MOLECULAR AND CELLULAR BIOLOGY, Aug. 1982, p. 881–889
0270-7306/82/080881-09$02.00/0

Vol. 2, No. 8

DNA-Mediated Transfer of Multiple Drug Resistance and Plasma Membrane Glycoprotein Expression

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Received 26 January 1982/Accepted 26 April 1982

Colchicine-resistant Chinese hamster ovary (CHO) cell mutants whose resistance results from reduced drug permeability have been isolated previously in our laboratories. This reduced permeability affects a wide range of unrelated drugs, resulting in the mutants displaying a multiple drug resistance phenotype. A 170,000-dalton cell surface glycoprotein (P-glycoprotein) was identified, and its expression appears to correlate with the degree of resistance. In this study we were able to confer the multiple drug resistance phenotype on sensitive mouse L cells by DNA-mediated gene transfer of DNA obtained from the colchicine-resistant mutants. P-glycoprotein was detected in plasma membranes of these DNA transformants by staining with an antiserum raised against membranes of mutant CHO cells. These results are consistent with a causal relationship between P-glycoprotein expression and the multiple drug resistance phenotype.

Mutant Chinese hamster ovary (CHO) cells selected for resistance to colchicine have been described previously by Ling and Thompson (19). Cells bearing the colchicine resistance (CH^R) phenotype are cross-resistant to a variety of unrelated drugs, including actinomycin D, adriamycin, cytochalasin B, emetine, puromycin, vinblastine, and melphalan (3, 10, 17, 19). The mechanism of this multiple drug resistance appears to be effected at the plasma membrane level, resulting in reduced drug permeability (7, 19, 24). A molecular alteration was identified as being associated with this membrane impermeability phenotype. This is the expression of a 170,000-dalton plasma membrane glycoprotein (P-glycoprotein), which is not detected in the drug-sensitive parent CHO cell line. The amount of P-glycoprotein expressed in the colchicine-resistant CHO plasma membrane correlates with the degree of drug resistance of the cell line (13, 22). Revertants in colchicine resistance, selected in a single step, have greatly reduced amounts of P-glycoprotein in their plasma membrane (22). The drug resistance phenotype is codominant in somatic cell hybrids between drug-resistant and drug-sensitive CHO cells, and the P-glycoprotein is expressed in the hybrids (18).

It appears that the expression of the P-glycoprotein is intimately associated with the ability of a cell to exclude a wide variety of drugs. However, direct proof that the P-glycoprotein is the causative molecular agent of the observed membrane permeability barrier has not yet been established, and the possibility remains that its expression could be secondary to an as-yet-unknown primary lesion. An approach which could provide a more definitive answer to this question is the isolation and characterization of the gene(s) coding for the colchicine resistance phenotype.

In this report, we have established a reproducible DNA-mediated transfer of the CHO cell colchicine resistance phenotype to mouse L-cell recipients as a first step towards the cloning of the putative drug resistance gene(s). In addition, we utilized a combination of methods that has allowed us to follow the expression of P-glycoprotein in drug-resistant transformants. These methods include a plasma membrane preparation, previously described (22), which highly enriches for the P-glycoprotein from whole cells; the development of a rabbit antiserum of sufficient titer to detect the P-glycoprotein at low levels; and a combination of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (11) and replica electroblotting (28) techniques which has allowed the sensitive, specific immunochemical detection of the P-glycoprotein in isolated plasma membranes of DNA-transformed cells.

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MATERIALS AND METHODS

Materials. Drugs used in this study were of the highest purity obtainable from Sigma Chemical Co. Fluorescamine (Fluram) was from Roche Diagnostics. Ultrapure sucrose was obtained from Schwarz/Mann. Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad, except for SDS, which was from BDH, and urea, which was from Schwarz/Mann. Protein A was from Pharmacia Fine Chemicals. $Na^{125}I$ (15 mCi/μg) was from Amersham Corp. Nitrocellulose filter paper (0.45-μm pore size) was from Schleicher & Schuell.

Horse serum was obtained from Microbiological Associates. Fetal calf serum was obtained from Flow Laboratories and α-minimal essential medium (α-MEM) (26) was obtained as a prepared powder from GIBCO Laboratories. Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.3 mM $MgCl_2$. Tris-saline consisted of 10 mM Tris-hydrochloride (pH 7.4), 0.9% NaCl, and 0.01% NaN_3. Bovine serum albumin-saline consisted of 3% bovine serum albumin (Sigma, fraction V) in Tris-saline buffer. HAT selection medium consisted of 0.1 mM hypoxanthine, 0.001 mM aminopterin, and 0.04 mM thymidine in α-MEM medium lacking nucleosides.

