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Reduced Drug Accumulation and Multidrug Resistance in Human Breast Cancer Cells Without Associated P-Glycoprotein or MRP Overexpression

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Abstract MCF-7 human breast cancer cells selected in Adriamycin in the presence of verapamil developed a multidrug resistant phenotype, which was characterized by as much as 100,000-fold resistance to mitoxantrone, 667-fold resistance to daunorubicin, and 600-fold resistance to doxorubicin. Immunoblot and PCR analyses demonstrated no increase in *MDR*-1 or MRP expression in resistant cells, relative to parental cells. This phenotype is similar to one previously described in mitoxantrone-selected cells. The cells, designated MCF-7 AdVp, displayed a slower growth rate without alteration in topoisomerase IIα level or activity. Increased efflux and reduced accumulation of daunomycin and rhodamine were observed when compared to parental cells. Depletion of ATP resulted in complete abrogation of efflux of both daunomycin and rhodamine. No apparent alterations in subcellular daunorubicin distribution were observed by confocal microscopy. No differences were noted in intracellular pH. Molecular cloning studies using DNA differential display identified increased expression of the alpha subunit of the amiloride-sensitive sodium channel in resistant cells. Quantitative PCR studies demonstrated an eightfold overexpression of the alpha subunit of the Na+ channel in the resistant subline. This channel may be linked to the mechanism of drug resistance in the AdVp cells. The results presented here support the hypothesis that a novel energy-dependent protein is responsible for the efflux in the AdVp cells. Further identification awaits molecular cloning studies. J. Cell. Biochem. 65:513–526.

Key words: mitoxantrone; drug resistance; non-Pgp MDR; rhodamine

Overexpression of P-glycoprotein reduces intracellular accumulation of chemotherapeutic agents and causes multidrug resistance. However, the role that P-glycoprotein plays in clinical resistance in oncology, and particularly in breast cancer, has been difficult to define. Whereas P-glycoprotein expression has been observed in clinical breast cancer samples, it has been difficult to determine whether or not this expression has prognostic significance [Veneroni et al., 1994; Decker et al., 1995; Schneider and Romero, 1995]. One possible explanation for the inability to define a role for P-glycoprotein is the existence of other prominent mechanisms of drug resistance. Both laboratory and clinical evidence suggest that mechanisms of drug efflux other than P-glycoprotein exist. First, cell lines exhibiting decreased drug accumulation without P-glycoprotein overexpression have been described [Center, 1993]. Second, discordance has been reported between drug efflux and P-glycoprotein expression in human leukemic cells [Marie et al., 1993; Leith et al., 1995].

A second transporter linked with drug resistance is the multidrug resistance-associated protein (MRP), which has been associated with either reduced accumulation, or increased efflux, of doxorubicin, rhodamine, vincristine, VP-16, calcein, and glutathione-conjugated substrates [McGrath and Center, 1988; Twentyman et al., 1994; Zaman et al., 1994; Feller et al., 1995; Schneider et al., 1995]. Increased accumulation, or decreased efflux, has been reported following ATP depletion or treatment with probenecid, BSO, the protein kinase C inhibitor GF 109203X, and the tyrosine kinase inhibitor genistein [Versantvoort et al., 1992; Versantvoort et al., 1994; Feller et al., 1995; Gekeler et al., 1995; Schneider et al., 1995]. For some of

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these compounds, the magnitude of the drug efflux in the MRP-overexpressing cells has been considerably less than that observed with P-glycoprotein-overexpressing cells. Both MRP and P-glycoprotein have in common an ATP-binding region and are thought to hydrolyze ATP coincident with drug transport. One hallmark of P-glycoprotein-mediated efflux is that ATP depletion completely inhibits efflux. Other apparent transporters containing ATP-binding regions have been reported, although a role in drug resistance has not been demonstrated [Allikmets et al., 1995].

To study an alternate mechanism of drug resistance in breast cancer, we examined in detail a multidrug resistant human breast cancer subline, MCF-7 AdVp, which had been selected in doxorubicin (Ad) in the presence of verapamil (Vp) in order to avoid selection for overexpression of P-glycoprotein [Chen et al., 1990]. This subline was previously shown to have reduced daunorubicin accumulation [Doyle et al., 1995c]. We observed that the drug resistance phenotype was similar to that previously reported in several mitoxantrone-selected cell lines [Dalton et al., 1988; Dietel et al., 1990; Taylor et al., 1991; Nakagawa et al., 1992] and confirmed that ATP-dependent drug efflux was present in the anthracycline-resistant MCF-7 AdVp cells. The decreased drug accumulation is not readily reversed by cyclosporine, a classical P-glycoprotein antagonist. The cells do not demonstrate higher expression of MRP or Pgp than parental cells and express Pgp at levels that are only detectable by PCR. Gene cloning experiments identified overexpression of the amiloride-inhibitable sodium channel.

