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Plasma Membrane Proteins and Glycoproteins From Chinese Hamster Cells Sensitive and Resistant to Actinomycin D

Robert H.F. Peterson and June L. Biedler

Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Plasma membrane proteins and glycoproteins have been isolated from Chinese hamster cells of the spontaneously transformed DC-3F parental cell line and the DC-3F/AD X line with a high level of acquired resistance to actinomycin D. Plasma membrane preparations from both cell lines band at 1.16 g/ml after isopycnic centrifugation. We present evidence to indicate differences in the leucylpeptide backbones of the antibiotic-sensitive cells and the drug-resistant DC-3F/AD X cells. In addition, there are differences in the plasma membrane glycopeptides of the two cell lines as revealed by sodium dodecyl gel electrophoresis. Drug-resistant cells synthesize a surface glycopeptide which is much larger than the major one present on the drug-sensitive cells. Both of these cell lines are devoid of 5′-nucleotidase and alkaline phosphatase activities. The role of plasma membrane protein differences in drug-resistant cells is discussed.

Key words: **plasma membranes, drug resistance, actinomycin D, glycoproteins**

Glycoproteins occupy a key role in the surface architecture of the cell. Marked changes in these macromolecules have been observed in virally transformed cells [1–3], in malignant cells [4, 5], and in drug-resistant cells [6, 7]. The precise mechanism(s) of these alterations is presently unknown. A prominent feature of cells in culture which display density-dependent inhibition of growth is a marked intracellular adhesiveness, and the role of complex carbohydrates in this process has been extensively studied by Roseman [8]. Earlier we provided evidence that acquired resistance to the antibiotic actinomycin D in our Chinese hamster cell lines was due to decreased permeability at the level of the plasma membrane [9, 10] and not to some alternative mechanism. This finding has recently been extended by us [10–12] and also by Poste [13]. These observations prompted us to examine the plasma membranes of the Chinese hamster cells in greater detail.

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In this report we demonstrate differences in the leucylpeptide backbones in plasma membranes of the spontaneously transformed DC-3F parental cell line and the actinomycin D-resistant DC-3F/AD X subline. In addition, we found differences in the major glycopeptides. The drug-resistant cells synthesize a surface glycopeptide which is much larger than the major one present on drug-sensitive parent cells.

An unusual observation is that neither of these cell lines appears to possess the plasma membrane marker enzyme 5′-nucleotidase nor the lysosomal enzyme alkaline phosphatase.

MATERIALS AND METHODS

Cells and Media

The origin of the Chinese hamster cells and the derivation of actinomycin D-resistant sublines have been described [9]. Substrate-attached cultures were maintained in Eagle's minimal medium (MEM) supplemented with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). Resistant sublines were grown without drug for 10–15 days prior to experimental analysis. All experiments were done with cells in the exponential phase of growth. Other details of cell preparation were given in an earlier communication [11].

Radioactive Labeling

Chinese hamster cells were grown for 72 h on radioactive fucose, [^{3}H]-L-fucose (New England Nuclear, 13.4 Ci/mmole) at a concentration of 2 μCi/ml, or with [^{3}H]-glucosamine (New England Nuclear, 10.13 Ci/mmole) at a concentration of 0.2 μCi/ml. When cells were grown on ^{3}H-L-leucine, 30 Ci/mmole, 0.2 μCi/ml, one-tenth the amount of leucine normally present in MEM was added to leucine-free medium.

