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Stability and Covalent Modification of P-Glycoprotein in Multidrug-Resistant KB Cells

Nancy D. Richert,[‡] Lois Aldwin,[§] Danute Nitecki,[§] Michael M. Gottesman,[‡] and Ira Pastan*[,‡]

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, 37/4E16, Bethesda, Maryland 20892, and Cetus Corporation, 1400 Fifty-Third Street, Emeryville, California 94608

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ABSTRACT: An antipeptide antibody (P7) to P-glycoprotein has been produced by immunizing rabbits with a synthetic peptide. Antibody P7 is directed against the amino-terminal region of P170 (residues 28–35). The antibody immunoprecipitates a 170-kDa P-glycoprotein from extracts of drug-resistant KB-V1 cells that is not present in the drug-sensitive cell line KB-3-1. Antibody P7 was used to quantitate the amount of P-glycoprotein present in drug-resistant KB lines at various levels of resistance and to demonstrate the presence of P-glycoprotein in NIH 3T3 cells transfected with a cloned *MDR*1 cDNA or human genomic DNA encoding *MDR*1. Pulse–chase labeling experiments demonstrated that P-glycoprotein is synthesized as a 140-kDa precursor which is slowly converted over 2–4 h to a 170-kDa glycoprotein. Tunicamycin treatment blocks the conversion of the precursor to the mature form, and removal of N-linked oligosaccharides with Endo F reduces the relative molecular weight of P-glycoprotein to 140K. The mobility of mature P-glycoprotein is unaffected by treatment with neuraminidase and Endo H. These data indicate that P-glycoprotein is N-glycosylated and contains little or no neuraminic acid. P-Glycoprotein is also phosphorylated, and the extent of phosphate incorporated is proportional to the amount of protein present in drug-resistant cells.

Multidrug resistance (MDR) is the tissue culture phenomenon whereby cells, selected for resistance to a single drug, develop cross-resistance to multiple, structurally unrelated agents (Pastan & Gottesman, 1987; Riordan & Ling, 1985). A similar multidrug resistance phenotype occurs in human tumors and presents a major obstacle to successful cancer chemotherapy. To study this problem, we have developed MDR tissue culture cell lines resistant to colchicine, adriamycin, and vinblastine (Akiyama et al., 1985; Shen et al., 1986a). The characteristic features of multidrug-resistant cells are (1) decreased drug accumulation due to increased drug efflux (Fojo et al., 1985a; Inaba et al., 1979; Willingham et al., 1986), (2) reversibility of multidrug resistance by verapamil (Inaba et al., 1979; Tsuruo et al., 1982; Willingham et al., 1986), and (3) increased expression of the *MDR*1 gene (Fojo et al., 1985b; Gros et al., 1986a; Riordan et al., 1985; Roninson et al., 1984, 1986; Scotto et al., 1986; Shen et al., 1986b; Van der Bliek et al., 1986). This gene encodes a 170000-dalton plasma membrane glycoprotein, termed P-glycoprotein or P170 (Kartner et al., 1983). Recently, multidrug resistance has been transferred to drug-sensitive cells by *mdr* gene sequences (Debenham et al., 1982; Gros et al., 1986b; Shen et al., 1986c) and *mdr* cDNA (Gros et al., 1986c; Ueda et al., 1987). These results indicate that the presence of P-glycoprotein confers multidrug resistance.

Sequence analysis of the *MDR*1 gene indicates that human P-glycoprotein is composed of 1280 amino acids and consists of 2 homologous halves (Chen et al., 1986; Gros et al., 1986d). Each half of the molecule has six hydrophobic transmembrane domains, and each has a nucleotide binding site. The homology of the nucleotide binding regions to several bacterial transport proteins (Chen et al., 1986; Gerlach et al., 1986; Gros et al., 1986d), taken together with the plasma membrane localization of the protein (Willingham et al., 1987) and its ability to bind chemotherapeutic agents (Cornwell et al., 1986), suggests that P-glycoprotein could function as an energy-dependent efflux pump.

To elucidate the steps in the synthesis of P-glycoprotein and to define functional domains of this protein, it would be useful to have a panel of monospecific antibodies to various regions of the molecule. To date, three monospecific antibodies have been reported: C219, which recognizes an intracytoplasmic region in the COOH-terminus (Kartner et al., 1985), and MRK16 (Hamada & Tsuruo, 1986) and 265/F4 (Lathan et al., 1985), which recognize external determinants of P-glycoprotein which have not been identified. In the current work, we have prepared an antipeptide antibody based on the deduced amino acid sequence of the cloned human P-glycoprotein and used it to study the biosynthesis of P-glycoprotein in multidrug-resistant human KB cells.

* Address correspondence to this author.
‡ National Institutes of Health.
§ Cetus Corp.

