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A SURFACE GLYCOPROTEIN MODULATING DRUG PERMEABILITY IN CHINESE HAMSTER OVARY CELL MUTANTS

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SUMMARY

Chinese hamster ovary cells selected for resistance to colchicine display pleiotropic cross-resistance to a wide range of amphiphilic drugs. The drug-resistant phenotype is due to a membrane alteration which reduces the rate of drug permeation. Surface labelling studies reveal that drug-resistant Chinese hamster ovary cell membranes possess a carbohydrate-containing component of 170 000 daltons apparent molecular weight which is not observed in wild type cells. Through studies of the metabolic incorporation of carbohydrate and protein precursors, and through the use of selective proteolysis, this component is shown to be a cell surface glycoprotein. Since this glycoprotein appears unique to mutant cells displaying altered drug permeability, we have designated it the P glycoprotein. The relative amount of surface labelled P glycoprotein correlates with the degree of drug resistance in a number of independent mutant and revertant clones. A similar high molecular weight glycoprotein is also present in drug-resistant mutants from another hamster cell line. Observations on the molecular basis of pleiotropic drug resistance are interpreted in terms of a model wherein certain surface glycoproteins control drug permeation by modulating the properties of hydrophobic membrane regions.

INTRODUCTION

We have isolated from Chinese hamster ovary tissue culture cells, colchicine-resistant (CH^R) mutants which exhibit reduced permeability to colchicine [1]. The degree of resistance observed in independently selected lines correlates strongly with the reduction in drug permeability. One distinguishing feature of these membrane-altered mutants is their pleiotropic cross-resistance to a wide range of apparently unrelated compounds such as vinblastine, colcemid, daunomycin, puromycin, cytochalasin B and others [1–3]. Several observations provide evidence that the mutation resulting in reduced colchicine permeability also results in a pleiotropic reduction in permeability to a wide variety of compounds. First, CH^R cells display reduced uptake of

some of the drugs to which they are cross-resistant [4]. Second, both the CH^R phenotype and the pleiotropic phenotype behave similarly in cell-cell hybrids, and are expressed as an incompletely dominant trait [2]. Third, revertants isolated in a single step from non-mutagenized cultures of highly colchicine-resistant lines possess greatly reduced resistance to colchicine and at the same time, display a reduction in resistance to other drugs [2].

Because of the marked pleiotropic nature of the membrane alteration(s) resulting in colchicine resistance, and because of the initial observations that colchicine permeates into the cell by unmediated diffusion [4, 5], we anticipated that some general alteration of the plasma membrane might be responsible for reduced drug permeability in the mutant cells. We examined the lipid composition of mutant cells, but observed no major changes (See, Y. P. and Ling, V., unpublished observations). On the other hand, we have recently made the preliminary observation that a new surface component, likely a glycoprotein, is associated with the plasma membrane of a mutant line [6]. The present study was undertaken to evaluate whether or not there is a correlation between the presence of this surface glycoprotein and the degree of colchicine resistance. Thus, we have examined the surface glycoproteins of a number of independently selected CH^R clones with different degrees of resistance, as well as two independently isolated revertant clones.

EXPERIMENTAL PROCEDURES

Cells. The maintenance of Chinese hamster ovary cells in culture has been detailed previously [1, 7]. Cells are normally maintained at 37 °C in monolayer cultures; however, for experiments involving the labelling of cell surfaces, cells are grown in suspension cultures for greater than seven generations before use. The isolation and growth characteristics of CH^R mutants have been described previously [1]. For this study, subclones of the original mutant lines have been employed. AUX B1 a glycine-, adenosine- and thymidine-requiring auxotroph [8], is the parental line from which the colchicine-resistant (CH^R) clones were originally selected [1]. S_n (where $n = 1, 2, 3, \ldots$ etc.) denotes the subclone number of the original mutant line. Clones I8-31 and I10-1 are revertants selected in a single step from the highly drug-resistant lines CH^RC4 and CH^RC5, respectively. The isolation of revertant cells will be communicated in detail elsewhere. A preliminary report on one method of revertant isolation has already been described [2].

