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Altered plasma membrane ultrastructure in multidrug-resistant cells

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Multidrug resistance is mediated by P-glycoprotein, an integral plasma membrane component which is thought to function as a drug export pump. This model can explain drug resistance, but fails to account for the broader pleiotropy of the multidrug resistance phenotype. We report here a freeze-fracture study revealing increases in the densities of protoplasmic face intramembrane particles in multidrug-resistant Chinese hamster ovary (CHO) and human leukemic cells. The intramembrane particle density in a CHO cell revertant which had lost the characteristics of the multidrug resistance phenotype was indistinguishable from that of the drug-sensitive parental cell line. This demonstration of a global multidrug resistance-linked change in plasma membrane architecture may have significant implications for understanding the variety of concurrent membrane-related changes which are not easily explained by the current model for multidrug resistance.

The multidrug resistance phenotype in mammalian cells is characterized by cross resistance to a spectrum of unrelated drugs as a result of reduced net drug accumulation [1–3]. Consequently, experimental multidrug resistance is regarded as a possible model of non-response in cancer chemotherapy [1,4,5]. Recently, it has been proposed that the increased expression of a 170 kDa plasma membrane glycoprotein, P-glycoprotein, mediates the phenotype through the extrusion of a variety of drugs from the multidrug-resistant cell. This is supported by considerable evidence; e.g.

(1) Multidrug-resistant cells accumulate cytotoxic drugs at a reduced rate, thereby implying an alteration in plasma membrane permeability or transport processes (reviewed in Refs. 2,3,5–8); and an increase in an energy-dependent drug efflux has been demonstrated with a number of multidrug-resistant cell lines [8].

(2) Increased expression of the highly conserved P-glycoprotein correlates with multidrug resistance in a variety of rodent and human cell lines [9,10] and is the most consistent alteration associated with the phenotype. Moreover, gene transfection studies indicate that increased expression of the transfected P-glycoprotein gene alone is sufficient to confer multidrug resistance on the recipient cell [11–13].

(3) Structural analyses of P-glycoprotein cDNA from mouse [14], human [15] and hamster [16] indicate that P-glycoprotein contains two homologous halves, each with a presumptive transmembrane region of six hydrophobic segments and a

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cytoplasmic ATP-binding domain. Thus, P-glycoprotein has features of a pore-forming, energy-transducing protein [17]. These structural features, along with striking homology to bacterial permeases, particularly to the *Escherichia coli* hemolysin export system [14,16,18], suggest that P-glycoprotein functions as an efflux pump. Drugs might be extruded from the cells either directly or indirectly by P-glycoprotein [16]. Such a model has been further supported by studies which show that P-glycoprotein binds some of the drugs involved in the multidrug resistance phenotype [19–21].

While the above model may explain the main feature of multidrug resistance, namely the extensive cross resistance to a number of unrelated cytotoxic drugs, it does not readily account for other pleiotropic changes frequently found in multidrug-resistant cells. These include collateral sensitivity to membrane active agents such as detergents, local anaesthetics and steroid hormones; resistance to channel-forming ionophores; changes in ion, amino acid and nucleoside transport; and changes in membrane physical properties, such as increased cellular fragility to shearing and osmotic shock, and altered membrane fluidity (for review, see Refs. 2 and 3). In highly drug-resistant cells, P-glycoprotein appears to be a major protein component of isolated plasma membranes [9,22,23], but little is known concerning the extent to which morphology of the plasma membrane is affected. The present study was undertaken to assess by freeze-fracture replication the plasma membranes of multidrug-resistant cells.

In the CHO tissue culture system, a series of cell lines selected for increasing resistance to colchicine is available where the correlation between cell surface P-glycoprotein expression and multidrug resistance is well established [9,10,24]. A revertant cell line is also available which, in a single step selection, has lost concomitantly P-glycoprotein expression, colchicine resistance and cross resistance to other drugs [6,9,10]. This genetically defined system is ideally suited to the investigation of whether cell membrane morphology can be correlated with a biochemically defined membrane alteration. A human multidrug-resistant cell line selected for resistance to vinblastine [25] and containing high levels of P-glycoprotein [10] was also examined. Characteristics of these cell lines are summarized in Table I.