Table I: Cross-Resistance of Multidrug-Resistant Cell Lines and Levels of P-Glycoprotein mRNA and Protein

cell line	selecting agent	concn (ng/mL)	*MDR*1 mRNA[a]	P-glycoprotein (ng/mg)[b]
	(I) KB Cell Lines			
KB-3-1	none	0	1	–[e]
KB-8	colchicine	5	14	–
KB-8-5	colchicine	10	42	–
KB-8-5-11	colchicine	100	240	143
KB-C4	colchicine	4000	1000	200
KB-C1-R1	revertant	0	1	–
KB-A1	adriamycin	100	900	200
KB-V1	vinblastine	1000	800	754
	(II) NIH-3T3 Cell Lines			
NIH-3T3	none	0	–	–
NIH-*mdr*[c]	colchicine	3000	>60[d]	–
T2C1 KB-C1.5 DNA	colchicine	1000	300[d]	140
NT12 *mdr*	colchicine	60	300[d]	55

[a] Fojo et al. (1987b); Shen et al. (1986b). [b] The radioactivity in the P170-kDa protein band was quantitated by solubilizing and counting in Aquasol. From the specific activity of total cell protein, the amount of P-glycoprotein (nanograms per milligram of extract) was made. [c] NIH 3T3 cells selected for multidrug resistance in colchicine have an amplified mouse *mdr* gene and are designated NIH-*mdr* (Shen et al., 1986c). [d] The *MDR*1 RNA expression for NIH 3T3 and NT-12 is estimated from Ueda et al. (1987) and for T2C1 and NI-*mdr* cells are from Shen et al. (1986c). The *mdr* RNA level in NIH-*mdr* may be underestimated, since a human *MDR*1 hybridization probe was used. NT-12 cells have two transcripts (11 and 6 kb) derived from the retroviral vector, and the total amount of *MDR*1 RNA is given. This may overestimate translatable *MDR*1 RNA. [e] Not detectable by this assay method.

Experimental Procedures

Synthetic Peptide Antibodies to P-Glycoprotein. The peptide representing an internal domain of P-glycoprotein was synthesized by the solid-phase method (Merrifield, 1985). Cysteine was added to the N-terminus of the peptide, and coupling to keyhole limpet hemocyanin (KLH) was performed with *N*-maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzenesulfonic acid as a coupling reagent (Aldwin & Nitechi, 1987). For rabbit immunizations, 500 μg of peptide–KLH conjugate was emulsified in complete Freund's adjuvant and injected intradermally at multiple sites. Injections were performed at 3-week intervals for a total of 9 weeks. Booster injections were performed in incomplete adjuvant. Rabbits were bled at weekly intervals, and antisera were screened for their ability to immunoprecipitate 170-kDa [^{35}S]methionine-labeled P-glycoprotein from labeled cell extracts. The positive antiserum was purified on an affinity column containing a peptide–bovine serum albumin (BSA) conjugate coupled to Affigel-10.

Cell Lines. The derivation and cross-resistance of the multidrug-resistant (MDR) KB sublines have been previously described (Akiyama et al., 1985; Shen et al., 1986a,b). KB-3-1 is the drug-sensitive parental cell line. KB sublines selected in colchicine, adriamycin, and vinblastine are summarized in Table I. The cell line designated T2Cl is a mouse NIH 3T3 cell line transformed in two steps with high molecular weight DNA originally derived from the multidrug-resistant subline KB-C1.5 (Shen et al., 1986c). The NIH 3T3 cell line transfected with a full-length *MDR*1 cDNA carried on a retroviral expression vector is designated NT12 (Ueda et al., 1987).

Metabolic Labeling of Cell Cultures. For metabolic labeling, cells were plated at 4 × 10^6 cells/100-mm dish, 6 h prior to use. Cells were labeled for 16 h with [^{35}S]methionine (250 μCi/mL; 800 Ci/mmol; 1 Ci = 37 GBq; Amersham) in methionine-free modified Eagle's medium with 5% fetal bovine serum. For [^{32}P]P_i labeling, cells were incubated for 16 h with [^{32}P]orthophosphate (1 mCi/mL; New England Nuclear) in phosphate-free Dulbecco's modified Eagle's medium (DME) with 5% dialyzed fetal bovine serum.

For labeling with ^{14}C sugars, cells were plated at the same density but were labeled the next day for 6 h in DME containing 2 mM glucose, 5% fetal bovine serum, and 10 μCi/mL [^{14}C]glucosamine (350 mCi/mol), [^{14}C]galactose (358 mCi/mmol), [^{14}C]mannose (238 mCi/mmol), or [^{14}C]fucose (55 mCi/mmol). All radioactive sugars were from New England Nuclear. Tunicamycin experiments were also performed 1 day after the cells were plated. The cells were treated with 10 μg/mL tunicamycin (Calbiochem) for 5 h and then labeled with [^{35}S]methionine for 3 h.

Immunoprecipitation. Labeled cells were lysed in RIPA buffer (1% Triton X-100/0.1% $NaDodSO_4$/1% sodium deoxycholate/0.15 M NaCl/20 mM Tris-HCl, pH 7.2), using 1 mL/dish, and the extracts were clarified at 100000*g* for 30 min. Cell extracts were precleared with 200 μL of formalin-fixed *Staphylococcus aureus* (10% w/v) at 4 °C for 15 min to reduce nonspecific binding to the immunoadsorbant. The total radioactivity of each cell extract was quantitated by trichloroacetic acid precipitation (Richert et al., 1985), and protein was determined by the Bio-Rad assay using bovine γ-globulin as a standard.