Surface labelling. The surface galactose and galactosamine residues of intact cells were labelled via the galactose oxidase-boro[^{3}H]hydride technique, as described previously [7, 9, 10]. Briefly, $1 \cdot 10^8$–$2 \cdot 10^8$ washed cells in 2 ml of Dulbeccos phosphate buffer were treated with 50 units of neuraminidase, followed by 30 units of galactose oxidase, both for 5 min at 37 °C. The enzymes were removed and the cells radiolabelled with approx. 1 mCi of tritiated borohydride for 5 min at 0 °C. Excess isotope was removed by two washes in buffer containing 1 % bovine albumin, followed by two further washes in buffer alone.

Metabolic labelling. Total cell protein was radiolabelled via the incorporation of ^{3}H-labelled amino acids (1–2 μCi/ml medium) during logarithmic growth. Cell oligosaccharides were labelled through the incorporation of [^{14}C]glucosamine (0.2 μCi/ml medium), also during logarithmic growth. Radioactive glucosamine is

employed here as a specific precursor for oligosaccharides, since there is probably little diversion of this substance into other biosynthetic pathways [11]. Metabolic incorporation with either isotope was continued for at least one doubling time (18–22 h).

Subcellular fractionation. Chinese hamster ovary cell plasma membranes were prepared and isolated using two cycles of an aqueous polymer separation system as previously described [12]. The characteristics of such membranes have been described in detail [7, 10].

Polyacrylamide gel electrophoresis and quantitation of radioactivity. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the methods of Fairbanks et al. [13]. Samples labelled via the galactose oxidase-boro[^{3}H]hydride method were usually analyzed on 7.5% acrylamide gel slabs. The radiolabelling patterns were visualized by impregnating the gels with 2,5-diphenyloxazole, followed by drying and autoradiography [14]. The autoradiograms were scanned with a Beckman Acta CII recording densitometer in order to quantitate the various labelled components fractionated by electrophoresis. Under these conditions, a linear correlation prevailed between the dpm in a single protein (^{3}H-labelled albumin) applied to the gel and the resulting peak area obtained from the densitometer scan, over the range 1000–65 000 dpm. The limit of detectability was about 500 dpm for a 1 week exposure. In the experiments described below, 5000–30 000 dpm were layered on the gel for each sample.

Samples labelled via the metabolic incorporation of ^{3}H-labelled amino acids and [^{14}C]glucosamine were analyzed on polyacrylamide disc gels which were sectioned, and incorporated radioactivity determined [10] using a Packard Tri Carb liquid scintillation counter with automatic external standardization. Corrections for quenching and calculation of the specific dpm of ^{3}H and ^{14}C in double label experiments were performed using a program developed for the Hewlett Packard programmable calculator. Apparent molecular weights of separated membrane components were estimated by comparison with a standard composed of ^{3}H-labelled β-galactoside- [15] (*Escherichia coli*), bovine albumin, ovalbumin and chymotrypsinogen, which was electrophoresed in parallel.

Materials. Neuraminidase, free of protease activity, was obtained from Behringwerke. Galactose oxidase was secured from Sigma or Kabi, Sweden (this material was tested for protease activity and freed of such activity by heat treatment [16], if necessary). Tritiated borohydride (100 Ci/M), [^{14}C]glucosamine (45–55 Ci/M), ^{3}H-labelled amino acid mixture (1.0 mCi/ml), and liquid scintillation supplies were obtained from New England Nuclear. Kodak RP/R14 X-ray film was obtained from Rutherford Scientific. Other chemicals were of reagent grade and were used as purchased.

RESULTS

Surface labelling patterns of wild type and drug-resistant cells

As we have previously reported, the galactose oxidase-boro[^{3}H]hydride labelling pattern of the surface membrane of wild type Chinese hamster ovary cells (AUX B1) consists of two major high molecular weight peaks of approx. 139 000

and 95 000–100 000, and a diffuse complex of smaller peaks in the range of 55 000–65 000 (refs. 6, 10 and Fig. 1a). The labelling patterns of a number of colchicine-resistant clones of increasing degrees of drug resistance (Table I) are shown in Figs. 1b–d, respectively. It can be seen that the most striking feature of these patterns is the increased labelling of a high molecular weight component of approx. 170 000