Freeze-fracture replicas were prepared from fixed, glycerinated cell pellets of the aforementioned cell lines and plates were taken of well preserved fields. Quantitative data were obtained by counting intramembrane particles within a template superimposed on membrane fields in numerous micrographs of freeze-fracture faces, and by measuring individual particles with a calibrated graticule. Representative electron micrographs of freeze-fracture replicas of drug-sensitive and drug-resistant cells are illustrated in Fig. 1. This figure portrays the immediate visual impact of a gross textural alteration in protoplasmic face morphology observed in both CHO and human leukemic drug-resistant cells. This effect is largely the result of a striking increase in intramembrane particle density throughout the inner leaflet of the plasma membrane of the multidrug-resistant cells. The fracture faces of all cells revealed a homogeneous intramembrane particle distribution without the occurrence of linear arrays or plaque-like aggregates. In both CHO and human cell lines the exoplasmic face was sparsely populated with intramembrane particles in comparison with the protoplasmic face. Thus, in spite of the striking differences in protoplasmic face morphology, there seemed to be little difference in the appearance of the exoplasmic faces examined (not shown), and therefore the present study was confined to protoplasmic faces.

The qualitative differences shown in Fig. 1 are supported by the quantitative data summarized in Fig. 2. In the drug-sensitive CHO cell line, AUXB1, the protoplasmic face revealed a moderate intramembrane particle density ($970 \pm 200/\mu m^2$). In striking contrast, the protoplasmic face of the drug-resistant CHO cells, CH^RB30, had an extraordinarily high intramembrane particle density ($2700 \pm 150/\mu m^2$), representing approximately a 3-fold increase over that in the drug-sensitive cells. Fig. 2B shows the size distributions of intramembrane particles in the various cell lines. In the drug-sensitive CHO cells 80% of the intramembrane particles measured 4–5 nm in diameter and 20% in the 6–9 nm range. A shift in the average intramembrane particle size was readily apparent in the drug-resistant CHO cell line,

