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Distinct P-glycoprotein precursors are overproduced in independently isolated drug-resistant cell lines

(multidrug resistance/vinblastine/taxol/colchicine)

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ABSTRACT **A family of P-glycoproteins are overproduced in multidrug-resistant cells derived from the murine macrophage-like line J774.2. To determine whether individual family members are overproduced in response to different drugs, the P-glycoprotein precursors in several independently isolated cell lines, which were selected for resistance to vinblastine or taxol, were compared. Individual cell lines selected with vinblastine overproduced P-glycoprotein precursors of either 120 or 125 kDa. Taxol-selected cell lines overproduced either the 125-kDa precursor or both precursors simultaneously. Two similar but distinct peptide maps for the mature P-glycoproteins were observed. These maps corresponded to each precursor regardless of the drug used for selection. One vinblastine-resistant cell line switched from the 125- to the 120-kDa precursor when grown in increasing concentrations of drug. This change coincided with the overexpression of a distinct subset of mRNA species that code for P-glycoprotein. It is concluded that precursor expression is not drug-specific. These data suggest that individual overproduced P-glycoprotein family members are translated as distinct polypeptides. The results may help to explain the diversity in the multidrug-resistant phenotype.**

Acquired or inherent resistance to antineoplastic drugs is likely to be a major reason for ineffective cancer chemotherapy (1). To understand the molecular basis of this phenomenon, tumor cells have been grown in tissue culture in the presence of antineoplastic drugs. This often results in the emergence of a population of cells that are resistant to the selecting drug as well as crossresistant to a variety of structurally and functionally unrelated drugs (see refs. 2 and 3 for review).

A second phenotypic characteristic of multidrug resistance is the overproduction of a plasma membrane-associated glycoprotein known as P-glycoprotein (4). Overproduction of the protein is mediated by overexpression of mRNA, as well as amplification of a multigene family that codes for the protein (5–10). Partial or complete sequence analyses of cDNA clones that code for P-glycoprotein from human, mouse, or hamster cells have suggested that the protein has 12 membrane-spanning domains, two nucleotide binding sites, a high degree of sequence similarity with bacterial transport proteins, and a polypeptide of 140 kDa (11–13). When a cDNA that codes for the protein is used to transfect cells, such cells become multidrug-resistant (14, 15). These findings, and the observations that photoaffinity analogs of vinblastine (16, 17) and ATP (18) bind to P-glycoprotein and that multidrug-resistant cells have low levels of intracellular drug (19), are consistent with the hypothesis that P-glycoprotein is an energy-dependent drug-efflux pump.

Multidrug-resistant cell lines can be distinguished by their resistance profiles; resistance is often greatest to the agent used for selection (2, 20–22). Since P-glycoprotein plays a central role in multidrug resistance, two related questions arise: do different types of P-glycoprotein molecules exist and, if so, can distinct types be selected in response to individual drugs? Characterization of the P-glycoproteins that were overproduced in J774.2 multidrug-resistant cells that were selected for resistance to colchicine, vinblastine, or taxol indicated that these cells overproduced antigenically related P-glycoproteins with distinct electrophoretic mobilities (23, 24). The basis for the difference between the colchicine- and vinblastine-selected cell lines was due, at least in part, to differential N-linked glycosylation of a common precursor of 125 kDa. In contrast, a taxol-selected cell line made two P-glycoproteins. The mature species were derived from two precursors, of 120 and 125 kDa, that remained distinct after N-linked-deglycosylation (25). To further study the relationship between the drug used for selection and the expression of P-glycoprotein precursors, we have examined several independently isolated cell lines selected for resistance to vinblastine or taxol. We report here that individual multidrug-resistant cell lines can express one or both precursors and that expression is not drug-specific. One cell line that switched expression of its precursor as the selecting drug concentration was increased provides insight as to how the expression of two precursors may have arisen.

MATERIALS AND METHODS

Cells. Independently isolated cell lines, selected from J774.2 mouse macrophage-like cells for resistance to vinblastine or taxol, were maintained in medium containing drug, as described (21). In some cases, drug-resistant cells were maintained at each stepwise increase in drug concentration. These steps were made at intervals of ≈5 weeks. Cell lines in this report are designated as in the following example: in J7.V1-1, "J7" indicates the parental cell line, "V1" indicates that it was the first cell line isolated that was resistant to vinblastine, and "1" indicates the micromolar drug concentration used for maintenance of the cell line. The first cell lines selected for resistance to colchicine (J7.C1-100), vinblastine (J7.V1-1), or taxol (J7.T1-50) were used in previous studies (21, 23–25). The relative resistances to the selecting drug in the multidrug-resistant cell lines compared to the parental cells were as follows: J7.C1-100, 2500-fold; J7.V1-1, 900-fold, J7.V2-1, 900-fold; J7.V3-1, 850-fold; J7-T1-50, >830-fold; and J7.T2-50, >830-fold.