Cell culture. Chinese hamster ovary cells CH^RC5 (19) are colchicine-resistant cells which were previously selected in three steps, with mutagenesis, from the glycine-adenosine-thymidine auxotrophic (GAT^-) line AUXB1 (20). The ouabain-resistant line $C5OUA^R$ was derived from CH^RC5. Murine L cells deficient in both thymidine kinase and adenine phosphoribosyltransferase (TK^-, $APRT^-$) are referred to as LTA cells. LTA cells were derived from L-M (TK^-) clone 1D cells (15), which are referred to as $LMTK^-$ in this paper, and were originally isolated and characterized by R. Hughes and P. Plagemann. $LMTK^-$ cells were provided by S. Silverstein, and LTA cells were obtained from C. P. Stanners. Cell numbers were determined by an electronic particle counter (Coulter Electronics, Hialeah, Fla). The plating efficiency (colony-forming ability) of various lines was determined as described (19). For the lines used in this study, the plating efficiencies were greater than 60% under normal conditions.

All $LMTK^-$ (21) cells and transformants were maintained in α-MEM supplemented with either 10% fetal calf serum or 10% horse serum (with no noticeable difference between the two). CHO cells and LTA cells were grown in α-MEM supplemented with 10% fetal calf serum. Cells were cultured in monolayers at 37°C in humidified air containing 5% CO_2. Plating efficiency of various lines in different drug concentrations was determined as previously described (19).

Mutagenesis was performed by exposing cells to 400 μg of ethyl methane sulfonate (EMS) per ml in complete medium for 24 h at 37°C. Under these conditions about half of the treated cells survived. After allowing 4 days for phenotypic expression, the cells were plated in control nonselective medium (approximately 60% plating efficiency) and in medium containing colchicine.

Two different protocols were employed in selection experiments. The first involved plating the cells in either 0.1, 0.3, or 0.5 μg of colchicine per ml. Growth of colonies was allowed in this medium without further changes. In the second protocol, cells were plated in 0.1 μg of colchicine per ml. After 3 days the medium was replaced with one containing 0.3 μg of colchicine per ml. After a further 3 days the medium was replaced again, with one containing 0.5 μg of colchicine per ml. Colonies were allowed to grow in the final medium for the duration of the experiment. This protocol is referred to as the three-step selection regime. Where HAT medium was used, the medium was replaced once after 3 days.

Hybrid cells were produced by plating CH^RC5 and $LMTK^-$ cells at a ratio of 10:1, 5 h before hybridization. Cells were then washed with PBS, exposed to 44% polyethylene glycol–10% dimethyl sulfoxide in α-MEM at 37°C for 1 min, washed again, and incubated for 1 day at 37°C in complete medium. The cells were then trypsinized, counted, and plated in selective HAT medium at 10^5 cells per 100-mm petri dish.

Colonies were picked by scraping and aspirating with a sterile Pasteur pipette and were transferred for further growth into medium containing the same concentration of colchicine as that from which they were taken. Plates to be scored for colonies were stained with methylene blue. Determination of cell number was done with a Coulter Counter, model F (Coulter Electronics), calibrated for the volumes of the cells counted.

DNA transfer. For transformation of $LMTK^-$ cells, the CHO donor DNA was prepared from washed nuclei as described by Pellicer et al. (21). For transformation of LTA cells, the CHO donor DNA was prepared by a modification of this procedure, in which the starting material for treatment with SDS and proteinase K was whole cells and the ethanol precipitation steps were replaced by extensive dialysis against Tris-EDTA buffer (0.01 M Tris-hydrochloride [pH 8.0], 0.01 M EDTA, and 0.01 M NaCl). In both procedures, DNA preparations were finally dialyzed against Tris-EDTA (pH 8.0) without sodium chloride and stored at 4°C with a few drops of chloroform. DNA was assayed by the method of Burton (6). $LMTK^-$ recipient cells were seeded at 10^6 cells per 75-cm^2 flask in complete medium 1 day before DNA transfer. LTA cells were seeded at 7×10^5 cells per 100-mm petri dish. DNA precipitation was performed by the method of Srinivasan and Lewis (25) except that the Ca^{2+}-DNA-Tris-EDTA mix was added to the freshly prepared HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-phosphate buffer in a round-bottom flask, rather than vice versa. Usually 2 ml of DNA-Ca^{2+} phosphate (10 μg of DNA per ml) was added to 20 ml of fresh medium overlying the cells. Adsorption was allowed for 8 h at 37°C; then 6 ml of 30% dimethyl sulfoxide in complete medium was added, and further incubation was allowed for 30 min. The medium was then aspirated off and replaced with fresh complete medium. Cells were incubated for 1 day, at 37°C, to allow phenotypic expression, then they were trypsinized, counted, and plated at 5×10^5 cells per 100-mm petri dish in medium containing colchicine, by the selection protocols described above, or in HAT medium.