For immunoprecipitation, (2–10) × 10^6 cpm of each cell extract was incubated with 20 μL of rabbit antipeptide serum at 4 °C for 1 h. Then 100 μL of *S. aureus* was added for an additional 30 min. The pellets were washed once with RIPA, then with RIPA containing 2.5 M KCl, and once again with RIPA. The pellets were resuspended in 50 μL of Laemmli sample buffer (Laemmli, 1970), and the samples were incubated at room temperature for 10 min to allow antibody–antigen complexes to dissociate from *S. aureus*. This procedure was adopted because the standard method of elution by boiling at 100 °C for 3 min causes aggregation of P-glycoprotein (S. B. Horwitz, personal communication). Samples were analyzed on 7% Laemmli gels or on modified Fairbanks gels (Debenham et al., 1982; Fairbanks et al., 1971). The gels were fluorographed (Bonner & Laskey, 1974) and exposed to X-ray film at −70 °C.

Glycosidase Treatment. Endo-β-*N*-acetylglucosaminidase H (Endo H) and Endo F were obtained from Genzyme. Neuraminidase type VIII was from Sigma. For neuraminidase digestion, immunoprecipitates were resuspended in 50 μL of 50 mM NaOAc (pH 5.5)/1 mM $CaCl_2$/2 mM phenylmethanesulfonyl fluoride (PMSF) and 5 milliunits of neuraminidase. For endoglycosidase digestion, immunoprecipitates were resuspended in 100 μL of 0.1 M Tris-HCl (pH 7.5)/6% $NaDodSO_4$, and immune complexes were eluted from *S. aureus* at 24 °C for 10 min. The *S. aureus* was removed by centrifugation, and the samples were acetone precipitated (Solari & Kraehenbuhl, 1984). For Endo H digestion, the precipitated samples were resuspended in 50 μL of 50 mM sodium phosphate (pH 6.1)/0.1% $NaDodSO_4$/0.1 mM PMSF and digested with 50 mIU of enzyme. For Endo F digestion, samples were resuspended in 50 μL of 0.1 M sodium phosphate (pH 6.1)/50 mM EDTA/0.5% Nonidet-P40 and 0.8 unit of enzyme. All digestions were performed at 37 °C for 20 h.

Western Blotting. Membranes were prepared by the method of Riordan and Ling (1979) and analyzed on $NaDodSO_4$/PAGE gels using the method of Fairbanks (Fairbanks et al., 1971) as modified by Debenham (Debenham et al., 1982). The proteins (50 μg/lane) were transferred to nitrocellulose

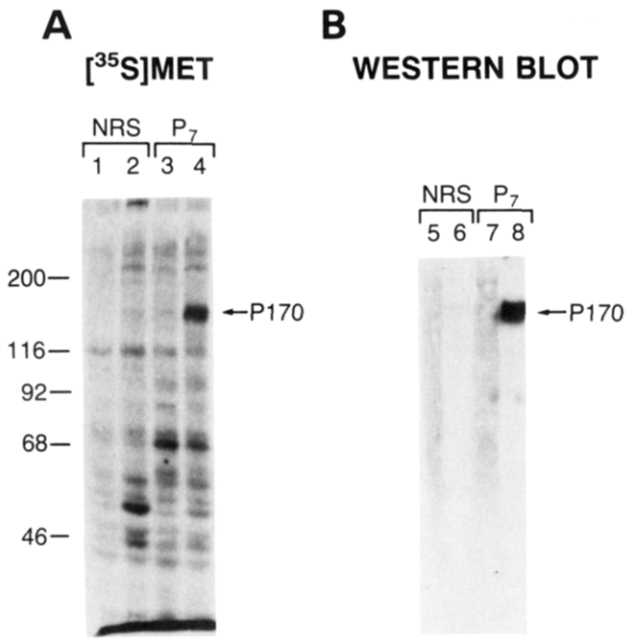

FIGURE 1: Immunoprecipitation and Western blotting with antipeptide antisera. (A) [^{35}S]Methionine-labeled cell extracts were prepared from drug-sensitive KB-3-1 cells (lanes 1 and 3) and from MDR KB-V1 cells (lanes 2 and 4) and immunoprecipitated with normal rabbit serum (NRS) or with antipeptide serum P7. Immunoprecipitates were analyzed on a 7% Laemmli gel. The molecular mass standards are as follows: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92 kDa), bovine serum albumin (68 kDa), and ovalbumin (46 kDa). (B) Membrane proteins (50 μg) from KB-3-1 (lanes 5 and 7) and KB-V1 cells (lanes 6 and 8) were electrophoresed on a modified Fairbanks gel (Debenham et al., 1982; Fairbanks et al., 1971) as described under Experimental Procedures and then transferred to nitrocellulose and immunoblotted with NRS or P7 antibody.

in Towbin buffer (Towbin et al., 1979) at 200 mA overnight at room temperature. The blots were incubated in "Blotto" blocking buffer (10% Carnation instant nonfat dry milk in Dulbecco's phosphate-buffered saline) at 24 °C for 1 h. Antibody incubation was carried out at 4 °C overnight using 2 μL of antibody/mL of "Blotto". Blots were washed 3 times for 5 min each time in the same buffer with 0.5% Tween-20 (Bio-Rad) and then incubated with ^{125}I protein A (5×10^5 cpm/mL) for 90 min at room temperature in "Blotto" containing 0.3% Tween-20. The wash procedure was repeated, and blots were exposed to film at −70 °C.

