homologous ASP54 epitope. The reason for the doublet in this region of the gel is not known but the lower molecular weight component probably occurs as a result of degradation of P20. Further studies also show that the antiserum ASP12 prepared against an extreme COOH-terminal sequence of P-glycoprotein (Tables I and II) reacts with a 20-kDa fragment (Fig. 8D). This fragment would thus correspond to the predicted formic acid cleavage product (fragment 5) which contains amino acids 1089–1280 (Table II). For reasons that are not known, antisera ASP21 and ASP23 used in these studies do not react with specific formic acid cleavage products (Fig. 8). Further studies have been carried out to analyze phosphorylation levels of formic acid cleavage products derived from P-glycoprotein. Thus in these experiments \(^{32}\)P-labeled P-glycoprotein was eluted from a polyacrylamide gel and thereafter treated with formic acid as described under “Experimental Procedures.” The labeled fragments were analyzed after polyacrylamide gel electrophoresis. The results reveal the presence of two fragments containing the major portion of radioactivity (Fig. 9). The major labeled fragment has a molecular mass of 35 kilodaltons while a second fragment with less radioactivity has a molecular mass of 42 kilodaltons (Fig. 9). The phosphorylated P55 corresponds exactly in molecular weight to fragment 2 which is detected with the ASP54 antiserum (Fig. 8). The nature of P42 is not known but this fragment could represent a partial digest product. Only very low levels of radioactivity were detected in a fragment which would correspond to fragment 5 detected with the ASP12 antiserum (Fig. 8). This suggests that amino acids 1089–1280 of P-glycoprotein have only low levels of phosphorylation.

**DISCUSSION**

Previous studies have shown that P-glycoprotein is phosphorylated (16–18) and that phosphorylation levels of this protein are increased when resistant cells are incubated in the presence of certain calcium channel blockers (17, 27) or phorbol esters (17). It has also been found that as verapamil enhances P-glycoprotein phosphorylation there is a concomitant increase in cellular drug levels (16). This raises the possibility that as P-glycoprotein is hyperphosphorylated its biological function is inactivated. However studies with verapamil are complicated since this agent may inactivate P-glycoprotein through its interaction with drug binding sites (28, 29). Studies with 12-O-tetradecanoylphorbol 13-acetate are also unclear since this agent which enhances P-glycoprotein phosphorylation does not affect the cellular accumulation of 32P-labeled P-glycoprotein contained in immunoprecipitates was prepared as described in the legend to Fig. 5. After polyacrylamide gel electrophoresis, P-glycoprotein was detected in the unfixed gel and the gel slice containing the protein was excised. P-glycoprotein was electroeluted from the gel and formic acid cleavage and electrophoresis was carried out as described under “Experimental Procedures.” The nature of the upper bands in this figure is not known. Prolonged incubation with formic acid does not remove this material. Possibly formic acid treatment can produce a P-glycoprotein aggregate which does not enter the gel.
of drug (17). In view of these studies we have attempted to gain additional information concerning the involvement of phosphorylation in the function of P-glycoprotein. Thus in the present study we have examined the effect of inhibiting P-glycoprotein phosphorylation on drug transport processes in the HL60/Vinc multidrug-resistant isolate. The results show that under a variety of conditions the protein kinase inhibitor staurosporine (24) induces an inhibition in the phosphorylation of P-glycoprotein and a concomitant increase in the cellular accumulation of [3H]daunorubicin. The increased levels of drug appears to be related to a staurosporine inhibition of drug efflux in resistant cells. Since staurosporine does not affect drug accumulation in sensitive cells the results suggest that altered drug levels in resistant cells are not due to nonspecific membrane changes occurring in the presence of this agent. The results thus taken together suggest that as phosphorylation levels of P-glycoprotein are decreased there is a parallel decrease in the ability of the protein to remove drug from the resistant cell. Recent studies have demonstrated that with drug-resistant human KB carcinoma cells protein kinase C is capable of phosphorylating P-glycoprotein in vitro (30, 31). It was also found that in this cell system staurosporine reverses drug resistance (30) and H-7 can bring about an inhibition of P-glycoprotein phosphorylation (31). H-7 has also been found to partially reverse resistance in an isolate obtained from murine fibrosarcoma cells (32). In other studies it was shown that cyclic AMP-dependent protein kinase is capable of phosphorylating P-glycoprotein prepared from a drug-resistant mouse cell line (18). These results taken together suggest that reversal of resistance by staurosporine or H-7 in some isolates occurs through an inhibition of protein kinase C or cyclic AMP-dependent protein kinase. Previously we have obtained evidence that protein kinase P (33) is capable of phosphorylating P-glycoprotein from HL60/Vinc cells (34). Whether this enzyme actually phosphorylates P-glycoprotein in vivo remains however to be determined. In the present system we find that, although staurosporine inhibits P-glycoprotein phosphorylation and induces an increase in cellular drug accumulation, H-7 does not affect these reactions. Although the effect of staurosporine on protein kinase P has not been reported it has been found that H-7 at 100 \mu M is only a very weak inhibitor of this enzyme (35). This latter result is therefore consistent with our present findings. We are unable, however, to rule out the possibility that staurosporine is inhibiting protein kinase C in vivo and for reasons that are not known H-7 is not reacting intracellularly with this enzyme. The phosphorylation of P-glycoprotein may also be quite complex and possibly more than one protein kinase is involved in this event. Also, as indicated above, different resistant cell lines may utilize different protein kinases to phosphorylate P-glycoprotein.