Isolation of Plasma Membranes

Isolation of plasma membranes was carried out essentially by the method of Atkinson and Summers [14] as modified in our laboratory for substrate-attached cultures. Briefly, the medium was aspirated, and the monolayers were washed three times with Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium or with saline alone when membrane marker enzymes were assayed. The cells were detached with 0.5 mM ethylenediaminetetraacetic acid (EDTA) and sedimented at 1,000g for 5 min. Cells were suspended in 10 mM Tris (pH 8.0) and allowed to swell for 10 min in an ice bath. The cells were homogenized with a Dounce homogenizer type B pestle for ten strokes, and breakage was monitored by phase microscopy. The homogenate was then adjusted to 10 mM NaCl and 5 mM $MgCl_2$. The suspension was layered on 45% sucrose in 10 mM Tris (pH 8.0) and centrifuged at 7,000g for 20 min in an HB-4 rotor (Sorvall). The top layer was removed and diluted with 10 mM Tris (pH 8.0) and sedimented at 16,000g for 10 min. The pellet was taken up in 10 mM Tris (pH 8.0) and overlaid on a discontinuous gradient containing 2 ml 45% sucrose and 7 ml 30% sucrose and was spun for 20 min at 7,000g as before. When sufficient material was available, another discontinuous gradient was run. Plasma membranes were collected at the 30–45 interface and again washed in 10 mM Tris (pH 8.0) and sedimented by centrifugation. The pellet was resuspended in 10 mM Tris (pH 8.0) and layered on a 28–42% sucrose gradient and spun in a 27.1 rotor for 16 h at 27,000 rpm. Polyacrylamide sodium dodecyl sulfate (SDS) gel electrophoresis was done according to the procedure of Laemmli [15]. Gels were sliced into 1-mm fractions with an Aliquogel fractionator (Gilson Medical Electronics, Middletown, Wisconsin 53562), and

counted in a dioxane-base scintillation fluid. For measurement of precursor incorporation into glycoproteins, aliquots were precipitated with equal volumes of 10% trichloroacetic acid in 2% phosphotungstic acid, and the precipitates were washed and collected on glass-fiber filters (Whatman GF/C). All samples were counted in a Packard liquid scintillation spectrometer (Packard Instruments, Downers Grove, Illinois).

Plasma Membrane Markers

5′-Nucleotidase (EC 3.1.3.5) was assayed by the method of Bodansky and Schwartz [16] and also by the method of De Pierre and Karnovsky [17]. (Na^{+}-K^{+})-dependent adenosine triphosphatase (ATPase: EC 3.6.1.4) was measured by the procedure of Wallach and Ullrey [18]. Alkaline phosphatase (EC 3.1.3.1) was measured by following the increase in absorbance of nitrophenol at 410 nm, as described in the Worthington manual [19]. Succinic dehydrogenase (EC 1.3.9.1) was assayed according to Green et al [20] and glucose-6-phosphatase (EC 3.1.3.9) as described by Swanson [21] Uridine diphosphatase (UDPase: EC 3.6.1.6) was measured according to the method of Plaut [22]. The phosphate determinations were all determined by Chen's modification [23] of the Fiske and Subbarow reaction [24]. Protein was estimated by the method of Lowry et al [25].