Biosynthesis of P-Glycoprotein. Cells were metabolically labeled with L-[^{35}S]methionine or L-[4,5-^{3}H]lysine (specific activity approximately 1500 Ci/mmol or 100 Ci/mmol, respectively; 1 Ci = 37 GBq; Amersham) at 250 μCi/ml, and P-glycoprotein was immunoprecipitated with a polyclonal antibody (24) as described (25). Proteins were resolved in a 4–10% gradient gel (26) and detected by fluorography (27).

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Abbreviation: endo H, endo-β-*N*-acetylglucosaminidase H.

Samples were not heated before $NaDodSO_4$/polyacrylamide gel electrophoresis because heating causes markedly diffuse resolution of the P-glycoprotein in this gel system (38). Prestained molecular mass standards were α_2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; and fructose-6-phosphate kinase, 84 kDa (Sigma). Digestion with either endo-β-*N*-acetylglucosaminidase H (endo H; EC 3.2.1.96) or peptide:*N*-glycosidase F (EC 3.5.1.52) (Genzyme, Boston, MA) was done as described (25). All gel lanes in a given figure panel were loaded with an equal amount of radioactivity.

Peptide Mapping. The method of Cleveland *et al.* (28) was used for peptide mapping of [^{35}S]methionine-labeled P-glycoprotein. In brief, radiolabeled, immunoprecipitated P-glycoprotein was localized in a nonfixed dried gel by comparison with an x-ray film of the gel. The material in the excised, hydrated gel slice was then coelectrophoresed with 20 μg of *Staphylococcus aureus* V8 protease (EC 3.4.21.19; Sigma) into a 4.5% stacking gel and resolved in a 15% gel. Methylated radioactive standards were carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21.5 kDa; cytochrome *c*, 12.5 kDa; and aprotinin, 6.5 kDa (Amersham).

Cyanogen bromide cleavage was done according to published methods (29). [^{3}H]Lysine-labeled, immunoprecipitated P-glycoprotein was electroeluted (30) from the gel slice known to contain it. After dialysis against water and lyophilization, the sample was resuspended in 50 μl of 70% formic acid containing cyanogen bromide (Fisher) at 5 mg/ml and was incubated for 4 hr at 25°C. (At least a 1000-fold molar excess of cyanogen bromide over P-glycoprotein was used.) Similar results were obtained after a 16-hr incubation. The sample was then diluted, lyophilized, resuspended in Laemmli sample buffer (26), and resolved in a 15% gel. The digestion was not complete since, in other experiments, [^{35}S]-methionine was present in some fragments.

RNA Blot Analysis. Total RNA was purified from subconfluent cells (31), denatured in formaldehyde/formamide, and size-fractionated by electrophoresis in 1.5% agarose gels (32). The RNA was transferred to nitrocellulose (33) and fixed by irradiation with a UV germicidal lamp (34). Prehybridization was carried out at 42°C in 50% (vol/vol) formamide/0.75 M NaCl/75 mM sodium citrate/50 mM sodium phosphate, pH 7.0/0.04% polyvinylpyrrolidone/0.04% Ficoll/0.04% bovine serum albumin containing denatured salmon sperm DNA at 125 μg/ml. Hybridization was at 42°C for 16 hr in 50% formamide/0.5 M NaCl/50 mM sodium citrate/50 mM sodium phosphate, pH 7.0/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin containing salmon sperm DNA at 125 μg/ml and 5×10^7 cpm (0.25 μg) of pCHP1 DNA (a gift of J. A. Riordan, Toronto, Ont., Canada) labeled with ^{32}P by nick-translation. In some hybridizations, 2×10^7 cpm of ^{32}P-labeled mouse β-actin cDNA was also included. For autoradiography, blots were exposed at −70°C to Kodak XAR film with a DuPont Cronex intensifier screen.