Results

Antibody P7, a site-specific antibody to P170, was prepared by immunizing rabbits with a chemically synthesized peptide ([Cys]-Lys-Glu-Lys-Pro-Thr-Val-Ser-Val-Phe-Ser). Antibody P7 is directed against amino acids lying near the amino terminus of P-glycoprotein (amino acids 28–35). On the basis of the most plausible model of the structure of P-glycoprotein, peptide 7 should be from an internal (cytoplasmic) domain (Chen et al., 1986).

Antipeptide antisera from several rabbits were screened for their ability to immunoprecipitate P-glycoprotein from [^{35}S]methionine-labeled extracts of drug-resistant cells. Figure 1 shows that antibody P7 precipitates a 170-kDa protein from resistant KB-V1 cells (lane 4) which is not present in the drug-sensitive parent line KB-3-1 (lane 3). No proteins in this molecular weight region are precipitated by normal rabbit serum (NRS) (lanes 1 and 2). Antiserum P7 also reacted with P-glycoprotein when Western blots were prepared from KB-V1 membranes (lane 8). No reaction was detected with KB-3-1

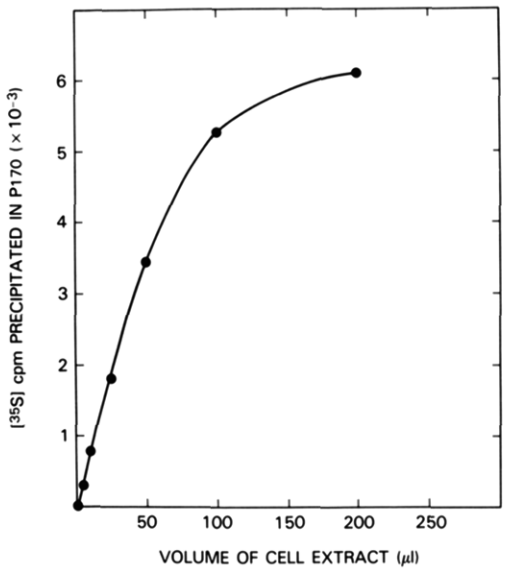

FIGURE 2: Titration of P-glycoprotein antipeptide antibody. KB-V1 cells were labeled with [^{35}S]methionine and extracted in RIPA (1 mL/100-mm dish of cells). Increasing concentrations of cell extract (6.2×10^7 acid-precipitable cpm/mL) were immunoprecipitated with 20 μL of P7 antibody. After electrophoresis, the radioactivity in the P-glycoprotein band was determined by solubilizing the gel slice in 2 mL of 30% H_2O_2/1% NH_4OH overnight at 39 °C and then counting in 15 mL of Aquasol. Background radioactivity was determined by counting another region of the same gel lane.

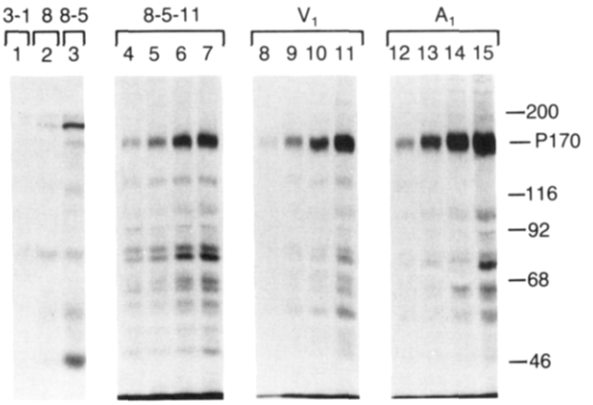

FIGURE 3: Immunoprecipitation of MDR KB cell lines with antibody P7. Radiolabeled KB cell lines were extracted in RIPA and immunoprecipitated with 20 μL of P7 antibody. For KB-3-1, KB-8, and KB-8-5 cells (lanes 1, 2, and 3), respectively, the entire extract from one dish of cells (90×10^6 acid-precipitable cpm) was incubated with antibody P7. Because of the large amount of protein, several background bands, also seen with NRS (data not shown), can be seen in lanes 1–3. For KB-8-5-11 extracts (35×10^6 cpm/mL), increasing concentrations of extract were immunoprecipitated with antibody. In lanes 4–7, the extract was 50, 100, 250, and 500 μL, respectively. For KB-V1 cell extract (47×10^6 cpm/mL), concentrations were 10, 25, 50, and 100 μL in lanes 8–11, respectively, and for KB-A1 cells (70×10^6 cpm/mL), extract concentrations were 25, 50, 100, and 200 μL in lanes 12–15, respectively.

membranes (lane 7) or with NRS on either cell type (lanes 5 and 6). The antiserum did not react with formalin-fixed permeabilized cells or living cells when examined by immunofluorescence.

To establish quantitative reaction conditions for immunoprecipitation, the binding capacity of the P7 antiserum was determined. Increasing concentrations of KB-V1 cell extract were incubated with a fixed volume of antiserum, and the amount of P170 precipitated was quantitated. The results (Figure 2) show that immunoprecipitation is linear at cell extract concentrations ranging from 5 to 100 μL.