MATERIALS AND METHODS

Cell Lines

MCF-7 AdVp cells were originally selected in increasing concentrations (up to 200 ng/ml) of doxorubicin in the presence of 5 μg/ml verapamil [Chen et al., 1990]. The selection pressure was increased in a stepwise manner, and cells were cultured in 100, 1,000, or 3,000 ng/ml doxorubicin with 5 μg/ml verapamil for the present studies. These sublines were delineated AdVp 100, AdVp1000, and AdVp3000, respectively. Control cell lines included a Pgp-overexpressing subline, SW620 Ad300, selected by continuous exposure to increasing concentrations of doxorubicin [Lai et al., 1991] and MRP-overexpressing UMCC cells selected in VP-16, generously provided by Austin Doyle [1995b]. MCF-7 cells and sublines were cultured in IMEM, whereas the SW620 and UMCC cells and sublines were cultured in RPMI. Both media were supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in the selecting agent and placed in drug-free medium one week prior to each experiment.

Cytotoxicity and Growth Assays

Assays were performed in 96-well plates using the colorimetric method described by Skehan et al., [1990]. Cells were seeded at a density of 1,000 cells/well, fixed after 4 days in 50% TCA, then stained in 0.4% sulforhodamine B (SRB) dissolved in 1% acetic acid. After washing, bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5). The number of viable cells was determined by measuring the OD at 570 nm.

Measurement of Intracellular pH

pH_i was measured as previously described [Altenberg et al., 1993] using the pH-sensitive dye BCECF. Cells were loaded at 21°C by a 15 min exposure to 5 μM BCECF AM in the presence of 0.07 mg/ml of pluronic acid F-127 (Molecular Probes, Eugene, OR). BCECF distribution, evaluated by confocal fluorescence microscopy, was indistinguishable between MCF-7 and MCF-7 AdVp cells and fairly uniform. Measurements were performed at 37°C, from 100–200 cells bathed for 30 min with control Ringer's containing 115 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1.5 mM Na phosphate, 8 mM glucose, equilibrated with 5% CO_2/95% O_2, pH 7.42–7.43. Calibration of the fluorescence signal was carried out by the high-[K+]/nigericin technique with solutions containing 150 mM K+ and 10 μM nigericin.

Confocal Fluorescence Microscopy

Images were taken using Noran Odyssey laser scanning digital imaging system (Noran Instruments, Middleton, WI), as described [Torres et al., 1996]. Excitation was at 488 nm and emitted light was measured at wavelengths longer than 515 nm.

Rhodamine Efflux Assay

A suspension of log phase cells was obtained by trypsinization. For ATP depletion experi-

ments, cells were incubated for 20 min prior to assay in 50 mM deoxyglucose and 15 mM Na Azide. As previously described, cells were incubated in 0.5 μg/ml rhodamine 123 in IMEM with 10% FCS at 37°C in 5% CO_2 for 30 min and then resuspended in rhodamine-free media for a 90-min efflux period. Cells were then washed in ice-cold HBSS and placed in HBSS with 10% FCS at 4°C and kept in the dark until flow cytometric analysis. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm Argon laser. The green fluorescence of rhodamine 123 was collected after a 530 nm band pass filter. Samples were gated on forward scatter vs. side scatter to exclude cell debris.

Immunoblotting

Nuclear extracts for topoisomerase IIα immunoblots were resolved on a 6% SDS-polyacrylamide gel. Transfer to an Immobilon-P membrane was followed by incubation with a 1:1,000 dilution of a polyclonal antibody against human topoisomerase IIα (TopoGEN, Columbus, OH) for 1 h at 24°C. For detection of MRP, a monoclonal antibody, MRPm6 (a gift of Rik Scheper) was used. For detection of P-glycoprotein, polyclonal antiserum 4007 (a gift of Michael Gottesman) was used. Membranes were washed and incubated with HRP-conjugated secondary antibody. The immunolabeled protein was detected by chemiluminescence, which was performed according to the manufacturer's directions (ECL kit, Amersham, Arlington Heights, IL).