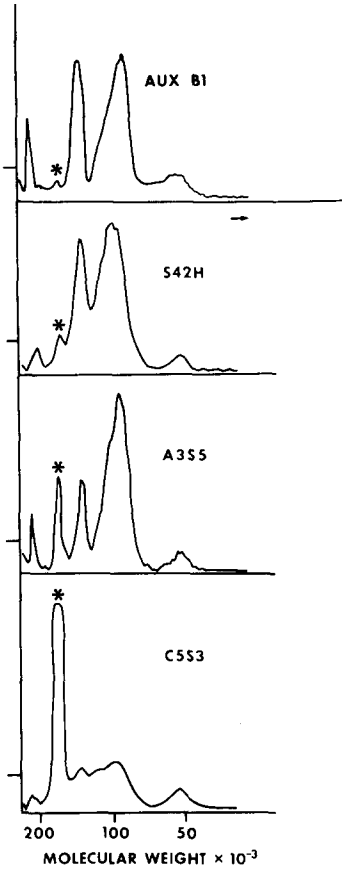

Fig. 1. Surface labelling of wild type and drug resistant Chinese hamster ovary clones. Clones AUX B1, $CH^{R}S42$ H52, $CH^{R}A3S5$ and $CH^{R}C5S3$ were surface labelled using the galactose oxidase-boro[^{3}H]-hydride technique. Plasma membranes were prepared from the labelled cells and were analyzed by gel electrophoresis in the presence of 1 % sodium dodecyl sulfate and mercaptoethanol, followed by autoradiography. Approximately equal amounts of radioactivity (6700 dpm) were layered on the gel for each cell type. The autoradiograms were scanned in a recording densitometer. The acrylamide concentration was 7.5 %. Since equal amounts of radioactivity were applied for each cell type, in the pattern for $CH^{R}C5S3$ where much of the label is associated with the 170 000 dalton peak, the other components appear reduced in magnitude. This is not the case, however, and d simply reflects the vast increase in the relative amount of labelled 170 000 dalton component, as compared to the other surface-labelled components. The component of apparent molecular weight greater than 200 000 is extremely labile and varies substantially in amount even between experiments on the same clone. Abscissa: apparent molecular weight in daltons. Ordinate: arbitrary units of absorbance (the scale is the same for all scans). (a) AUX B1. (b) $CH^{R}S42HS2$. (c) $CH^{R}A3S5$. (d) $CH^{R}C5S3$.

TABLE 1

CORRELATION BETWEEN THE AMOUNT OF SURFACE-LABELLED GLYCOPROTEIN AND THE DEGREE OF DRUG RESISTANCE

The relative amount of surface-labelled P glycoprotein is represented by the parameter F. This parameter is calculated from densitometer scans of autoradiograms by dividing the area under the half height of the 170 000 dalton peak by the area of the entire scan between 40 000 and 200 000 daltons*. As indicated in Experimental Procedures, we have established that the peak area is linearly related to the amount of radioactivity in a particular component. Thus, the F parameter is a reflection of the relative amount of radiolabel introduced into a particular component by the surface label technique. The relative resistance data reflect the degree of resistance of the variant clones as compared to AUX B1 in terms of plating efficiency in the presence of colchicine [1]. The results of individual experiments (F), as well as an average ($\bar{F}$) are indicated. Spearmans rank coefficient = 0.84. Significant at the 0.01 level.

Clone	F	$\bar{F}$	Relative resistance
AUX B1 (parent)	0.027, 0.009, 0.021	0.019	1.0
I10-1 (revertant)	0.022	0.022	3.0
CH^RS_42HA	0.034, 0.053	0.044	16.0
CH^RS_42HS2	0.051, 0.057	0.059	3.0
CH^RA3S5	0.057, 0.076	0.067	5.0
I8-31 (revertant)	0.072	0.072	6.0
CH^RC4S4	0.213, 0.232	0.223	74.0
CH^RC5S3	0.378, 0.540	0.460	184.0

* In the case of AUX B1, where no distinct peaks were detectable in some cases, we simply measured the area over the 160 000–175 000 dalton region and used this as the numerator.