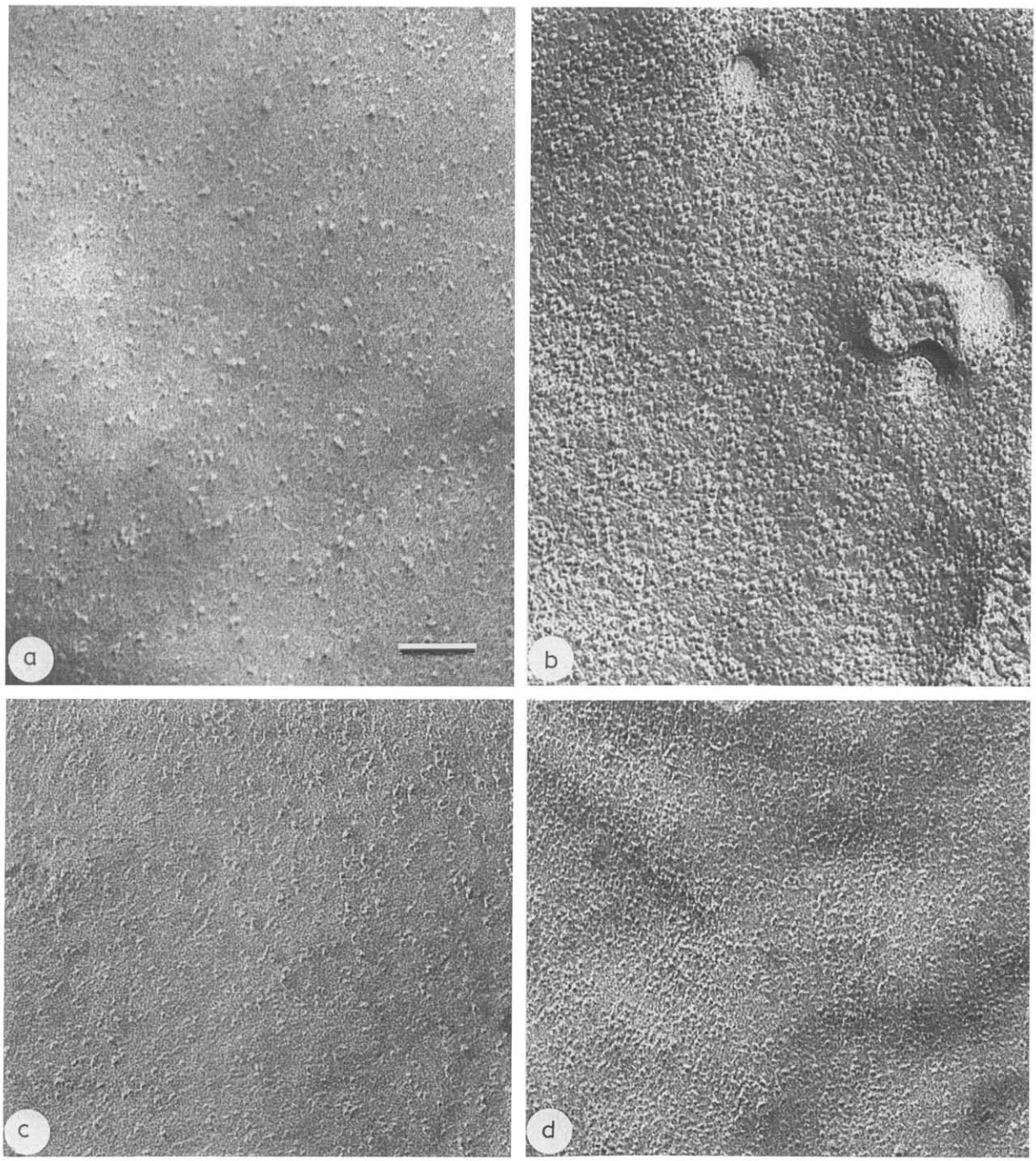

Fig. 1. Freeze-fracture replicas of drug-sensitive and multidrug-resistant CHO and human leukemic cell lines. Cell suspensions were pelleted and fixed in 5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.3) containing 5% sucrose, for 30 min on ice. Cells were rinsed in buffer, glycerinated in buffer containing 20% glycerol for 5–10 min, frozen in liquid Freon 22, and transferred to liquid nitrogen. Freeze-fracturing was performed using a Balzers 301 device at −105 °C, and replication was done at a 45° angle with platinum and carbon, then stabilized with carbon. Replicas were cleansed in chromic acid, placed on grids, examined in a Philips 300 electron microscope and recorded at 16000× magnification. All aspects of freeze-fracture replication, tissue fixation and cryoprotection, and photographic reproduction were standardized to avoid apparent intramembrane particle alteration due to technical variability. The fracture planes of protoplasmic faces are revealed in (a) drug-sensitive, AUXB1, and (b) highly multidrug-resistant, $CH^{R}B30$, CHO cell lines, each illustrating their characteristic intramembrane particle densities. The highly elevated intramembrane particle density on the protoplasmic face of the resistant cell (b) is due to the selective amplification of the 6–9 nm particles. Protoplasmic faces of (c) a drug-sensitive human leukemic cell (CCRF-CEM) and (d) a highly multidrug-resistant derivative (CEM/VLB_{4500}) illustrate their relative intramembrane particle densities with the resistant cell possessing a 2-fold increase in particles. The exoplasmic faces of each cell line revealed a sparse intramembrane particle distribution (not shown) with no significant differences observed between sensitive and resistant cells. The scale bar in (a) is 100 nm.

TABLE I

DESCRIPTION OF CELL LINES

The CHO cell line CH^RB3 was clonally selected, stepwise, from the drug-sensitive parental line, AUXB1 [24]. CH^RB30 was derived from CH^RB3 by stepwise selection (giving an intermediate cell line, CH^RC5), and continuous culture in increasing colchicine concentrations. I10 is a revertant cell line, selected in a single step from CH^RC5 [6]. Note that the reversion of I10 is not complete, in that a low level of residual resistance remains. CEM/VLB_{4500} was derived from the parental line CCRF-CEM by continuous culture in increasing vinblastine concentrations via the intermediate cell line CEM/VLB_{100} [10]. Relative resistance was determined as described previously [26]. The apparent increase in P-glycoprotein relative to the drug-sensitive parent was determined from fluorescence flow-cytometric studies with a P-glycoprotein-specific monoclonal antibody [10]. The numbers given should be regarded as minimum values, since the baseline for the drug-sensitive parent cell is raised by indeterminate non-specific antibody binding. In the cross resistance column, drugs are listed in order of decreasing relative cross resistance. Since cross resistance is shown only for those drugs tested, absence of a drug from the cross resistance column does not imply lack of cross resistance to that drug. Drug abbreviations: ACR, acriflavine; CCH, colchicine; CMD, colcemid; CYB, cytochalasin B; DNR, daunorubicin; DOX, adriamycin; EME, emetine; ERY, erythromycin; ETB, ethidium bromide; PUR, puromycin; VCR, vincristine; VLB, vinblastine.