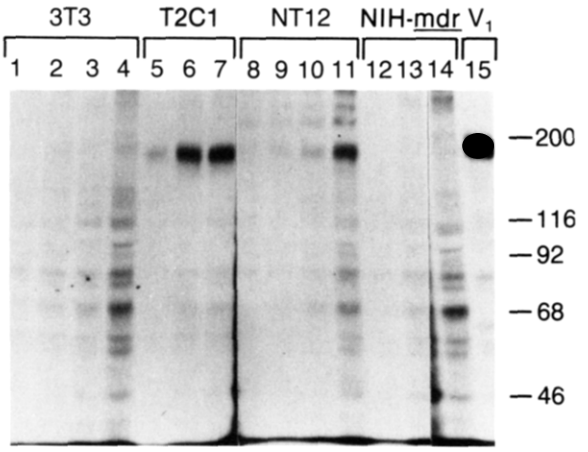

FIGURE 4: Immunoprecipitation of P-glycoprotein from mouse NIH 3T3 cells. Cells were labeled with methionine overnight and extracted into 1 mL of RIPA. Increasing concentrations of cell extract were immunoprecipitated with P7 antibody. For NIH 3T3 and NT12 (35×10^6 cpm/mL), extract concentrations were 50, 100, 250, and 500 μL (lanes 1–4 and lanes 8–11, respectively); for T2Cl (33×10^6 cpm/mL), 25, 50, and 100 μL were used (lanes 5–7); for NIH-*mdr* (32×10^6 cpm/mL), 50, 100, and 500 μL were used (lanes 12–14); for KB-V1 cell extract (47×10^6 cpm/mL), 100 μL was used (lane 15).

To determine if the amount of protein precipitated by antiserum P7 is correlated with the degree of drug resistance, extracts of various KB sublines were immunoprecipitated with anti-P7. Figure 3 shows that the 170-kDa protein could not be detected in KB-3-1 cells, in KB-8 cells (2-fold resistant to colchicine), or in KB-8-5 cells (4-fold resistant to colchicine). In KB-8-5-11 cells which are 40-fold resistant to colchicine and in vinblastine- and adriamycin-resistant cells which are several hundredfold resistant (KB-V1 and KB-A1, respectively), the protein is readily detected. The 170-kDa protein is not detected in revertant cell lines which have almost completely lost the drug-resistant phenotype (Table I).

Previous studies demonstrated that NIH 3T3 cells were rendered drug resistant after transformation with high molecular weight DNA from multidrug-resistant KB cells (Shen et al., 1986c) or by transfection with a full-length cDNA derived from the human *MDR*1 gene (Ueda et al., 1987). To demonstrate that human P-glycoprotein was synthesized in these cells and that the protein produced was of the appropriate size, antibody P7 was used to immunoprecipitate labeled cell extracts. The results in Figure 4 demonstrate that detectable levels of P-glycoprotein are present in both T2C1 and NT12 cells. The protein could not be detected in the parent NIH 3T3 cells by antibody P7. The P7 antibody does not cross-react with mouse P-glycoprotein as evidenced by the failure to precipitate P170 from extracts of mouse cells (NIH-*mdr*) which have been selected in colchicine and have amplified copies of the endogenous mouse *mdr* gene. This confirms that the P-glycoprotein expressed in the transfectants is encoded by the human genomic DNA or *mDR*1 cDNA transferred into these cells.

Table I summarizes the results of all cell lines tested and demonstrates that at high levels of resistance there is an association between the amount of P-glycoprotein present and the degree of drug resistance in these various cell lines.

Biosynthesis and Turnover of P-Glycoprotein. To study the biosynthesis and turnover of P-glycoprotein in multidrug-resistant KB cells, pulse–chase labeling experiments were performed. The results presented in Figure 5 demonstrate that P-glycoprotein is synthesized as a 140-kDa precursor during a 15-min pulse labeling and is subsequently converted to the

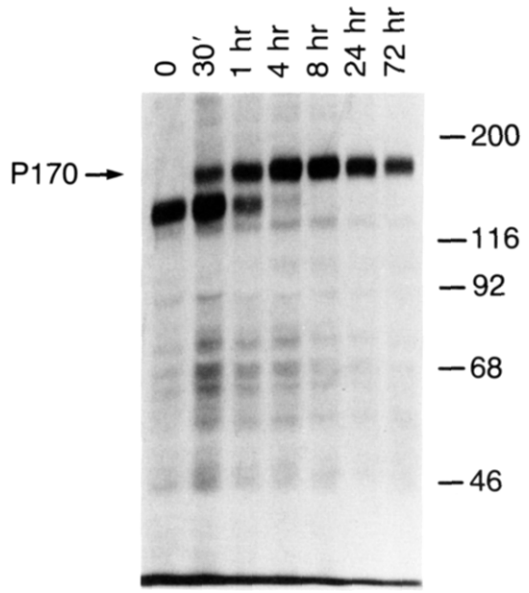

FIGURE 5: Pulse–chase labeling of P170 in KB-V1 cells. KB-V1 cells were plated at 6×10^5 cells/35-mm dish 1 day prior to use. The cells were rinsed twice with phosphate-buffered saline and then labeled for 15 min at 37 °C with [^{35}S]methionine. After being labeled, the cells were either harvested immediately (T_0 or rinsed with phosphate-buffered saline and transferred into standard growth medium and harvested at various times thereafter.