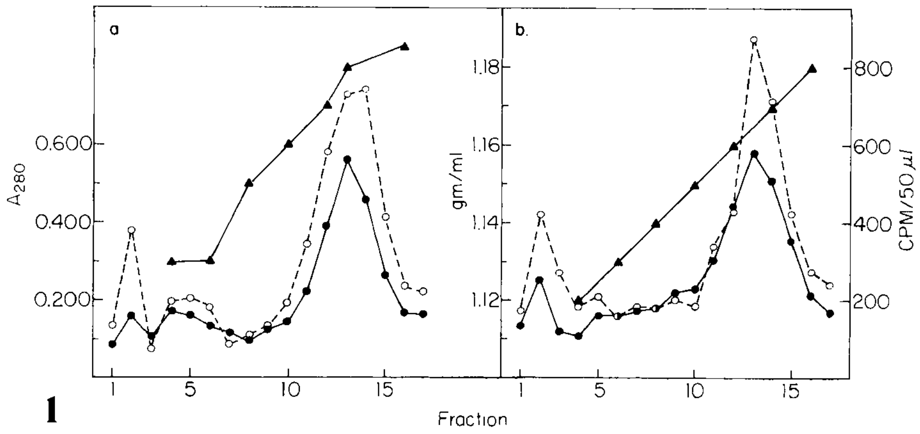
RESULTS

Isopycnic Banding of Chinese Hamster Plasma Membranes

Drug-sensitive DC-3F and actinomycin D-resistant DC-3F/AD X cells were grown in medium containing one-tenth the normal amount of leucine in the presence of 0.20 μCi/ml tritiated leucine. After 72 h, plasma membranes were isolated as described in Materials and Methods. The plasma membranes from both cell lines banded sharply at a density of 1.16 g/ml (Fig. 1). Similar gradient profiles were obtained when cells were grown in the presence of fucose or glucosamine. A small amount of radioactivity was associated with a less dense material located at the top of the gradient with a density of 1.11 g/ml, which may be fragmented membranes or possibly membrane vesicles [14]. This minor fraction was usually observed also when the cells were grown on glycoprotein precursors.

SDS Gel Electrophoresis of the Leucyl-Labeled Plasma Membrane Peptides

The pooled membrane peptides obtained from the gradient fractions 12, 13, and 14, as shown in Figure 1, were separated on SDS gel electrophoresis (Fig. 2). A major leucyl peptide of 46,000 daltons was present in both DC-3F and DC-3F/AD X plasma membranes, which is most likely actin, a known constituent of fibroblasts. A large peptide of 100,000 daltons was present in the membranes from the antibiotic-resistant cells, but was not found in membrane preparations of the drug-sensitive parental cells. The DC-3F plasma membranes show a minor component with a molecular weight just above 200,000 daltons (Fig. 2). Most of the other polypeptides are coincident in the two cell lines and exhibit a marked degree of relatedness. A comparison of total membrane proteins on gels stained with Coomassie blue and then scanned at 540 nm is shown in Figure 3. These profiles compare favorably with the radioactivity profiles shown in Figure 2 for both cell lines. The glycopeptide profiles from plasma membranes obtained from cells grown on labeled glucosamine are shown in Figure 4. The predominant glycopeptide from membranes of the DC-3F cells has an apparent molecular weight of 93,000 daltons, whereas the actinomycin



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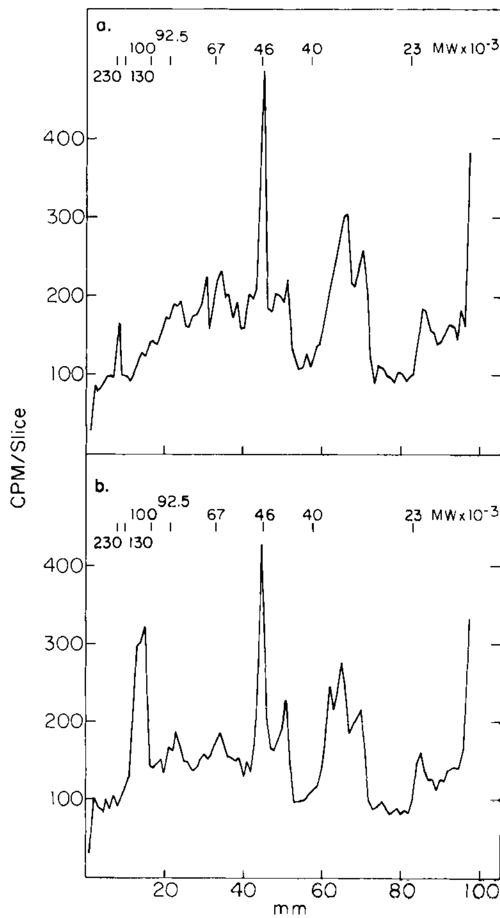