Species	Parent line	Resistant line	Drug of selection	Selection concn. (μg/ml)	Relative resistance	P-glyco-protein increase	Cross resistance	Refs.
Chinese hamster	AUXB1	CH^RB3	CCH	3.0	31	3.5	PUR ETB DNR CCH EME ACR CYB ERY	24,26
		CH^RB30	CCH	30	640	18	CCH PUR DOX	10
		I10	–	–	2	1.1	CCH CMD VLB DNR PUR	6
Human	CCRF-CEM	CEM/VLB_{4500}	VLB	4.5	$\approx$ 3500	5.1	VCR VLB PUR DNR DOX	10,25

where only 2% were in the 4–5 nm range and 90% measured 6–9 nm in diameter. It is this quantitative difference in the larger particles which accounts for the obvious visual difference observed between Fig. 1a and Fig. 1b, since the protoplasmic face of the drug-resistant cell line showed a 14-fold increase in 6–9 nm intramembrane particles in comparison with the drug-sensitive cell line. This increase was similar to that seen for P-glycoprotein in the same cells (Table I).

Further evidence of a quantitative relationship between intramembrane particle density and multidrug resistance in CHO cells was obtained on investigation of a cell line of intermediate resistance, CH^RB3, and a drug-sensitive revertant, I10. The series of CHO cells (Fig. 2A, a–d) illustrates a significant stepwise increase in protoplasmic face intramembrane particles with increasing drug resistance (intermediate resistance yielded $1590 \pm 180/\mu m^2$), and a decrease in intramembrane particles for the revertant ($1000 \pm 180/\mu m^2$) to a level indistinguishable from that of drug-sensitive cells. It is of interest that the 6–9 nm particles in the CHO cell line of intermediate resistance constituted 64% of the total protoplasmic face intramembrane particles (Fig. 2B). This size group showed a 6-fold increase in density over that in the sensitive CHO cell line, confirming the intermediate appearance of the micrographs (not shown). The total number of protoplasmic face intramembrane particles in the revertant cell line was essentially the same as that in the sensitive cell line, but the size distribution in the revertant cells, where 6–9 nm intramembrane particles comprised 62% of the total, was more like that in the cells of intermediate resistance (Fig. 2B). Again, the quantitative differences in the 6–9 nm intramembrane particles resembled those seen for P-glycoprotein (Table I).

Similar results were obtained for the two human leukemic cell lines (Figs. 1c and 1d), as shown in Fig. 2A (e and f). The multidrug-resistant cells, CEM/VLB_{4500}, had a significantly increased amount of intramembrane particles ($3020 \pm 560/\mu m^2$) in comparison with the drug-sensitive cells, CCRF-CEM ($1670 \pm 500/\mu m^2$), but unlike the CHO cells, the human cells showed no significant differences in intramembrane particle size distribution, 95% of the particles being within the 4–5 nm range in both cases (Fig. 2B).

The approximately 2-fold increase in intramembrane particles was not as dramatic as in the CHO system, possibly due to a higher basal level of intramembrane particles in the drug-sensitive human cells. The apparent discrepancy with the comparable increase in P-glycoprotein of approximately 5-fold (Table I) requires some explanation. We speculate that only a fraction of the