(marked by an asterisk). This component appears to be largely absent in the parental line (Fig. 1a) but it is the predominant labelled entity in the highly resistant clone CH^RC5S3 (Fig. 1d). The surface components labelled in the parental line (Fig. 1a) are also present in the mutant lines (Figs. 1b–1d), but vary somewhat in their relative amounts. These data are consistent with the previous observation [6] that a unique surface glycoprotein of high molecular weight was present in a highly colchicine-resistant mutant. Since this glycoprotein (denoted by asterisk in Fig. 1) appears to be associated with mutants displaying altered drug permeability, we have designated it as the "P glycoprotein".

In order to quantitatively evaluate the relationship between the presence of surface-labelled P glycoprotein and the degree of drug resistance, we have estimated the relative amount of galactose oxidase-boro[^{3}H]hydride-induced labelling in this glycoprotein in several independent mutant and revertant clones, and have compared these values with the relative resistance of these clones to colchicine (Table I). As can be seen from Table I, a significant correlation exists between the relative amount of labelled P glycoprotein and the relative resistance to colchicine. The Spearman's rank correlation coefficient is 0.84 and is significant at the 0.01 level [17]. Thus, this provides persuasive evidence that the relative amount of surface-labelled P glycoprotein is related to the degree of drug resistance. The correlation, however, is not exact; for example, clone CH^RS42HA has a relatively small amount of labelled P glycoprotein in relation to its degree of resistance to colchicine (Table I). This might indicate that other mechanisms for regulating drug resistance may come into play.

Metabolic incorporation of carbohydrate and protein precursors

The increased galactose oxidase-boro[^{3}H]hydride-induced labelling of the P glycoprotein observed in our mutant lines (Fig. 1) could result from any one of several possible causes. For example, it could stem from changes in the membrane conformation of mutant cells, resulting in greater accessibility of the P glycoprotein [18], from alterations in the composition of the oligosaccharide side chains of the P component [9], or even from selective adsorption of material from the growth medium by mutant lines. Alternatively, the increased surface labelling of the P glycoprotein might result from the introduction of increased amounts of this glycoprotein into the mutant membranes via normal biosynthetic routes.

We have attempted to discriminate among these possibilities by metabolic labelling studies, employing precursors to proteins (^{3}H-labelled amino acids) and oligosaccharides ([^{14}C]glucosamine) [11]. As can be seen in Fig. 2, the membrane components of a highly colchicine-resistant mutant, CH^{R}C5S3 and of the parental

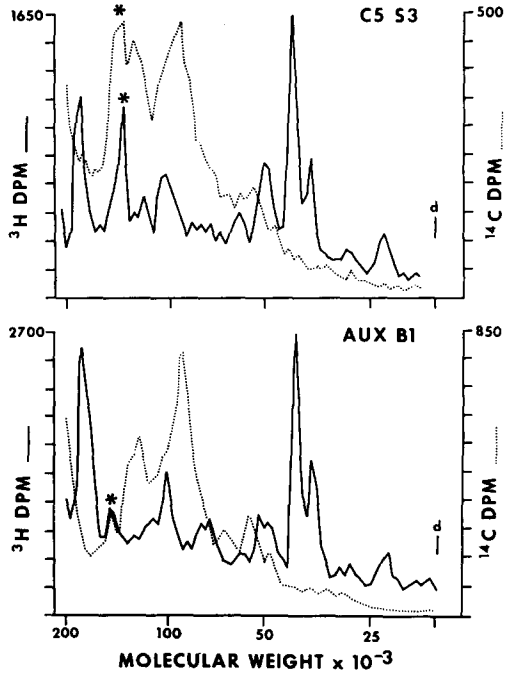

Fig. 2. Metabolic labelling of wild type and drug-resistant clones. Individual cultures of clones C5S3 and AUX B1 were labelled by metabolic incorporation of [^{14}C] glucosamine or of ^{3}H-labelled amino acids and membranes were prepared from the labelled cells as described in Experimental Procedures. For each cell type, membranes labelled with ^{3}H and with ^{14}C were pooled, dissolved in sodium dodecyl sulfate and analyzed by electrophoresis on a single disc gel in the presence of 1 % sodium dodecyl sulfate plus mercaptoethanol. The gels were sectioned and analyzed for radioactivity as described in Experimental Procedures. The vertical scales were chosen for convenience of display. The parameters of interest are the relative peak heights and not the absolute dpm values, since different amounts of material were layered on each gel. It should be noted that the prominent peak of molecular weight 200 000 is extremely labile and varies substantially from experiment to experiment within a single clone. Abscissa: molecular weight scale. Ordinate: dpm per gel slice.