Electrophoretic analysis of plasma membrane proteins. The isolation of plasma membranes was accomplished by the method of Riordan and Ling (22). Membrane protein was assayed by the method of

Bohlen et al. (5), using bovine serum albumin as a standard. Where necessary, plasma membranes were concentrated by centrifugation at 130,000 × *g* for 20 min in a Beckman Airfuge. Electrophoresis was performed by a slight modification of the method of Fairbanks et al. (11). The modifications made to this system were the inclusion of 2% SDS in the sample solubilizing buffer; heating of the solubilized sample at 100°C for 5 min; the addition of urea (to 4.5 M final concentration) to the sample after cooling on ice; and the inclusion of urea (to 9 M final concentration) in the polyacrylamide gel. Samples were fractionated on 1.5-mm-thick slab gels (10 by 14 cm) in a Bio-Rad slab gel apparatus. Electrophoresis was performed at 5 W per gel constant power, without cooling, until the Pyronin Y dye front reached the bottom end of the gel. Gels were stained by the sensitive silver staining method of Switzer et al. (27).

Replica electroblotting (Western blotting). In the Western blotting procedure, a replica blot (on nitrocellulose paper) of a protein profile, fractionated by SDS-polyacrylamide gel electrophoresis, is stained with specific antiserum. The method that we used was essentially that of Towbin et al. (28).

Rabbit antiserum was prepared as follows: male Reiman rabbits of about 2 to 3 kg were injected with plasma membrane vesicles isolated from the colchicine-resistant CHO cells, CH^RC5. An antiserum of excellent titer was obtained after six injections of about 1 mg of protein each, at 2-week intervals. The specificity of the antiserum for P-glycoprotein was improved by preabsorbing it with SDS-solubilized plasma membrane proteins isolated from the drug-sensitive parent CHO cell line, AUXB1. These proteins were immobilized on nitrocellulose particles to facilitate absorption of the antiserum. The absorbed antiserum was used at a 400-fold dilution in bovine serum albumin-saline. A more detailed account of the preparation and a characterization of the antiserum and absorbed antiserum are to be described elsewhere (Kartner, Ling, and Riordan, manuscript in preparation).

^{125}I-protein A was used to visualize antibody binding to protein bands. *Staphylococcus aureus* protein A was radiolabeled by dissolving 100 μg of lyophilized protein in 30 μl of PBS. To this was added 5 μl of dimethyl sulfoxide, 10 μl of $Na^{125}I$ (0.1 Ci/ml, carrier free), and 5 μl of chloramine T (0.5 mg/ml in PBS). After 30 min at 20°C the reaction was stopped with 5 μl sodium metabisulfite (1 mg/ml in PBS). The reaction mixture was desalted on a 4-ml Sephadex G-25M column equilibrated in PBS. The pooled void-volume peak was adjusted to 2.5 ml with PBS and was batch eluted with 3.5 ml of buffer through a prepacked Pharmacia column PD-10 equilibrated with 50 mM NaH_2PO_4 adjusted to pH 4.0 with acetic acid. The eluate volume was adjusted to 6 ml in the latter buffer, 4 ml of reagent grade ethanol was added, and the solution was stored at −20°C. A specific activity of approximately 5 to 10 mCi/mg is typically obtained, with good stability for about 1 month.