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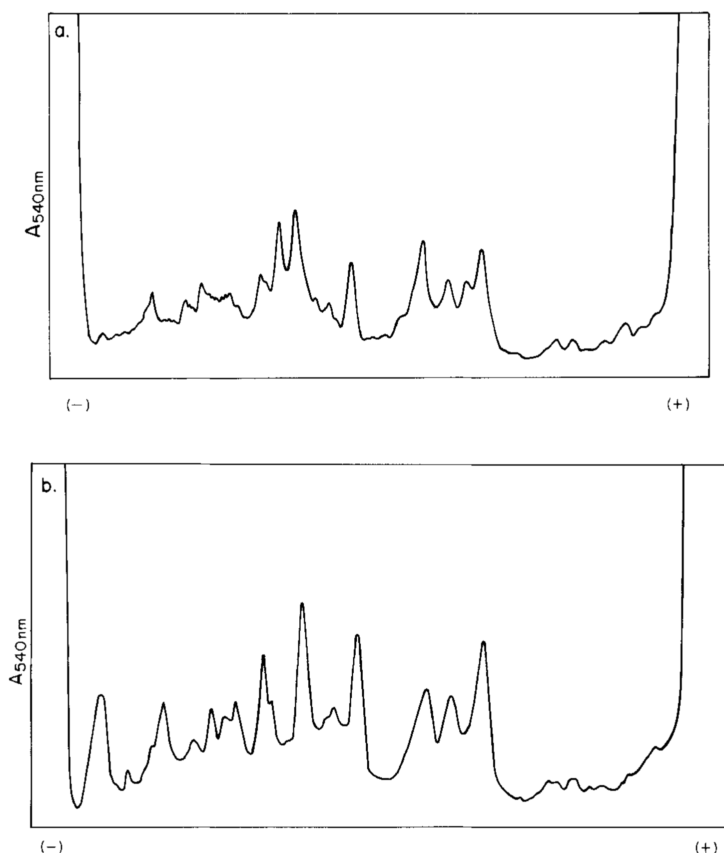

Fig. 3. Sodium dodecyl sulfate gel electrophoresis on 10% polyacrylamide of plasma membranes from a) DC-3F and b) DC-3F/AD X. The gel was stained with Coomassie blue, and the gels were scanned in a Beckman spectrophotometer Acta CV at 540 nm. Top of the gel is to the left.

Fig. 1. Isopycnic banding of Chinese hamster cell plasma membranes from DC-3F and DC-3F/AD X labeled with ^{3}H-leucine 0.2 μCi/ml of medium containing one-tenth the normal amount of leucine. The material was taken from a discontinuous gradient at the 30–45% w/w interface and after washing and concentrating was applied to an 18-ml 28–42% w/w linear sucrose gradient and banded by centrifugation in a Spinco 27.1 rotor at 27,000 rpm for 16 h at 4°C. Densities were measured with a refractometer. •) A_{280nm}, ○) cpm/50 μl, ▲) g/ml. a) DC-3F, b) DC-3F/AD X.

Fig. 2. Sodium dodecyl sulfate gel electrophoresis on 10% polyacrylamide of plasma membranes from Chinese hamster cells grown on tritiated leucine and pooled and concentrated from fractions 12, 13, and 14, as shown in Figure 1. a) DC-3F, b) DC-3F/AD X. The standard reference proteins were myosin 230,000, β-galactosidase 130,000, phosphorylase a 92,500; bovine serum albumin 67,000; actin 46,000; aldolase 40,000, and IgG light chain 23,000.