RESULTS

Biosynthesis of P-Glycoproteins Made by Independently Isolated Cell Lines. It was shown previously that J7.V1-1 cells make a 125-kDa precursor, whereas J7.T1-50 cells make two precursors of 120 and 125 kDa (25). Two additional independent cell lines selected for resistance to vinblastine, J7.V2-1 and J7.V3-1, made a 125- or a 120-kDa precursor, respectively (Fig. 1 *Upper*, 5-min pulse). J7.T2-50 cells made only the 125-kDa precursor. Each P-glycoprotein precursor matured to a single distinct molecular size ranging from 130 to 143 kDa (Fig. 1 *Upper*, 5-min pulse, 3-hr chase). Only J7.T1-50 cells, which made two precursors, had two mature P-glycoproteins. The lower molecular mass species incorporated less radioactivity than the higher molecular mass species. This was related to the pulse interval, since after 15- to 30-min labeling periods, the two species were equal in intensity (see ref. 25). The P-glycoproteins that were immunoprecipitated by the polyclonal antibody had the same molecular size as the only overproduced silver-staining protein(s) in each cell line (data not shown).

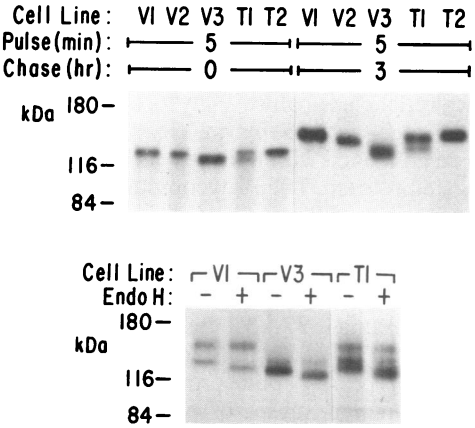

FIG. 1. P-glycoproteins synthesized by independently derived multidrug-resistant cell lines. (*Upper*) Cells were metabolically labeled in methionine-free medium containing [^{35}S]methionine for 5 min (pulse) with or without a subsequent 3-hr incubation in normal medium containing no radioactive methionine (chase). The P-glycoprotein was immunoprecipitated, resolved in gels, and detected by fluorography. Cell line abbreviations are V1, J7.V1-1; V2, J7.V2-1; V3, J7.V3-1; T1, J7.T1.50; T2, J7.T2-50. Control immunoprecipitations with preimmune serum were negative (data not shown). (*Lower*) After a 30-min pulse with radiolabel, the P-glycoprotein was immunoprecipitated, incubated at 37°C for 16 hr with or without endo H (5 milliunits in 70 μl), and then analyzed by gel electrophoresis and fluorography. The T1 lanes were exposed twice as long as the other gel lanes in this panel.

In previous studies (25), it was concluded that the difference in size between the two precursors made by J7.T1-50 cells was not due to differential N-linked glycosylation, since (*i*) two distinct species were observed after removal of all N-linked carbohydrate from the mature species by peptide:N-glycosidase F and (*ii*) a broad band at 115–120 kDa, possibly representing two species, was observed after deglycosylation of the precursors by endo H. Since the 125-kDa and 120-kDa precursors made by J7.V1-1 and J7.V3-1 cells, respectively, comigrated with the two precursors observed in J7.T1-50 cells, it was hypothesized that individual deglycosylated precursors in the J7.V1-1 and J7.V3-1 cells would behave like deglycosylated precursors expressed in J7.T1-50 cells. To test this hypothesis, the sizes of the deglycosylated precursors made by J7.V1-1 and J7.V3-1 cells were compared with the sizes of those made by J7.T1-50 cells.

Cells were metabolically labeled for 30 min to incorporate sufficient [^{35}S]methionine into each precursor (Fig. 1 *Lower*, − endo H). The half-time for processing the 125-kDa precursor in J7.V1-1 cells was 20 min (25); therefore, some mature form was also observed. Less of the mature form was observed in the J7.V3-1 cells, since the half-time for processing was >30 min. When the precursors were deglycosylated by endo H, the 125-kDa precursor from J7.V1-1 cells decreased to 120 kDa (Fig. 1 Lower, + endo H). This form was distinct from the 120-kDa precursor in J7.V3-1 cells, which decreased to 115 kDa. Both mature forms of the protein were resistant to endo H. The molecular sizes of the deglycosylated species in the J7.V1-1 and J7.V3-1 cells spanned the major deglycosylated species of 115–120 kDa in J7.T1-50 cells. In the latter cells, the two higher molecular mass species are most likely the two mature P-glycoprotein molecules, which are endo H-resistant (25).