RESULTS

DNA-mediated transfer frequency versus spontaneous mutation frequency. A major consideration in this study was to establish that the expression of drug resistance in putative transformants results from bona fide DNA-mediated transformation, effected by DNA isolated from drug-resistant CHO cells, rather than from de novo mutation in the recipient cell genome. The frequency of DNA-mediated transformation would be expected to be low, possibly approaching the frequency of spontaneous occurrence of drug-resistant membrane permeability mutants in mouse L cells. It has been reported previously that drug-resistant mouse cells, phenotypically similar to membrane permeability mutants of CHO cells, can be selected in vivo (9, 14) and in vitro (8). Thus, at first it was important to define selection conditions where the colchicine concentration was high enough to give a negligible spontaneous mutation frequency, yet low enough to permit the growth of drug-resistant transformants. Different variables were examined: drug concentration during selection; selection protocol, namely, selection at a single dose or with a three-step regime employing three increasing doses, stepped up at 3-day intervals; and the application of EMS mutagenesis as a means of increasing mutation frequency. Table 1 summarizes the results of the various selection conditions examined with $LMTK^-$ cells. It is apparent that these concentrations of colchicine represent very stringent selection conditions even if gradually imposed as a three-step regime,

TABLE 1. Spontaneous and EMS-induced CH^R frequency in $LMTK^-$

Condition[a]	Selection in colchicine (μg/ml)	EMS	Cells plated	Colonies	Colonies picked	Continued growth in colchicine
1	0.3	−	1×10^8	0	—	—
	0.3	+	5×10^7	2	2	0
2	0.5	−	5×10^7	0	−	−
	0.5	+	5×10^7	0	−	−
3	0.1 → 0.3 → 0.5	−	7.5×10^7	0	−	−
	0.1 → 0.3 → 0.5	+	2.5×10^7	2	2	2

[a] In conditions 1 and 2, cells were plated in growth medium supplemented with the indicated concentrations of colchicine. In condition 3, drug concentration was increased according to the three-step regime described in the text. The number of cells plated was approximately 5×10^5 per 100-mm tissue culture dish. Colonies were screened after 3 weeks. The four colonies observed were picked 2 weeks after screening.

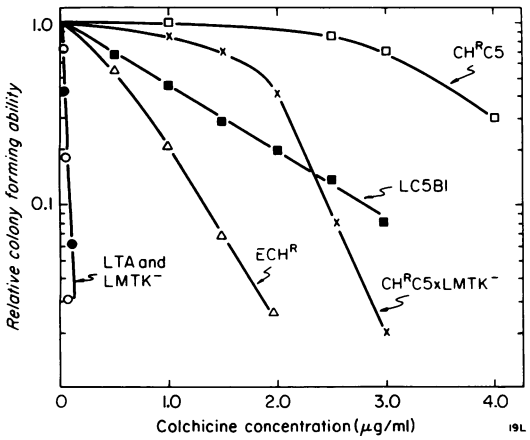

FIG. 1. Dose response of various cell lines to colchicine. Relative colony-forming ability in the presence of colchicine was calculated as the number of colonies scored for each drug concentration divided by the number of colonies scored in the absence of the drug. Colonies were scored after 10 to 12 days incubation at 37°C. Details of the cell lines are given in the text. LTA and LMTK$^-$ are drug-sensitive mouse lines; ECH^R is a colchicine-resistant mutant selected from LMTK$^-$; CH^{R}C5 × LMTK$^-$ is a hybrid line; LC5B1 is a DNA transformant of LTA using CH^{R}C5 DNA; CH^{R}C5 is a colchicine-resistant CHO cell line. The parental drug-sensitive line of CH^{R}C5 is AUXB1. It has a dose response similar to LTA and LMTK$^-$ (data not shown).

since no mutants were obtained without mutagenesis among 5×10^7 to 5×10^8 cells plated. Two colonies that bred true were obtained under the three-step selection regime only after treatment with the mutagen EMS. Thus we conclude that under the conditions described in Table 1, the spontaneous frequency of colchicine-resistant mutants was low enough for us to work out conditions to screen for putative DNA-mediated transformants.

Previously we established that the CH^R phenotype is dominant in intraspecific CHO hybrids (18). To establish that the CHO drug resistance phenotype could, in fact, also be expressed in L cells, interspecific somatic cell hybridization was performed with LMTK$^-$ and the colchicine-resistant CHO cell line CH^{R}C5. That the drug resistance phenotype is expressed codominantly in the hybrid CH^{R}C5 × LMTK$^-$ is shown in Fig. 1. DNA was then isolated from both CH^{R}C5 and C5OUA^R, a ouabain-resistant line selected from CH^{R}C5, to transform LMTK$^-$ or LTA recipient cells for resistance to colchicine.