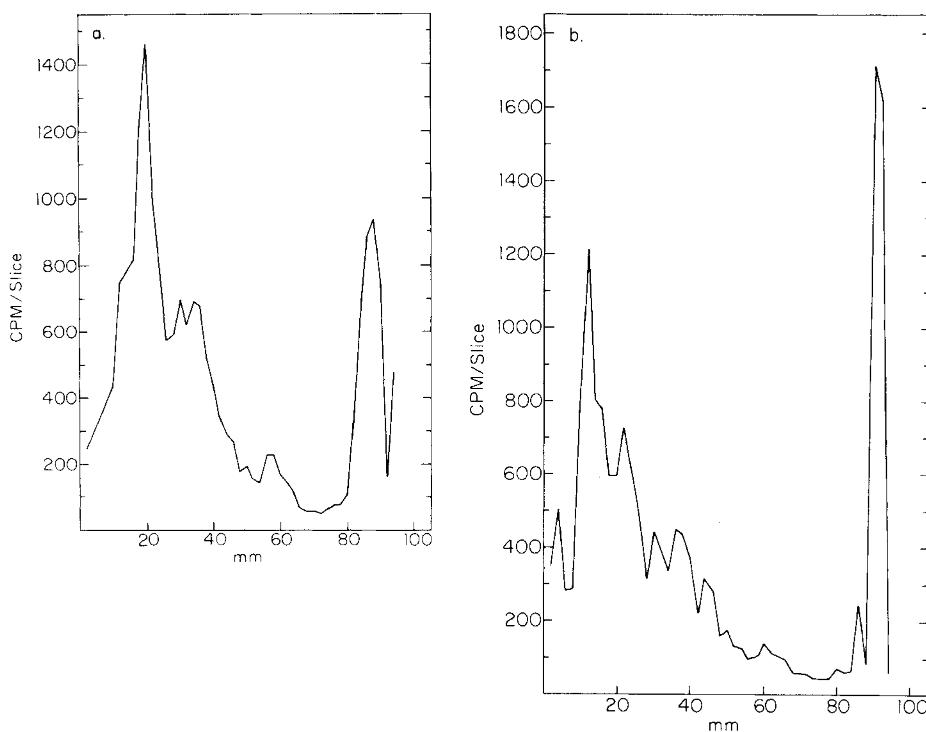

Fig. 4. Sodium dodecyl sulfate gel electrophoresis on 10% polyacrylamide of plasma membranes grown on tritiated glucosamine for 72 h and purified on two cycles of discontinuous gradients, as described in Materials and Methods. a) DC-3F, b) DC-3F/AD X.

D-resistant cells synthesize a major glycopeptide with a molecular weight of approximately 150,000 daltons. Similar profiles were obtained with cells grown on radioactive fucose. The minor components at fractions 30 and 35 mm were present in both drug-sensitive and drug-resistant cell populations. The rate of glycoprotein turnover of membrane proteins is shown in Figure 5. The cells were grown on labeled glucosamine for 24 h, the medium was aspirated, the cultures were washed with serum-free medium, and one series of flasks was harvested and membranes prepared at "time zero." Conditioned medium was added to the remaining flasks, which were harvested at 24, 48, and 72 h. These values are expressed as percentages of cpm/mg protein. The rates of turnover of the actinomycin D-resistant membrane proteins are shown in Figure 5. Similar results were obtained with preparations from the parental cells. From the specific activity curves a half-life of 14.4 h was observed for both cell lines. The individual glycopeptide profiles do not reveal any differential loss of peptides over the 72-h period; that is, there is no preferential loss of high-molecular-weight material. Therefore, it appears that there is a heterogeneous turnover of membrane glycopeptides from both cell lines. We do, however, find shedding of peripheral glycoproteins into the medium during growth (data not shown).

The specific activities of plasma membrane marker enzymes are shown in Table I. There was a 9- to 11-fold enrichment in the $(Na^{+}\text{-}K^{+})$-dependent ATPase obtained from discontinuous gradients, and values were greatest in the 1.16 g/ml fraction shown in Figure 1. There is evidence of mitochondrial contamination as revealed by the presence of succinic