line AUX B1, labelled via metabolic incorporation, yielded different patterns when analyzed by polyacrylamide gel electrophoresis. The main difference is the greatly increased labelling of a high molecular weight component (denoted by asterisk in Fig. 2) in the mutant line. This component displays a relative increase, both in the pattern derived from labelling with carbohydrate precursors and in the pattern derived with protein precursors. Moreover, this component has an apparent molecular weight similar to the P glycoprotein identified by surface labelling, namely, 170 000. It should be noted that, aside from the difference in the relative amount of the 170 000 dalton component, the labelling patterns of the mutant and the parental lines resemble each other in most respects (Fig. 2). Furthermore, a similar result prevails in the case of another independently selected highly colchicine-resistant clone CH^RC4S4 (unpublished observation). Thus, these data appear consistent with the concept that the P glycoprotein is a biosynthetic cellular product which can be labelled by metabolic incorporation of isotopic precursors and that it is present in increased amounts relative to other membrane components in the mutant cells.

To further substantiate that the new 170 000 dalton peak observed by metabolic labelling in mutant cells (Fig. 2) is identical with the P glycoprotein (Fig. 1), we have double-labelled the membrane carbohydrate of a highly colchicine-resistant mutant by metabolic incorporation of [^{14}C]glucosamine and by external labelling with galactose oxidase and NaB^3H_4. Analysis of membranes from these cells demonstrates that the P glycoprotein component visualized by surface labelling coincides with that labelled via metabolic incorporation (data not shown). These results, taken with those described above, indicate that the primary cause of the increased galactose oxidase-induced labelling of P glycoprotein in drug-resistant clones is an increase in the relative amount of this particular class of cell surface glycoprotein in the membranes of mutant cells.

Selective proteolysis

As a further test of the chemical nature and subcellular localization of the P glycoprotein, we determined whether or not labelled P glycoprotein was affected when intact cells were treated by various proteases. As can be seen in Fig. 3, when highly resistant C5S3 cells are treated with proteases, subsequent to labelling with galactose oxidase-boro[^{3}H]hydride, the amount of surface-labelled material in the range of 40 000–200 000 daltons is substantially reduced. This was the case when C5S3 cells were treated, either with pronase (100 μg/ml) or chymotrypsin (1.0 mg/ml). Since proteolytic dissection, as employed here, cleaves primarily cell surface polypeptides and does not affect intracellular components [10], the results from Fig. 3, provide evidence for a cell surface location for the 170 000 dalton component. Furthermore, the proteolytic susceptibility of the galactose oxidase-boro[^{3}H]hydride-labelled material confirms that the components visualized by this surface label technique, including the P component, are glycoproteins, rather than another form of carbohydrate-containing molecule. The P glycoprotein, although reduced by proteolytic cleavage, seems to be more resistant to this process than some of the other membrane glycoproteins (e.g. the 200 000 dalton component).