Transfer of drug resistance was observed only under the least stringent protocols, which appear to be the two- and three-step, stepped-up selection regimes (Table 2). No colchicine-resistant colonies were obtained with control DNA

TABLE 2. Selection conditions

DNA source	Selection[a]	Colonies	Frequency (10^6)
DNA transfer to LMTK$^-$			
CH^{r}C5	0.1 → 0.3	5	0.58
	0.1 → 0.3 → 0.5	2	0.27
	0.3	0	<0.1
	0.5	0	0.1
	HAT	10	2.6
AUXB1	0.1 → 0.3	0	<0.18
	0.1 → 0.3 → 0.5	0	<0.21
DNA transfer to LTA			
CH^{r}C5	0.1 → 0.3 → 0.5	8	0.76
	HAT	16	11.4
AUXB1	0.1 → 0.3 → 0.5	0	<0.09
	HAT	3	2.1

[a] Numbers represent concentrations (micrograms per milliliter) of colchicine used in single-step selection or selection with sequential changes of medium containing increasing concentrations of colchicine, as described in the text.

derived from AUXB1 under any of the selection regimes. Although the frequency of putative transformants is low even under the three-step selection regime, this proved to be a practical selection procedure that apparently ruled out the appearance of spontaneous mutants. Transfers were also achieved with LTA recipients (Table 2). Quantitatively similar results were obtained in further transformation experiments which are tabulated in Table 3.

Included in these tables are control experiments, performed in parallel, in which the transfer of thymidine kinase (the TK$^+$ locus) was monitored with the same DNA preparations as in the colchicine resistance selection. TK$^+$ transformation was scored as the number of colonies growing in HAT medium. For unknown reasons a number of transfer experiments gave poor or no TK$^+$ transformation, and in such instances we have excluded those experiments from our evaluation of colchicine resistance. Invariably, no colchicine-resistant transformants were observed in experiments where TK$^+$ transformants were not observed. In successful experiments the DNA-mediated transfer of the TK$^+$ phenotype was observed with a frequency approximating that reported elsewhere for CHO genomic DNA transfer into LMTK$^-$ (16). No spontaneous TK$^+$ revertants were observed in any experiments with LMTK$^-$ cells or LTA cells.

Also in Table 3 are data showing that no colchicine-resistant transformants were observed with the three-step selection regime without DNA, with AUXB1 DNA, with LMTK$^-$

TABLE 3. Transfer frequencies for HAT and colchicine resistance

DNA source	HAT colonies[a]	HAT frequency (10^6)	Colchicine colonies	CH frequency (10^6)	HAT frequency/ CH frequency
C5OUA^R	20	8	4	0.8	10
C5OUA^R	23	6	2	1.2	5
C5OUA^R	62	7	4	0.5	14
C5OUA^R	21	5	7	0.8	6
C5OUA^R	15	5	2	0.7	7
CH^{R}C5	—	—	11	0.9	—
None	0	<0.2	0	<0.1	—
AUXB1	67	5	0	<0.1	—
LMTK$^-$	0	<0.2	0	<0.2	—
MTX^{RIII}	4	1.1	0	<0.6	—

[a] In each row the same DNA precipitate was used in both the selection for transfer of HAT resistance and in the selection for transfer of colchicine (CH) resistance. Selection conditions for colchicine resistance were according to the three-step regime outlined in the text. The recipient cells were LMTK$^-$.

DNA, or with the colchicine-sensitive, methotrexate-resistant CHO MTX^{RIII} *pro*$^-$ (12) DNA. AUXB1 DNA was effective, however, for the transfer of the TK$^+$ phenotype. Both TK$^+$ and methotrexate resistance (data not shown), but not colchicine resistance, were transferred using MTX^{RIII} *pro*$^-$ DNA. Methotrexate resistance was not transferred by C5OUA^R DNA (data not shown), nor was TK$^+$ transferred with LMTK$^-$ DNA.

Thus, the data shown in Tables 2 and 3 support the conclusion that we are able to perform reproducibly the DNA-mediated transfer of the colchicine resistance phenotype under conditions which preclude the selection of new mutants. Although the overall transformation efficiency for colchicine resistance is low, possibly due to the high stringency of the selecting condition, nevertheless, the selection is appropriate since no drug-resistant colonies were observed in five different experiments where DNAs from colchicine-sensitive cells were used, yielding an estimated frequency of less than 3×10^{-8}. In contrast, in seven experiments in which DNAs from colchicine-resistant cells were used, a total of 38 drug-resistant colonies were observed with an overall frequency of 71×10^{-8}. These results are completely consistent with the hypothesis that the transformants are indeed expressing the colchicine resistance of the CHO cell donors. A number of independent transformant colonies were picked into medium containing selecting concentrations of colchicine. In all cases they were able to grow to mass cultures and were maintained in the presence of the drug. A typical dose-response curve for a transformant (LC5B1) is shown in Fig. 1.