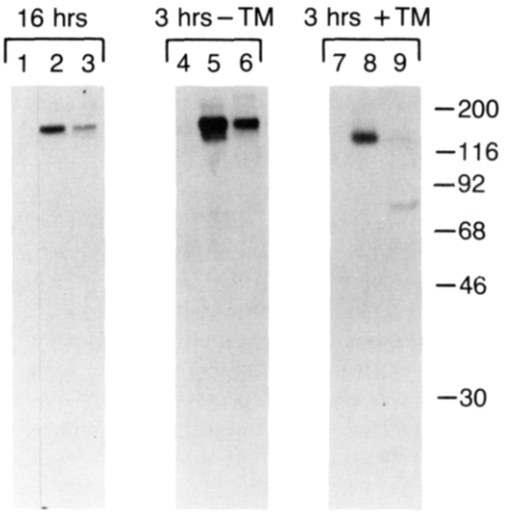

FIGURE 6: Tunicamycin treatment of KB-V1 cells. KB-V1 cells were labeled with [^{35}S]methionine for 16 h in the standard manner (lanes 1–3), or the cells were labeled for 3 h after treatment with tunicamycin (10 mg/mL) for 5 h (lanes 7–9) or without tunicamycin (lanes 4–6). Immunoprecipitations were performed with NRS (lanes 1, 4, and 7) or with P7 antibody (lanes 2, 5, and 8) or with antibody to the EGF receptor previously described (Van der Bliek et al., 1986) in lanes 3, 6, and 9. The samples were run on a 10% Laemmli gel.

170-kDa form during the next 2–4 h. Approximately half of the label present at 4 h remains in the protein 68 h later, indicating that P-glycoprotein is a very stable protein. In other experiments, the half-life of P-glycoprotein in KB-8-5-11 cells was measured and found to be very similar to that in KB-V1 cells (data not shown).

Glycosylation of P-Glycoprotein. The amino acid sequence of human P-glycoprotein indicates that it contains 10 potential sites of N-glycosylation (Chen et al., 1986) but only 3 of these sites are oriented on the external surface of the plasma membrane if the published models are correct (Chen et al., 1986). To study the glycosylation of P-glycoprotein, KB-V1 cells were treated with tunicamycin for 5 h prior to labeling (Figure 6).

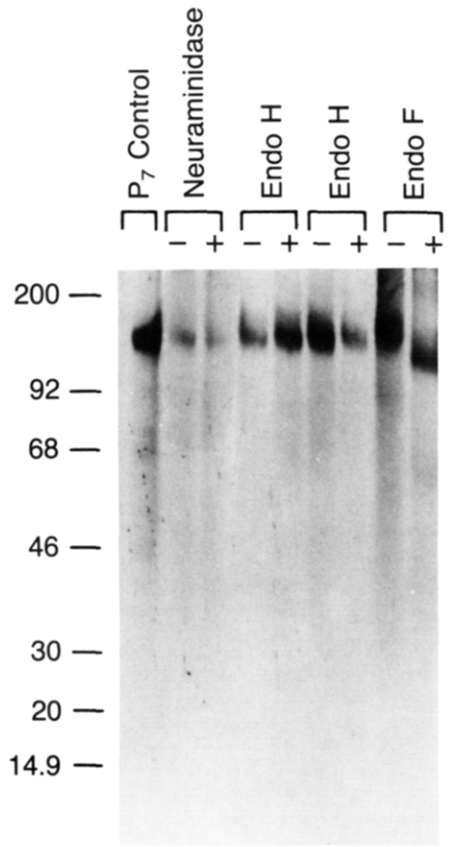

FIGURE 7: Glycosidase treatment of P-glycoprotein in KB-V1 cells. [^{35}S]Methionine-labeled P170 from KB-V1 cells was isolated by immunoprecipitation with P7 antibody. The immunoprecipitates were subjected to no treatment (P7 control) or were incubated for 20 h at 37 °C in the presence or absence of neuraminidase. Endo H digestion was performed on samples that were heat denatured at 100 °C for 3 min (the fourth and fifth lanes) or on samples that were not heated (the sixth and seventh lanes). Samples were analyzed on modified Fairbanks gels (Debenham et al., 1982; Riordan & Ling, 1979). Ovalbumin showed a reduction in molecular weight after a parallel Endo H digestion (data not shown).

In the absence of N-glycosylation, P-glycoprotein migrates as a 140-kDa protein, its predicted molecular mass in the absence of glycosylation (Chen et al., 1986) (Figure 6, lane 8). The nonglycosylated form comigrates with the EGF receptor precursor (lane 9) which has a molecular weight of 139K. It also comigrates with the 140-kDa P-glycoprotein precursor seen in pulse–chase labeling experiments (lane 5, lower band and data not shown).

The nature of the glycosylation was studied using glycosidase digestion. Figure 7 shows that [^{35}S]methionine-labeled P-glycoprotein did not shift in mobility after treatment with neuraminidase or endoglycosidase H. The molecular weight was shifted by Endo F. *O*-Glycanase had no effect (data not shown). The incorporation of ^{14}C-labeled sugars into P-glycoprotein is shown in Figure 8. *N*-Acetylglucosamine (lanes 3 and 4) and galactose (lanes 5 and 6) effectively label P170. With fucose and mannose, the labeling is poor (data not shown).