Peptide Mapping of P-Glycoprotein Variants. To further assess the relationship between P-glycoproteins expressed in these cell lines, peptide mapping was performed (Fig. 2). Metabolically labeled mature P-glycoprotein was immunoprecipitated and resolved by gel electrophoresis, and the polypeptide contained within the gel slice was digested with *S. aureus* V8 protease. The P-glycoprotein made by J7.V1-1 cells had a peptide profile similar to that of the larger species (upper band) in J7.T1-50 cells. J7.C1-100 cells, which made a 125-kDa P-glycoprotein precursor (25), also had a similar peptide profile. In contrast, the peptide profiles of the P-glycoprotein found in J7.V3-1 cells and the smaller species (lower band) in J7.T1-50 cells were similar to each other. Therefore, two types of peptide profiles were observed; each contained a common subset of peptides but also unique peptides. The unique peptides were not directly attributed to glycosylation, since the carbohydrate-containing peptide (which was identified by metabolically incorporating [^{3}H]glucosamine into each glycoprotein) was resolved as a broad smear at >30 kDa (data not shown). Furthermore, to ensure that differences in the peptide maps were not due to N-linked glycosylation, the immunoprecipitated P-glycoproteins were deglycosylated with peptide:N-glycosidase F prior to peptide mapping. Whereas this enzyme shifted the 143-kDa mature species found in J7.V1-1 cells to 125 kDa (25), it shifted the mature 130-kDa species found in J7.V3-1 cells to 120 kDa (data not shown). Differences in the peptide maps were still observed when the deglycosylated molecules were compared (Fig. 2*B*). Distinct peptide patterns were also obtained when 5-fold more protease was used (data not shown) or the P-glycoproteins were chemically digested with cyanogen bromide (Fig. 2*C*).

Analysis of the RNA Coding for P-Glycoprotein. Since differences in precursor size could be observed after a 5-min pulse and no known posttranslational modification could account for these results (see *Discussion*), it was hypothesized that distinct precursors were derived from different translation products. If this were true, more than one mRNA that encodes the P-glycoprotein should exist. The following data demonstrate that multiple mRNA species encode the P-glycoprotein in these drug-resistant cells. Expression of a subset of these mRNA species correlates with the expression of distinct precursors. However, the data are not sufficient to prove that these multiple mRNAs encode distinct precursors.

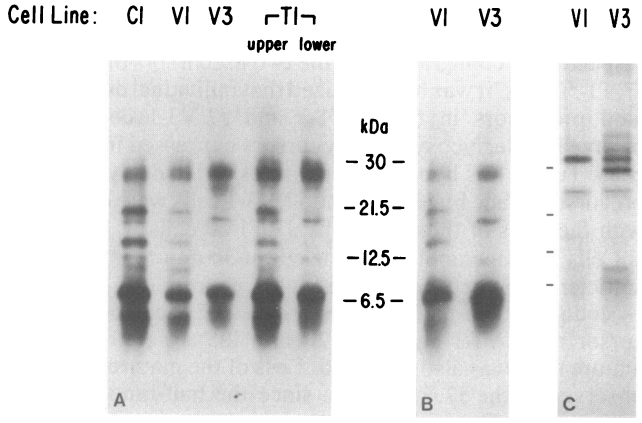

FIG. 2. Peptide mapping of P-glycoproteins made by independently derived multidrug-resistant cell lines. (*A*) [^{35}S]Methionine-labeled mature P-glycoprotein was immunoprecipitated, resolved in gels, and subjected to peptide mapping by limited proteolysis with *S. aureus* V8 protease (20 μg per lane). (*B*) As in *A*, except that immunoprecipitated P-glycoprotein was deglycosylated with peptide:*N*-glycosidase F (10 units/ml at 37°C for 16 hr) prior to peptide mapping. (*C*) [^{3}H]Lysine-labeled mature P-glycoprotein was immunopurified, electroeluted from gels, and digested with cyanogen bromide. Cell line abbreviations are described in the legend to Fig. 1; an additional cell line, J7.C1-1, is abbreviated C1.