Cross-resistance pattern. To further characterize the colchicine resistance phenotype transferred, the responses of the transformants to different drugs were determined. This was undertaken because selection for colchicine resistance alone does not constitute a selection for the membrane impermeability phenotype which we wish to study, and we have shown previously that in membrane-altered mutants of this class, cross-resistances to unrelated drugs are a characteristic trait (1). As can be seen in Table 4, independent transformants all displayed this pleiotropic phenotype, which confirms that the site of resistance in these cells is at the membrane level. Furthermore, the fact that both the colchicine resistance and the multiple drug resistance phenotypes are concordantly transferred in independent transformants supports our original hypothesis that resistances to these multiple unrelated drugs are coded by a single gene (1). Cotransfer of unlinked genes with genomic DNA is rare. For example, in this present system, colchicine resistance, HAT resistance, ouabain resistance, and methotrexate resistance do not cotransfer at a detectable frequency (data not shown).

It should be noted that the L-cell mutant, ECH^R, selected directly for colchicine resistance from a mutagenized culture (see above) also expressed a pleiotropic phenotype similar to that of the CHO cell mutant (Table 4). This strongly indicates that ECH^R is a membrane-altered mutant.

Membrane analysis. As indicated above, our previous studies have clearly shown that the expression of a 170,000-dalton glycoprotein (P-glycoprotein) correlated with the multiple drug resistance phenotype of the colchicine-resistant mutants. It was therefore of interest to determine whether this protein is also expressed in the DNA-mediated transformants. Figure 2 shows membrane components of various cell lines separated by polyacrylamide gel electrophoresis and stained by the sensitive silver staining procedure of Switzer et al. (27). The P-

TABLE 4. Cross-resistance[a]

Cell line	Colchicine	Puromycin	Actinomycin D	Emetine	Cytochalasin B
AUXB1	1 (2.5×10^{-2})	1 (1.3)	1 (3.0×10^{-3})	1 (1.4×10^{-2})	1 (4.2×10^{-1})
C5OUA^R	76	45	13	10	4.8
CH^{R}C5[b]	184	105	10	29	11
LMTK$^-$	1 (2.2×10^{-2})	1 (2.6)	1 (3.0×10^{-3})	1 (1.9×10^{-2})	1 (6.0×10^{-1})
1B	37	26	6.3	5.7	5.6
1C	33	20	6.7	5.5	4.8
2	31	19	7.3	5.5	4.8
ECH^R	26	21	8.8	2.6	11
LTA	1 (5.3×10^{-2})	1 (2.9)	1 (9×10^{-3})	1 (1.3×10^{-2})	1 (6.0×10^{-1})
LC5B1	43	60	9.4	25	11
LC5B3	57	60	14	28	12

[a] Data were obtained by interpolating and averaging duplicate survival curves. The numbers given in this table are relative resistance values. These represent the fold increase in D_{10} of the tested cell line over that of the drug-sensitive parent line. D_{10} is the concentration of drug which reduces the relative plating efficiency to 10%. For the parent lines the actual D_{10} values in micrograms per milliliter are indicated in parentheses for each drug. Lines 1B, 1C, and 2 are colchicine-resistant transformants of LMTK$^-$ with C5OUA^R DNA. Lines LC5B1 and LC5B3 are colchicine-resistant transformants of LTA with CH^{R}C5 DNA.

[b] Data for CH^{R}C5 were taken from Bech-Hansen et al. (3).

glycoprotein is visualized as a broad, heavily stained band in the CHO cell mutant CH^{R}C5 but was not observed in the drug-sensitive parental line AUXB1 nor in the mouse L cell. Faintly stained bands which could correspond to the P-glycoprotein, however, could be observed in DNA-mediated transformants and in the L-cell mutant ECH^R.