Phosphorylation of P170. To determine whether P-glycoprotein is phosphorylated in multidrug-resistant KB cells and whether the extent of phosphorylation correlates with drug resistance, KB-3-1 cells, KB-8-5-11 cells, and KB-V1 cells were labeled with [^{32}P]orthophosphate for 16 h, and the extracts were immunoprecipitated with affinity-purified P7. The results in Figure 9 demonstrate that P-glycoprotein is phosphorylated in both multidrug-resistant cell lines. The amount of phosphorylation is proportional to the amount of *P*-glycoprotein

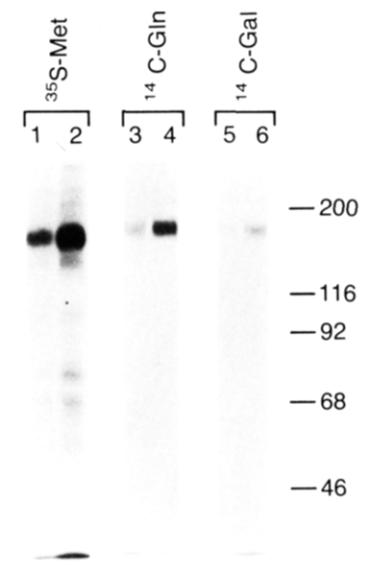

FIGURE 8: Metabolic labeling with ^{14}C sugars. KB-V1 cells were labeled for 6 h with [^{35}S]methionine (200 μCi/mL) or with 10 μCi/mL ^{14}C-labeled *N*-acetylglucosamine (^{14}C-Gln) or [^{14}C]galactose (^{14}C-Gal) as described under Experimental Procedures. Cells were extracted and immunoprecipitated using 0.1 mL of extract (10 000 acid-precipitable cpm) in lanes 3 and 5 or 0.5 mL of extract in lanes 4 and 6. For [^{35}S]methionine extracts, 0.1 mL (5.4×10^6 cpm) was used in lane 1, and 0.5 mL was used in lane 2. After fluorography, the gel containing methionine-labeled proteins was exposed to film for 5 h at −70 °C, and the ^{14}C-labeled proteins were exposed for 2 weeks at −70 °C.

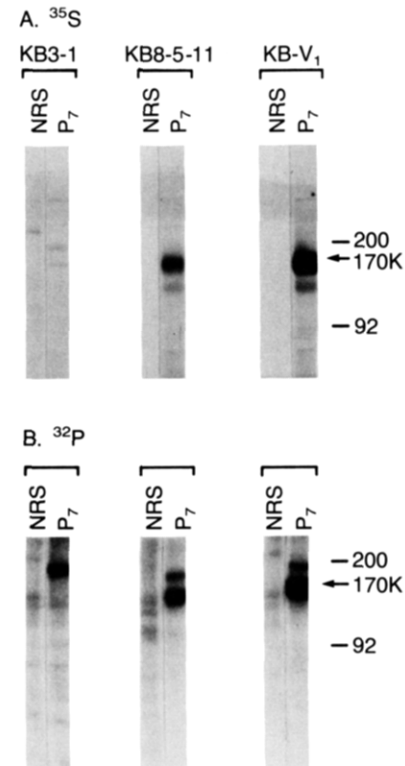

FIGURE 9: Phoshorylation of P-glycoprotein. Duplicate plates of KB-3-1, KB-8-5-11, and KB-V1 were seeded at 4×10^6 cells/dish and then labeled for 16 h with [^{35}S]methionine (panel A) or [^{32}P]-orthophosphate (panel B). Extracts were immunoprecipitated with affinity-purified P7 antibody (20 μg) or with NRS. For [^{35}S]-methionine extracts, 4×10^6 acid-precipitable cpm were used, and for [^{35}P]orthophosphate, 28×10^6 acid-precipitable cpm were used. This represented about 0.25 mL of each extract.

present (compare ^{35}S in the upper panel with ^{32}P labeling in the lower panel). P7 also precipitates a 200-kDa phospho-

protein from these extracts which is poorly labeled with methionine. This protein is not related to drug resistance because it is also present in KB-3-1 cells. Presumably, the 200-kDa phosphoprotein is a minor normal cell protein with an epitope which is recognized by antibody P7.

Discussion

To study the biosynthesis of the product of the *MDR*1 gene, P-glycoprotein, a rabbit antiserum to a peptide (P7) located in an intracellular portion of P-glycoprotein was prepared. The peptide contains amino acids 28–35 which lie between the first and the second methionines in the deduced amino acid sequence for P-glycoprotein (Chen et al., 1986). Since an antiserum against P7 detects P-glycoprotein in drug-resistant cells, we conclude that the first methionine in the deduced sequence is the initiating methionine. The anti-P7 serum is especially useful for investigation of the biosynthesis of P-glycoprotein because it reacts with a defined sequence close to the amino terminus of the protein. Other antibodies to P-glycoprotein have been prepared, but the precise region of P-glycoprotein with which they react either is not known (Hamada & Tsuruo, 1986; Lathan et al., 1985) or is in the carboxy terminus (Kartner et al., 1985).