Presence of altered cell surface components in other drug-resistant cells

The work thus far has described variants of the Chinese hamster ovary cell

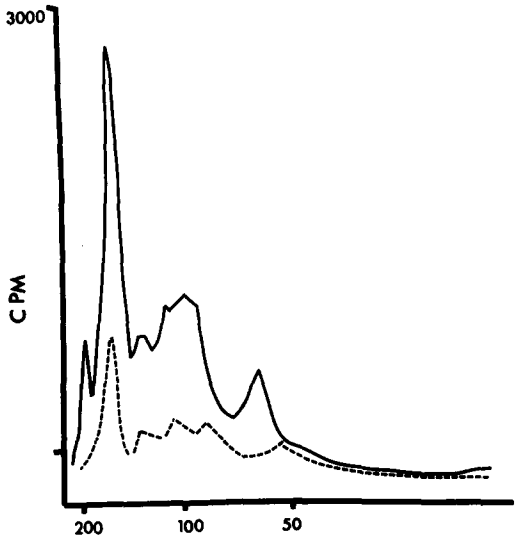

Fig. 3. Proteolytic digestion of cell surface proteins. Cells of clone $CH^{R}C5S3$ were surface labelled via the galactose oxidase-boro[^{3}H]hydride technique. An aliquot of labelled intact cells was treated with pronase (100 μg/ml) for 5 min at 35 °C. This sample was then washed four times in Dulbeccos buffer plus 1 % albumin. Membranes were prepared from control and pronase-treated cells and were analyzed by electrophoresis on 7.5 % polyacrylamide gels in the presence of sodium dodecyl sulfate and reducing agent. An equal amount of membrane (A_{280nm}) was layered on each gel. The gels were sectioned and analyzed for radioactivity, as described in Experimental Procedures. Abscissa: apparent molecular weight $\times 10^{-3}$. Ordinate: cpm/gel slice. — — —, control cells; -------, pronase-treated cells.

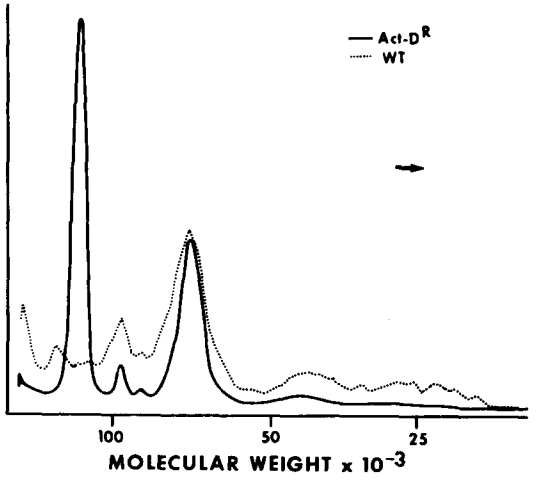

Fig. 4. Surface labelling of wild type and actinomyein D resistant SV40 transformed Syrian hamster cells. Wild type and actinomycin D-resistant SV40-transformed Syrian hamster cells were surface labelled via the galactose oxidate-boro[^{3}H]hydride technique as described in Experimental Procedure. Membranes prepared from the labelled cells were analyzed by electrophoresis on a 7.5 % acrylamide gel slab in the presence of sodium dodecyl sulfate. Approximately equal amounts of radioactivity (3000 cpm) were layered on the gel for each sample. Abscissa: apparent molecular weight scale. Ordinate: arbitrary units of absorbance.

line. It is of interest to know whether or not pleiotropic changes in drug resistance are accompanied by cell surface changes in other cell types. In this regard, we have examined the galactose oxidase-boro[^{3}H]hydride-induced labelling patterns of wild type and actinomycin D-resistant clones from an SV40-transformed Syrian hamster line [19] isolated in another laboratory. This resistant variant also exhibits pleiotropic cross-resistance to other drugs. Both wild type and actinomycin D-resistant lines were recloned prior to surface labelling studies. As seen in Fig. 4, the galactose oxidase-induced labelling pattern of wild type SV40-transformed hamster cells is different from that of wild type CHO cells, with the two most prominent peaks having a lower apparent molecular weight (c.f. Fig. 1a). However, as in the case of the Chinese hamster ovary system, the drug-resistant SV40-transformed cells possess a large labelled peak of high molecular weight which is not present in wild type cells (Fig. 4). Thus, the evidence suggests that, in hamster cells at least, a pleiotropic pattern of drug resistance is accompanied by an alteration in the array of cell surface glycoproteins.

DISCUSSION

The results presented here indicate that drug-resistant Chinese hamster ovary cells possess a component of high molecular weight, the P glycoprotein, which is not readily detectable in the wild type cells (Figs. 1 and 2). Evidence for the glycoprotein nature and cell surface localization of this component includes the observations that the component is reactive with the galactose oxidase-boro[^{3}H]hydride technique for labelling surface carbohydrates, that the component can be labelled by metabolic incorporation of precursors for either carbohydrates or proteins, and that the component can be cleaved by proteolytic digestion of intact cells under conditions where only cell surface peptides are attacked (Figs. 2 and 3).