The P-glycoprotein is usually observed as a rather diffuse band in polyacrylamide gels, and when it is present in low amounts, its detection is difficult over a background of other components migrating in the same region of the gel. To increase the sensitivity and specificity of detecting the P-glycoprotein, we employed an antiserum raised against CHO cell mutant membranes in a replica Western blot procedure (see above). The results in Fig. 3 show that by using this method, the P-glycoprotein was not observed in the drug-sensitive CHO and L cell lines but was

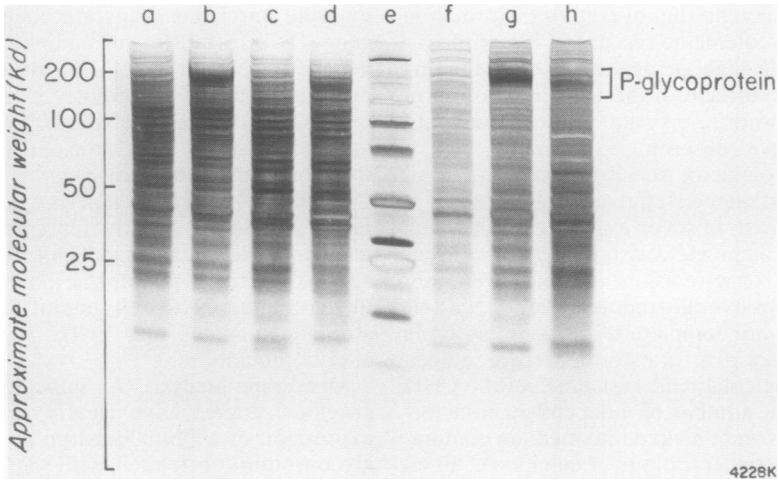

FIG. 2. Fractionation of membrane components by SDS-polyacrylamide gel electrophoresis. Conditions for membrane preparation, gel electrophoresis, and silver staining are described in the text. Each sample contained approximately 15 μg of protein. The region of the gel where P-glycoprotein migrates is indicated. This figure is a composite of two experiments (a through d and e through h) performed on different days. The samples are from (a) AUXB1; (b) CH^{R}C5; (c) LTA; (d) ECH^R; (e) molecular weight standards from Bio-Rad containing myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme; (f) AUXB1; (g) CH^{R}C5; and (h) LC5B1. See the legend to Fig. 1 for more details concerning these lines.

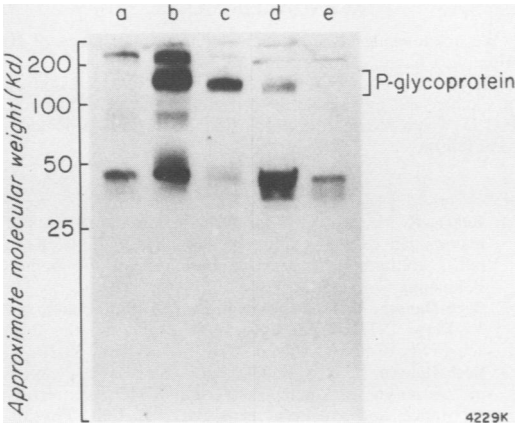

FIG. 3. Immunostaining (Western blot) of membrane components. Membrane components fractionated by gel electrophoresis were transferred onto nitrocellulose and stained with an antiserum against CH^RC5 membranes. Antibody binding was visualized by staining with ^{125}I-protein A followed by radioautography. Details are in the text. Membrane preparations, 50 μg per sample, are from (a) AUXB1, (b) CH^RC5, (c) LC5B1, (d) ECH^R, and (e) LTA. See the legend to Fig. 1 for more details concerning these lines.

detected in mutants (CH^RC5 and ECH^R) from both lines, the amounts in CH^RC5 being considerably more than in ECH^R. The DNA-mediated transformant LC5B1 also displayed the presence of the P-glycoprotein, and this component was consistently observed in a number of independently derived colchicine-resistant DNA-mediated transformants. Thus, expression of the P-glycoprotein is associated with the expression of multiple drug resistance in the transformants. Using this antiserum, increased staining of components migrating at 50,000 and 200,000 daltons was also observed in the CHO cell mutant (Fig. 3). These components, however, were not consistently observed in DNA transformants (see lane c, Fig. 3); moreover, the amount of such components observed in different cell lines (either mutant or wild type) varied with different membrane preparations (unpublished data). Because of these observations, we think it is unlikely that the 50,000- and 200,000-dalton components play important roles in mediating the multiple drug resistance phenotype.

That the antiserum cross-reacts with the putative P-glycoprotein of mouse L cells in the ECH^R line has two implications. First, this result indicates that the P-glycoprotein may be a conserved protein and that this mechanism of resistance may be common to different species of mammalian cells. Second, since it is not possible with this approach to clearly discriminate between the hamster P-glycoprotein and that of the mouse, we cannot say at this point whether it is, in fact, the mouse or the hamster P-glycoprotein that is expressed in the DNA-mediated transformants.