Initial assessment of the expression of distinct mRNA species was based on RNA blot analysis. A cDNA probe, pCHP1, that specifically recognizes mRNA coding for the P-glycoprotein in hamster multidrug-resistant cells was used (6). The probe hybridized to a major mRNA species of ≈4.6 kilobases (kb) in all cell lines (Fig. 3). However, J7.V3-1 and J7.T1-50 cells overexpressed an additional species of 5.0 kb, whereas J7.V1-1 and J7.V2-1 cells overexpressed an additional species of 5.1 kb.

J7.T1-50 and J7.V3-1 cells expressed both the 120-kDa precursor and the 5.0-kb mRNA. The relationship between the precursor and this mRNA was examined in the J7.V3 cell line as it was developed by continual selection in increasing concentrations of drug (Fig. 4). The relative amounts of the 120- and 125-kDa precursors within cells maintained at each drug concentration were assessed by loading an equal amount of immunoprecipitable radioactivity in each gel lane. This does not reflect the actual amount of the precursor produced by each cell line. Cells maintained in 10 and 20 nM vinblastine made a 125-kDa precursor and overexpressed, at low levels, the 4.6-kb mRNA species. Trace amounts of the 125-kDa P-glycoprotein precursor were detected in the parental cell line. Cells maintained in 40 nM vinblastine made both precursors. At that concentration, both the 4.6- and the 5.0-kb mRNA species were detected. Cells grown in higher concentrations of drug made predominantly the 120-kDa precursor and overexpressed both mRNA species. At the highest vinblastine concentration used, 1 μM, only the 120-kDa precursor was observed, whereas marked overexpression of both the 4.6- and the 5.0-kb mRNA species was detected.

DISCUSSION

The results demonstrate that multidrug-resistant cell lines derived from J774.2 cells are able to make two P-glycoprotein precursors; the 120- or 125-kDa precursor can be overproduced individually or simultaneously. Further diversity in the family of P-glycoproteins can be created when the 125-kDa precursor undergoes differential N-linked glycosylation (25). Cells also are able to switch the expression of the precursor. In J7.V3-.01 cells, only the 125-kDa precursor was overproduced. This most likely reflects overproduction of a precursor that the normal cell makes, since a trace amount of the 125-kDa precursor was detected in parental cells. When

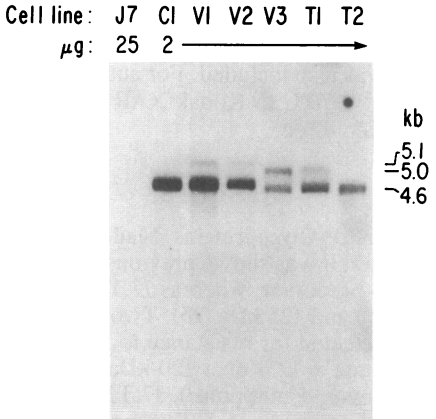

FIG. 3. Blot analysis of mRNA coding for the P-glycoprotein in independently derived multidrug-resistant cell lines (abbreviated as in Figs. 1 and 2). Total RNA samples (25 μg or 2 μg) were electrophoresed in an agarose gel, blotted, and probed with ^{32}P-labeled insert of pCHP1. Autoradiographic exposure time was 36 hr.

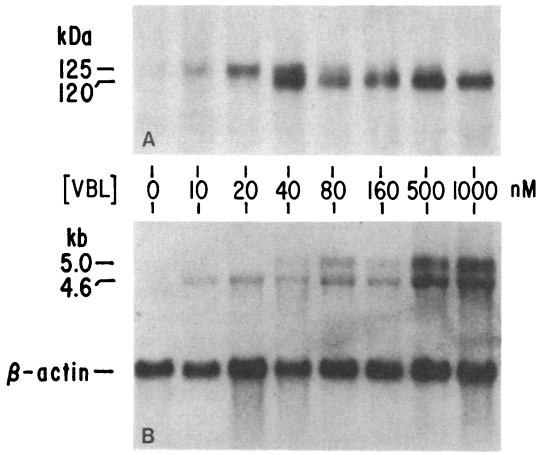

FIG. 4. Analysis and correlation between P-glycoprotein and its mRNA in J7.V3-1 cells selected by stepwise increases in vinblastine concentration. (*A*) P-glycoprotein precursor made during a 5-min labeling period as in Fig. 1. (*B*) RNA blot analysis was done as in Fig. 3 with 10 μg of total RNA per lane. Blots were also probed with ^{32}P-labeled β-actin probe to ensure equal loading of RNA in each lane. The vinblastine (VBL) concentration used for cell maintenance is shown; 0 nM drug indicates the parental cell line.