Cell fractionation showed that all of the P-glycoprotein in KB-V1 cells was localized in the 100000*g* pellet fraction, when cells were homogenized in hypotonic buffer and subjected to differential centrifugation (data not shown). Subsequent experiments demonstrated that P-glycoprotein was totally solubilized by RIPA extraction of whole cells. To quantitate P-glycoproein and to estimate the sensitivity of the immunoprecipitation assay, P-glycoprotein was measured in seven KB sublines with various degrees of drug resistance as well as in NIH 3T3 transfectants which expressed the MDR phenotype. In general, there was an association between the degree of resistance and the amount of P-glycoprotein precipitated. As we have previously noted (Ueda et al., 1986), using monoclonal antibody C219 which recognizes a carboxy-terminal antigen (Kartner et al., 1985), P-glycoprotein was not detected in the drug-sensitive parent (KB-3-1) and could not be detected in the KB-8 or KB-8-5 cell lines which have low levels of resistance even though high concentrations of these cell extracts were used. In contrast, *MDR*1 RNA could be detected in KB-8 and KB-8-5 cells. The immunoprecipitation assay is therefore not as sensitive as the RNA blot assay (Table I) in detecting *MDR*1 gene expression in cells with low levels of drug resistance. To rule out the possibility that our inability to detect P-glycoprotein in KB-8 and KB-8-5 cells after [^{35}S]methionine labeling was due to a low synthetic rate or a high turnover rate of newly synthesized protein, membranes from KB-3-1, KB-8, KB-8-5, and KB-V1 cells were reacted with antibody P7 on immunoblots to determine steady-state levels of protein. Only KB-V1 cells gave a strong positive reaction. Thus, it is unlikely that antibody P7 will be as useful for measuring P-glycoprotein expression in human tumors as RNA blotting, because the level of *MDR*1 gene expression in many tumors is often comparable to that in KB-8-5 cells (Fojo et al., 1987a,b).

The biosynthesis and turnover of P-glycoprotein in KB-V1 cells was examined by pulse–chase labeling using a short 15-min labeling period. P-Glycoprotein is synthesized as a 140-kDa precursor during the 15-min pulse, and the precursor size is close to the molecular weight predicted by the amino acid sequence of P-glycoprotein. The precursor is converted to the 170-kDa mature form in a slow process requiring 2–4 h. The conversion appears to be due to the addition of N-linked oligosaccharides, because treatment with tunicamycin or Endo F reduces P-glycoprotein to the 140-kDa precursor molecular weight. The slow conversion is surprising because N-linked sugars are generally added cotranslationally in the endoplasmic reticulum, and the EGF receptor in these cells is N-glycosylated to its 170-kDa mature form within 15 min (Beguinot et al., 1985). A possible explanation for the slow conversion of P-glycoprotein to its mature form may be related to how the 12 hydrophobic segments are inserted into the membrane or how a protein with this many hydrophobic segments travels from the endoplasmic reticulum to the plasma membrane. Only one 140-kDa precursor and one major 170-kDa glycosylated form of P-glycoprotein were seen in the multidrug-resistant KB cells, in contrast to a recent report showing multiple forms of P-glycoprotein in taxol-selected multidrug-resistant mouse macrophage lines (Greenberger et al., 1987).

The half-life of P-glycoprotein is very long (48–72 h) in KB-V1 cells. The EGF receptor is degraded in the same cells with a half-life of about 4 h (Beguinot et al., 1985; data not shown). Thus, P-glycoprotein is an extremely stable protein. Previous electron microscopic studies suggested that P-glycoprotein was found all over the cell surface except in coated pits (Willingham et al., 1987). It also was not found in endocytic vesicles (Willingham et al., 1987), suggesting it might be relatively immobile in the plasma membrane and not readily internalized. The stability of P-glycoprotein at the cell surface suggests that when possible biological effects of biosynthesis and glycosylation inhibitors on P-glycoprotein function are being evaluated, prolonged incubation with the inhibitor will be essential, to allow complete turnover of preexisting protein.

From the labeling experiments and glycosidase digestions, the following features of P-glycoprotein glycosylation are apparent. First, all of the oligosaccharide side chains appear to be N-linked because tunicamycin and Endo F treatments convert P-glycoprotein to a lower molecular weight form that comigrates with the 140-kDa nonglycosylated precursor. The probable absence of O-linked sugars is also supported by the inability of *O*-glycanase digestion to affect the molecular weight of P170. Second, the Endo H resistance of P170 suggests that the oligosaccharides are of the "complex" rather than the "high mannose" type. Such side chains often consist of terminal trisaccharide units of GlcNac-Gal-NANA (*N*-acetylneuraminic acid). The presence of GlcNac and Gal was confirmed by metabolic labeling with [^{14}C]galactose and glucosamine. NANA appears not to be present or to be present in only small amounts since the mobility of P-glycoprotein is not affected by neuraminidase. Therefore, the side chains could be truncated disaccharide units or could contain fucose instead of NANA. P-Glycoprotein was poorly labeled with fucose and mannose, and further experiments are needed to confirm in detail the structure of its sugar moiety.

The phosphorylation of P-glycoprotein in KB cells was examined by labeling with [^{32}P]orthophosphate. The phosphate incorporation into P-glycoprotein was roughly proportional to the amount of protein present. Thus, in KB-V1 cells, phosphorylation is 5-fold higher than in KB-8-5-11 cells, and this is consistent with the difference in protein levels. Recent results by Hamada et al. (1987) demonstrate that agents, e.g., verapamil and trifluoperizine, which reverse the drug-resistant phenotype also stimulate the phosphorylation of P-glycoprotein. Their data suggest that phosphorylation may regulate the activity of P-glycoprotein. Our results, in which the specific activity of phosphate incorporation into P-glycoprotein remains constant for two cell lines of varying resistance, suggest that the amount of P-glycoprotein, rather than the extent of its

phosphorylation, determines the multidrug resistance of these cell lines in the absence of verapamil.

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