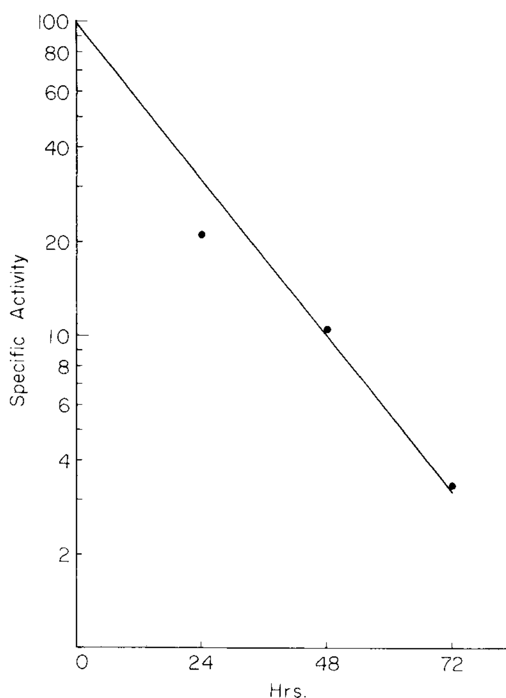

Fig. 5. Turnover of plasma membrane glycoproteins in Chinese hamster cells grown in the presence of tritiated glucosamine for 72 h. The medium was then aspirated, the cells washed with fresh MEM without serum, and the "zero-time" set was harvested. The remaining flasks were harvested at times 24, 48, and 72 h. Plasma membranes were prepared as described in Materials and Methods. Results are expressed as percentage cpm of membrane mg/protein. DC-3F.

dehydrogenase in both the upper and lower phases of the gradients. When we use a post-mitochondrial supernatant [14] before applying sample to the gradients, a considerable loss in plasma membranes occurred (data not shown). There was no detectable 5′-nucleotidase or alkaline phosphatase in either cell line when up to 3 mg of membrane protein was assayed. These two markers copurify with plasma membranes in L1210 cells from our laboratory (data not shown). We thank Dr. C.H. Yang for verification of these preliminary results.

DISCUSSION

The parental Chinese hamster cells described in this report are spontaneously transformed and are tumorigenic in a heterotransplantation system [26]. The actinomycin D-resistant subline DC-3F/AD X is nontumorigenic and displays morphologic and growth properties of putatively normal cells, eg, increased adhesiveness, greater density-dependent inhibition of growth, and reduced agglutination by plant lectins [26, 27]. Actinomycin D-resistant Chinese hamster cells derived from the DC-3F line are cross-resistant to a variety of agents within a given molecular weight range [9]. Earlier studies with the detergent Tween 80 [10] demonstrated a potentiation of uptake of actinomycin D, thereby substantiating the evidence [9] that the major barrier to drug effect is the plasma membrane. In a later communication, it was shown that over 250 times more actinomycin D was

TABLE I. Distribution of Plasma Membrane Marker Enzymes of Chinese Hamster Cells

Cell line	Fraction	(Na^+-K^+)-ATPase[a] (μmoles·mg^{-1}·h^{-1})	5'-AMPase (μmoles·mg^{-1}·h^{-1})	Alkaline phosphatase	Succinic dehydrogenase (nmoles·mg^{-1}·h^{-1})	Glucose-6-phosphatase (μmoles·mg^{-1}·h^{-1})	UDPase (μmoles·mg^{-1}·h^{-1})
DC-3F	Crude homogenate	0.094 ± 0.066	ND[a]	ND	0.458	0.159 ± 0.015	0.954 ± 0.412
	Upper phase	0.421 ± 0.067	ND	ND	1.66	1.11 ± 0.159	3.77 ± 0.212
	Lower phase	0.820 ± 0.048	ND	ND	3.74	0.743 ± 0.112	1.88
	Linear	1.61 ± 0.491	ND	ND			
DC-3F/ AD X	Crude homogenate	0.056	ND	ND	0.562	0.105	0.780
	Upper phase	0.636 ± 0.217	ND	ND	1.49	0.680	13.39
	Lower phase	0.607 ± 0.055	ND	ND	4.99	0.640	10.29
	Linear	2.25 ± 0.880	ND	ND			9.62

[a] Not detectable.