DISCUSSION

The data obtained in this study allow us to draw some conclusions concerning the role of the P-glycoprotein in the multiple drug resistance phenotype. Based on the relatively high frequency of DNA-mediated transformation of L cells over appropriate controls (Tables 2 and 3) and spontaneous appearance of drug-resistant mutants (Table 1), it is almost certain that the surviving colonies of our selection regime are expressing the drug resistance of the donor CHO cell line. The fact that P-glycoprotein (Fig. 3) and multiple drug resistances (Table 4) are concordantly expressed in independent transformants strongly supports the notion that their expression is mediated by the same gene since it is unlikely that two unrelated phenotypes would be transferred and selected for simultaneously in the present study.

Whether or not the P-glycoprotein within the drug-resistant cells is, in fact, the causative molecular agent mediating membrane impermeability or merely the accumulated byproduct of some as-yet-uncharacterized mechanism is not clear from this study. The basis for this uncertainty lies in the fact that our assay could not discriminate between the CHO cell P-glycoprotein and the putative mouse cell P-glycoprotein. Thus the data from the DNA-mediated transformants can be rationalized in one of two ways. If independent transformants all express the CHO P-glycoprotein, then it is likely that the transferred DNA codes for this gene product, whose expression results in the pleiotropic drug resistance phenotype. However, if the mouse P-glycoprotein is expressed in the transformants, then the possibility exists whereby the transferred CHO DNA codes for an as-yet-unidentified function(s) which mediates the expression of the mouse resistance phenotype. Although it is not possible at present to discriminate between these two possibilities, nevertheless, the fact that the drug resistance phenotype can be transferred via isolated DNA is gratifying since it allows further manipulation of the transformation assay towards the isolation of the relevant gene(s). Assays for restriction enzyme sensitivity are under way in our laboratories as a further step towards this goal.

This study has also provided some further insights into the possible origin of the P-glycoprotein. First, the fact that the L-cell mutant ECH^R apparently also expresses a P-glycoprotein on the cell surface of approximately the

same molecular weight as and antigenically similar to that of the CHO cell (Fig. 3) raises the possibility that this is a conserved membrane protein. Second, in the drug-sensitive parental cell lines (CHO and L cells), no cross-reactive membrane proteins at any molecular weight are detected which could quantitatively represent the wild-type counterpart of the P-glycoprotein. Third, the multistep selection for increased drug resistance results in the concomitant increased expression of the P-glycoprotein (13, 22). Taken together, these points suggest that it is unlikely that a point mutation could account for the de novo synthesis of such a functional protein. Rather, it seems more likely that the P-glycoprotein is present in normal cells in amounts below the limits of detection by our present methods. Thus, the mutant phenotype could represent a state of overproduction of the P-glycoprotein, possibly as the result of amplification of the gene. This mechanism for drug resistance has been implicated in different systems (23; V. Ling *in* N. Bruchovsky and J. H. Goldie, ed., *Drug and Hormone Resistance in Neoplasia*, in press).

Drug resistance as a result of a membrane permeability mutation has been described in a number of mammalian systems (1, 4, 17). Phenotypically these mutants are very similar, and it is conceivable that the pleiotropic membrane barriers which they express are the result of similar or identical molecular mechanisms. Thus, the colchicine-resistant CHO cell mutant described here has served as a useful model for the study of this phenotype in our laboratories. Further study, at the molecular level, is of interest for a number of reasons. Colchicine resistance may serve as a useful model of the molecular control of passive permeability at the cell surface. Presently no other well-characterized models are available for this purpose. The system also lends itself to the study of a unique integral membrane glycoprotein which may have an important regulatory function in this respect. Although the lesion at the level of the genome is still unknown, there is certainly a close association with the expression of the P-glycoprotein. Thus, this system may lend itself to the study of control of gene expression or possibly gene amplification. Moreover, many of the drugs to which these mutant cells are cross-resistant are used widely in the treatment of cancer. It is possible that the recurrence of malignancy with acquired multiple drug resistance, after apparent remission with multiple drug chemotherapy, is the result of selection for a drug-resistant tumor cell subpopulation bearing the membrane impermeability phenotype (2). Therefore, the study of this system is of broad clinical relevance to cancer chemotherapy.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of N. Alon, A. Chase, S. Fahim, and M. Naik.

This work was supported by the Medical Research Council of Canada and by the National Cancer Institute of Canada. P.G.D. was a Medical Research Council of Canada postdoctoral fellow.

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