Additional studies using pulse-chase experiments show that the phosphate groups of P-glycoprotein are metabolically active and that the protein is undergoing phosphorylation and dephosphorylation in the cell. The results of these studies thus provide further evidence that phosphorylation of P-glycoprotein plays an important role in its biological activity. Understanding further the protein kinases and phosphatases which regulate this phosphorylation event should provide important insight into the development of strategies for reversing drug resistance.

In view of the above findings we have carried out studies to identify sites of phosphorylation in P-glycoprotein. One approach taken has been to analyze phosphorylated cleavage products produced after treatment of P-glycoprotein with formic acid. Based on the deduced sequence of P-glycoprotein (20) formic acid which cleaves at asp-pro linkages (26) should produce five distinct fragments. To identify these cleavage products we have used antisera prepared against P-glycoprotein synthetic peptides (22). Four of the predicted formic acid cleavage products should contain epitopes detected by these antisera. Studies with Western blot analysis revealed that the antisera could detect 2 of the 4 predicted fragments and these could be unambiguously identified as those containing amino acids 446–744 (fragment 2, Table II) and amino acids 1089–1280 (fragment 5, Table II). Analysis of 32P-labeled formic acid cleavage products of P-glycoprotein yields a major component which corresponds exactly to fragment 2 identified by the ASP54 antisera. These results thus strongly suggest that a major phosphorylated domain of P-glycoprotein resides within the sequence containing amino acids 446–744. The results of these studies also suggest that since no major phosphorylated fragment 2 could be detected the extreme COOH terminus of P-glycoprotein (amino acids 1089–1280) contains only low levels of phosphorylation. A major phosphorylation domain in P-glycoprotein has also been identified through an analysis of tryptic peptide cleavage products. Thus it has been found that mild trypsin treatment of 32P-labeled P-glycoprotein results in the formation of a highly phosphorylated 85-kDa fragment which is selectively immunoprecipitated by P-glycoprotein site specific antisera. Mapping of P55 with these antisera strongly suggests that this fragment is derived from the COOH terminus of P-glycoprotein. Since this sequence is not predicted to be glycosylated P55 should represent a region extending from amino acids of about 775–1280. Based on an analysis of 32P-labeled formic acid cleavage products it is indicated that amino acids 1089–1280 have only very low levels of phosphorylation. It is thus suggested that the P55 phosphorylation sites are contained within amino acids of about 775–1088. Using a similar procedure for trypsin cleavage of membranes from resistant cells Yoshimura et al. (36) have also identified a P55 COOH-terminal fragment of P-glycoprotein which contains a binding site for azidopine. Recent studies indicate that P-glycoprotein and the cystic fibrosis transmembrane conductance regulator have a minor sequence homology and share certain structural features particularly with regard to their multiple transmembrane domains (37). The cystic fibrosis transmembrane conductance regulator has a major region of internal phosphorylation sites which may be similar to the location of phosphorylation domains in P-glycoprotein (37). Further localization of P-glycoprotein phosphorylation sites and subsequent studies using insertional mutagenesis should clarify the role of the phosphate groups in the biological function of P-glycoprotein.

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