For a description of the fractions, see Materials and Methods.

required to inhibit RNA synthesis than was required by the sensitive cells [11]. More recently]28], it was reported that lipid vesicles could mediate the transversion of actinomycin D across the plasma membrane of antibiotic-resistant cells and restore inhibition of RNA synthesis to the sensitive control levels. We thus initiated investigations of the plasma membrane. In this first study we have examined the composition of leucyl-labeled proteins and glycoproteins of membranes isolated from the antibiotic-sensitive and -resistant cells. Indeed, we have observed differences in the nonglycosylated proteins as well as the glycoproteins. Although there are differences in the protein backbones and in the glycoproteins, there is some degree of relatedness between the membrane components of sensitive and resistant cells as indicated by SDS gel electrophoresis profiles.

An unusual finding for our Chinese hamster cells is the absence of the plasma membrane marker enzyme 5′-nucleotidase. This enzyme is considered by many to be a ubiquitous enzyme marker for plasma membranes. However, McKeel and Jarett [29] were not able to detect 5′-nucleotidase activity in any of their fractions from rat fat cells. More recently, Trams and Lauter [30] reported the apparent absence of both 5′-nucleotidase and p-nitrophenyl-phosphatase in L-929, a mouse fibroblast line. Juliano and Gagalang [31] observed 5′-nucleotidase in Chinese hamster ovary cells, although there was no enrichment in their plasma membrane fraction. Therefore, this enzyme may not be reliably used as plasma membrane marker in all cells. Another unusual observation was that our cell lines lack alkaline phosphatase activity as do the L-929 cells reported by Trams and Lauter [30]. Alkaline phosphatase has been shown to copurify with L1210 plasma membranes [32]; we also demonstrated alkaline phosphatase activity in isolated membranes from L1210 cells (unpublished observations).

Our actinomycin D-resistant cells have a surface glycoprotein with an apparent molecular weight of 150,000 daltons which is considerably smaller than the 205,000-dalton peptide on the surface of the chick embryo fibroblast cells described by Teng and Chen [33]. The latter glycoprotein was implicated in cellular adhesion, since mild proteolytic treatment causes loss of this peptide with concomitant loss of adhesiveness [34]. We do not as yet know whether the 150,000-dalton peptide is involved in the increased adhesiveness of our actinomycin D-resistant Chinese hamster cells.

No observable differences were noted in turnover rate in the individual glycopeptides of either cell line DC-3F or DC-3F/AD X. However, the total turnover rate was similar to that reported by Kaplan and Moskowitz [35] for both growing and nongrowing M-K_2 cells. Roberts and Yuan [36] measured similar turnover rates in Chinese hamster ovary cells; these authors also failed to find a preferential turnover of individual membrane peptides. Warren and Glick [37] determined the rate of turnover of plasma membranes in mouse L cells and found the rate of synthesis was equal to the rate of degradation in nongrowing cells. Under our experimental conditions, actinomycin D-sensitive and -resistant cells were in exponential growth phase.

Our results are compatible with those reported by Juliano et al [6] for colchicine-resistant mutants of Chinese hamster ovary cells. An altered surface component with an apparent molecular weight of 165,000 daltons was found in drug-resistant cells. This glycopeptide may be similar to, or the same as, the one we measured at 150,000 daltons. In addition, their drug-revertant cell line 18–31 showed only modest amounts of the 165,000-dalton peptide. It is notable that these CH^RC4 cells are cross-resistant to actinomycin D [7]. Although our plasma membrane preparations do have some mitochondrial contamination, the glycopeptides found in the plasma membranes have been shown to be present in other subcellular fractions as well [3]. We are presently attempting to determine whether

the 150,000 dalton-glycopeptide present on the surface of the DC-3F/AD X cells is responsible for resistance to actinomycin D. The regulation and control of glycoproteins and their participation in drug resistance are currently under investigation in our laboratory.

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