Evidence that the presence of the P glycoprotein is functionally related to the drug-resistant phenotype, rather than due to clonal variation, differences in growth rates, or other adventitious effects, is in the form of a strong correlation between the relative amount of surface-labelled P glycoprotein and the degree of drug resistance (Table I). The correlation prevails in a number of independent clones where stepwise increases in resistance are accompanied by concomitant increases in the proportion of labelled P glycoprotein. This observation parallels the genetic characterization of the CH^R phenotype, where the stepwise selection of mutants with increasing drug resistance seems to suggest the existence of multiple alleles controlling the degree of expression of a particular trait [2, 3]. The correlation also holds in drug-sensitive revertants which display a marked decrease in the relative amount of P glycoprotein (c.f. Table I), as would be expected if the P glycoprotein were functionally related to the drug-resistant phenotype. Moreover, a selective alteration of the surface glycoprotein pattern, similar to that seen in the Chinese hamster ovary system, has also been observed in a completely independent line (actinomycin D-resistant SV40-transformed Syrian hamster cells) (Fig. 4). These results suggest that the molecular basis for a pleiotropic drug-resistant phenotype may frequently be an alternation in the array of cell surface glycoproteins. However, at least one possible exception to this pattern (clone CH^{R}S42HA) has been detected indicating the possibility of other mechanisms for the control of drug resistance.

Our observations on the relation between drug resistance and the augmented

levels of P glycoprotein are consistent with an earlier report [20] where drug-resistant cells were observed to display an increase in glycosyltransferase activity, a decrease in glycosidase activity and complex alterations of the membrane glycoproteins as visualized by periodic acid-Schiff staining. In contrast to the present report, however, only one resistant line was examined, a line which had originated by continuous selection in the presence of increasing amounts of drug [20, 21]. Thus, the complex changes observed may have been the end result of multiple genetic alterations.

The pleotropic drug-resistant phenotype has a number of interesting features which when considered together suggest a model having pertinence to many areas of membrane biology. Some of these features are summarized below. (a) The mutation(s) leading to colchincine resistance also lead to a pattern of cross-resistance to other amphiphilic drugs, and the resistance is due, in all cases tested, to reduced drug permeation in the mutant cell types [2]. (b) Since the uptake kinetics for amphiphilic drugs suggest passive permeation [4, 5], and since degree of resistance to different agents appears to correlate to some extent with the hydrophobicity of the drugs [3], it seems likely that many amphiphilic drugs permeate into the cell via diffusion through hydrophobic regions of the plasma membrane. (c) However, the permeability of such regions must be under metabolic control, since the rate of drug uptake appears to be controlled in a reversible manner by the intracellular ATP levels [4]. These observations together with the present results concerning the presence of a novel surface glycoprotein in the resistant cells, are consistent with the following model. We propose that cell surface proteins, such as the "P glycoprotein", modulate the fluidity of the plasma membrane lipids, either globally or locally within restricted domains. Alterations of membrane lipid fluidity are reflected by changes in the permeation rate of drugs entering the cell via hydrophobic pathways [22]. The conformation and thus the function of the P-type proteins themselves are, in turn, influenced by cellular ATP levels, either directly or through the agency of as yet unidentified cytoplasmic components.

Alternative explanations of the observed results might include the notion the P-type components provide a glycoprotein "fuzz" which screens the membrane, or that P-type components bind and immobilize drugs at the cell surface and thus prevent their permeation; however, neither of these suggestions in tenable. If the entire cell surface were effectively screened by a glycoprotein mesh, then the permeation of all solutes should be reduced. However, this is not the case, since passively diffusing hydrophilic moieties such as erythritol enter the mutant cells as readily as wild type cells [4]. The possibility that P -type components specifically bind drugs also seems rather unlikely, since the mutant cells are simultaneously resistant to a number of structurally unrelated compounds, whose only common characteristic is their amphiphilic nature, as manifested by a relative high oil/water partition coefficient [3].

It is of considerable interest to determine if P-type glycoproteins exert their effect on a restricted membrane domain concerned with drug permeation, or whether they influence the overall state of the membrane lipid bilayer. If the latter were true, then P-type glycoproteins might play a role in modulating the activity of those membrane enzymes, and transport systems which are susceptible to fluidity changes [2]. Thus, P-type glycoproteins could serve as transducers, linking events at the cell surface to metabolic activity within the cell interior.

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