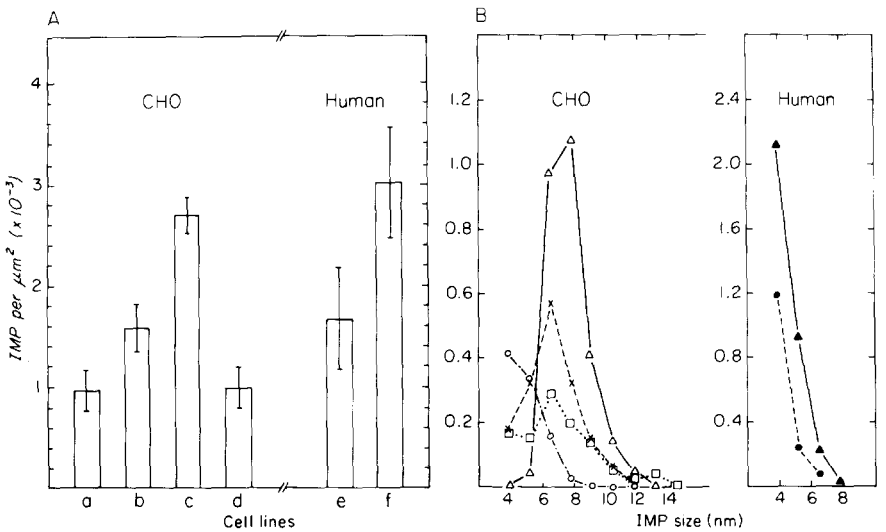

Fig. 2. Quantitation of protoplasmic face intramembrane particles of CHO and human leukemic plasma membranes. Representative micrographs printed at 77000× magnification were prepared from replicas of the different cell lines (see Fig. 1). Flat areas of protoplasmic faces were chosen at random and overlayed with a template revealing 0.04 μm^2 of membrane surface area. All particles of about 4 nm or greater diameter were counted. The normal granular background of the membrane surface did not allow discrimination of smaller particles. For statistical calculations, the intramembrane particle counts taken from four fields in each sampled cell were summed. Variances among fields from any one cell were small compared with variances among cells of a given cell line. Data for the CHO and human groups were treated separately. The numbers of cells analysed were, for CHO cells (panel A) a, b, c and d: 10, 7, 17 and 12, and for human leukemic cells (panel A) e and f: 11 and 9, respectively. The Kruskal-Wallis test applied to the pooled data of the CHO group confirmed that there were highly significant differences among the cell lines ($P < 0.005$). Mann-Whitney tests confirmed highly significant differences (two-tailed $P < 0.01$) for all pairs except the drug-sensitive and revertant CHO cells (panel A, a and d; two-tailed $P = 0.4$). No difference was found between the Z-score for the c vs. a increment (highly resistant versus sensitive) for CHO cell intramembrane particle density and the Z-score for the f vs. e increment (highly resistant versus sensitive) for human leukemic cell intramembrane particle density (two-tailed Z test, $P = 0.4$). All results were normalized to give numbers of intramembrane particles per μm^2 of plasma membrane surface. Error bars represent 95% confidence limits calculated from pooled standard deviations. In order to determine the intramembrane particle size distribution, areas of electron micrographs were examined using a 7× magnifying lens with graticule calibrated in 0.1 mm increments. Five hundred particles were measured to the nearest 0.1 mm for each cell line. Sizes were determined by measuring along the shadow line of each particle. Particles were typically globular in appearance, apparently approximating a spherical shape. Data were plotted on a scale representing the calculated true particle sizes in nanometres. (Panel A) Intramembrane particle density in protoplasmic faces of drug-sensitive and multidrug-resistant CHO and human leukemic cell lines. CHO, mean number of intramembrane particles per μm^2 associated with protoplasmic face of plasma membranes of different CHO cell lines; (a) drug-sensitive parental cell line, AUXB1; (b) cell line of intermediate drug resistance, CH^RB3; (c) highly drug-resistant cell line, CH^RB30; (d) drug-sensitive revertant cell line, I10. In (c), the high density of large intramembrane particles may have masked the smaller intramembrane particles and, consequently, particles smaller than 6 nm may be under-represented. Human, mean number of intramembrane particles per μm^2 associated with protoplasmic face of plasma membranes of drug-sensitive and drug-resistant human leukemic cell lines; (e) drug-sensitive parental cell line, CCRF-CEM; (f) highly drug-resistant cell line CEM/VLB_{4500}. For description of cell lines see Table I. (Panel B) Density of intramembrane particles (IMP), according to size, associated with protoplasmic face in drug-sensitive and multidrug-resistant CHO and human leukemic cell lines. CHO, series of CHO cells of different resistance. Drug-sensitive cell line, AUXB1, O·–·–·O; cell line of intermediate drug resistance, CH^RB3, ×–––×; highly drug-resistant cell line, CH^RB30, △———△; drug-sensitive revertant, I10, □- - - - -□. Human, drug-sensitive and multidrug-resistant human leukemic cell lines. Drug-sensitive cell line, CCRF-CEM, ●–––●; highly drug-resistant cell line CEM/VLB_{4500}, ▲———▲.