selective pressure is increased by growing the cells in higher concentrations of drug, cells can overproduce the two precursors simultaneously. They then have the ability to overproduce the 120-kDa precursor exclusively. Therefore, alternative expression of the 120-kDa precursor seems to be a regulated event that is related to drug concentration. However, precursor selection *per se* is not directly related to the selecting drug, since (*i*) the overproduction of the 120-kDa precursor occurred in only one of three independently isolated cell lines selected with vinblastine and (*ii*) a cell line selected for resistance to taxol (J7.T1-50) made both precursors simultaneously.

The basis for differences in size of precursor molecules may be due to either the expression of different translation products or differential co- or posttranslational modification. Differential N-linked glycosylation cannot account for the results. Since unique precursors were labeled within 5 min and were endo H-sensitive, it is reasonable to suggest that distinct molecules are created in an early processing compartment at or before the medial Golgi cisternae. Although it is known that the mature forms incorporate radiolabeled phosphate, under the experimental conditions used the P-glycoprotein precursor was not labeled (35). Although no other modification of the protein has been reported, different half-times for maturation of each precursor to the mature form may suggest that some, as yet undetermined, co- or posttranslational modification differences exist.

An alternative hypothesis is that the precursors may be derived either from different initiator codons on a single mRNA (12) or from distinct mRNAs. Previously reported findings would be consistent with the latter possibility. For example, multiple mRNAs could result from expression of more than one gene of the multigene family coding for P-glycoprotein (7, 8, 36, 37) or from alternative splicing of a single P-glycoprotein gene (37). In J774.2-derived multidrug-resistant cells, the evidence for more than one mRNA encoding two P-glycoproteins is correlative in nature. A 4.6-kb mRNA species must encode a 125-kDa precursor, since J7.C1-1 cells synthesize only one precursor and one mRNA for P-glycoprotein. The presence of a second mRNA species in J7.V3 cells is suggested by the expression of the 5.0-kb mRNA, since the appearance of this mRNA is correlated with the switch from the 125- to the 120-kDa precursor. However, the 5.0-kb mRNA species must not be the only species that codes for the 120-kDa precursor because (*i*) overexpression of the 4.6-kb mRNA increased as the J7.V3-1 cells switched to the synthesis of the 120-kDa precursor exclusively and (*ii*) both the 4.6- and the 5.0-kb mRNA in J7.V3-1 cells were associated with polysomes (L.L., unpublished results). Therefore, the relationship between the 5.0-kb mRNA and the 120-kDa precursor cannot be specified at this time. Since the 5.0-kb mRNA species is always expressed with high levels of the 4.6-kb mRNA species, this suggests that if mRNAs with different translation products exist, there may be heterogeneity in the 4.6-kb mRNA species. Recently, it was found that two different cDNA probes selectively hybridize to different 4.6-kb mRNA species; both mRNA species were detected only at the drug concentration at which both precursors were overproduced in J7.V3 cells (S. I. Hsu and L.L., unpublished observations).

It is not known whether production of different relative amounts of individual P-glycoprotein family members may explain why multidrug-resistant cells have diverse resistance profiles. To help evaluate this hypothesis, a detailed comparative analysis of resistance profiles for each cell line will be needed. Interestingly, compared to the two vinblastine-selected cell lines expressing the 125-kDa precursor, J7.V3-1 cells, which overproduce the 120-kDa precursor, have 1/30th as much DNA amplification, express one-fourth as much mRNA that codes for the protein, and contain half as much P-glycoprotein as detected by staining with Coomassie blue (L.L., unpublished observations). Since these three cell lines are equally resistant to vinblastine, it may be that P-glycoprotein derived from the 120-kDa precursor is more efficient in reducing intracellular drug accumulation than that derived from the 125-kDa precursor. Further quantitative assessment of P-glycoprotein function will be necessary to critically evaluate this hypothesis.

In conclusion, J774.2 multidrug-resistant cells can overproduce P-glycoprotein family members with unique precursors independent of the selecting drug. Such precursors may arise from the expression of more than one mRNA species. Evaluation of the primary structure of these proteins by amino acid or cDNA sequence analysis is likely to be important for the further understanding of the diversity of the P-glycoprotein molecules and the basis of multidrug resistance.

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