basal level of intramembrane particles in the drug-sensitive human leukemic cells is likely to be related to those that are amplified in the drug-resistant cells, so the increase in that specific class of intramembrane particles may be considerably greater than 2-fold. With the present methods it is impossible to determine the increase specifically in the resistance-related intramembrane particles. It is interesting to note that the relative increase in P-glycoprotein itself is not as dramatic in the human cells as in the CHO cells (Table I). Nevertheless, an absolute increase in intramembrane particle density of approx. 10^3 per μm^2 of plasma membrane surface in the highly drug-resistant human leukemic cells is remarkable, and not significantly different from the absolute increase in intramembrane particles observed in the highly resistant CHO cells. Thus, based on similar increases in the density of intramembrane particles associated with the protoplasmic face, both the CHO and human leukemic cell lines displayed dramatic morphological modifications of the inner leaflet of the plasma membrane which correlated with the expression of the multidrug resistance phenotype.

Clearly the present correlation between protoplasmic face-associated intramembrane particles and multidrug resistance is consistent with that observed previously between P-glycoprotein and multidrug resistance (Table I and Refs. 9, 10, 27). The relationship between the degree of drug resistance (Table I) and the intramembrane particle density in the CHO cells (Fig. 2A) appears exponential, rather than linear, similar to that observed for drug resistance and P-glycoprotein expression in another multidrug resistance model [28]. Furthermore, the correlation of increased intramembrane particles with drug resistance holds true for the human leukemic cell lines. This is a significant finding as the only consistent molecular alteration found between the plasma membranes of multidrug-resistant CHO and human cells is the increased expression of P-glycoprotein [9,10]. Thus, the increased numbers of intramembrane particles in the resistant cells (approx. 10^6/cell) likely represent either P-glycoprotein molecules, or localized perturbations of membrane bilayer organization mediated by P-glycoprotein molecules.

The modulation of membrane physical properties, which could directly affect diffusional drug uptake, or indirectly affect transport processes, has been postulated as a possible functional role for P-glycoprotein in multidrug-resistant cells [6]. The high density of intramembrane particles observed in the highly drug-resistant cells in Fig. 1 is consistent with this view, since it was reminiscent of that seen in naturally occurring specialized membrane structures, such as junctional complexes, where cell surface permeability is known to be altered. Examination of thin sections prepared by slam freezing and freeze substitution of drug-sensitive and drug-resistant cells, however, revealed no apparent differences in the thickness, density, or glycocalyx of their plasma membranes, and no underlying cytoplasmic density, such as that seen in association with junctional complexes, was observed (data not shown). Moreover, in the drug-resistant cells the entire plasma membrane was affected, with no evidence of patching or clustering of intramembrane particles. Thus, the phenomenon described here appears to be unrelated to known specialized membrane structures.

P-glycoprotein likely functions directly in the active transport of a variety of drugs from the cell, but the large amount of P-glycoprotein required to maintain a high level of multidrug resistance must also significantly perturb the cell membrane. This perturbation is presumably reflected in the dramatic alteration of membrane ultrastructure, as revealed by freeze-fracture, and may account for membrane-related changes that are not explained by a simple export-pump model for multidrug resistance. Perturbation and possibly reorganization of lipid domains by the high density of intramembrane particles in the highly drug-resistant cells could account for alterations in unrelated membrane transport systems which are affected by local membrane composition, and might also explain altered sensitivity to surface active agents, and altered membrane physical properties, all of which have been seen in association with multidrug resistance [2,3]. It is interesting to note that although P-glycoproteins of different species are very similar in size and structure [9,10,14–16], the resistance-associated intramembrane particles of CHO and human leukemic cells appear to be different in size. As intramembrane particle mor-

phology is likely to be dependent on lipid and protein interaction, this difference in intramembrane particle size may reflect inherent differences in membrane composition or membrane microenvironments in the two cell types. Such differences in the membrane domains in which P-glycoprotein resides may in turn account for the specific pleiotropy expressed by a particular multidrug-resistant clone. Recently, the human P-glycoprotein gene has been functionally transfected into rodent cells, indicating the cross-species conservation of function [11]. It would be of interest to examine the intramembrane particle morphology of such transfectants in the light of the present findings.

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