Drug Accumulation Assay

Accumulation studies were performed as previously described [Fojo et al., 1985]. Briefly, cells were plated in 6-well dishes on the day prior to the assay. For ATP depletion assays, cells were incubated in glucose-free media, sodium azide, and deoxyglucose for 20 min prior to initiation of the assay. Cells were incubated in 2×10^6 dpm ^{3}H-daunomycin for 30 min, continuing in ATP depleting conditions.

DNA Topoisomerase II Activity

Crude nuclear extracts were prepared from 1×10^8 cells in early log phase culture as previously described [Duget et al., 1983]. The decatenation reaction was carried out with serial dilutions of nuclear extract and 0.1 μg of kinetoplast DNA (TopoGEN) in 50 mM Tris-HCl (pH 7.5), 85 mM KCl, 10 mM MgCl2, 5 mM dithiotreitol, 0.5 mM EDTA, 0.03 mg/ml bovine serum albumin, and 1 mM ATP at 30°C for 30 min. Samples were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and photographed [Takano et al., 1991].

Northern Analysis and RNase Protection

RNA was isolated by solubilization in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion [Maniatis et al., 1987]. RNA expression was determined by Northern analysis using standard methods, or by RNase protection, as previously described [Mickley et al., 1989]. Topoisomerase IIα cDNA, residue 2814–3433, was labeled by random priming for use in the RNase protection assay for protection of a 620 bp fragment.

Differential Display Assay, Subcloning, and Sequencing

Parental MCF-7 and the two resistant sublines, MCF-7 AdVp100 and MCF-7 AdVp1000, were used as sources of RNA for differential display, which was performed using RNAmap Kit A (GeneHunter Corp, Brookline, MA) as previously described [Liang and Pardee, 1992; Liang et al., 1993]. Briefly, 0.2 μg of total RNA from the parental cells and the two resistant sublines were reverse transcribed, respectively with T12MG, T12MA, T12MT, or T12MC (where M may be dG, dA, or dC) as a primer, followed by PCR amplification in the presence of ^{35}S-ATP (Amersham). PCR-amplified fragments from the three RNA samples were loaded in adjacent lanes in a 6% denaturing polyacrylamide gel. The dried gel was exposed to Kodak XAR film. The reaction showed a highly expressed fragment in MCF-7 AdVp cells. The PCR product was again loaded on a gel, the band excised, and then subcloned for sequencing using the Sequenase Version 2.0 DNA sequencing kit from U.S. Biosciences.

Quantitative PCR Assay

Cells were solubilized in guanidinium isothiocyanate, RNA was isolated as previously described, and checked for quality by ethidium bromide staining after separation in a formaldehyde gel [Chomczynski and Sacchi, 1987; Maniatis et al., 1987]. Quantitative PCR was per-

formed as previously described, with minor modifications [Murphy et al., 1990; Kang et al., 1995]. Reverse transcription of 1 μg of total RNA using 0.5 μM of specific 3′ primer was performed. Amplification was performed for 30 cycles. Water was amplified a total of 40 cycles to detect possible contamination; 40 μl of each PCR product was separated by gel electrophoresis and stained with 2 μg/ml ethidium bromide for analysis on a Fotoeclipse densitometer (Fotodyne, MI). The primers used for PCR are as follows:

	5′ primer:
MDR-1:	5′GCCTGGCAGCTGGAAGACAAATAC ACAAAATT3′
MRP:	5′CGGAAACCATCCACGACCCTAATCC3′
hαENaC:	5′TTCTCCTCAGACCACCTGGGCTGTT TCACC3′
hβENaC:	5′GGTCAGCGTCTCCCTCTCCGTAG 3′
hγENaC:	5′CGCCCTCCTCGTCTTCTCCTTCT3′
	3′ primer:
MDR-1:	5′CAGACAGCAGCTGACAGTCCAAGAA CAGGACT3′
MRP:	5′ACCTCCTCATTCGCATCCACCTTGG3′
hαENaC:	5′GGAGACCAGTATCGGCTTCGGAACC TTCG3′
hβENaC:	5′CAGATGGAGAAGTTCAGGTTCC3′
hγENaC:	5′CGGTGGGAGAATCTAGGCTGC3′

RESULTS

MCF-7 AdVp cells display a multidrug-resistant phenotype, but do not overexpress P-glycoprotein or MRP. Increasing the selection pressure by increasing doxorubicin concentrations in MCF-7 AdVp cells while maintaining the cells in 5 μg/ml verapamil resulted in cells with increasing levels of resistance to doxorubicin. Table I demonstrates the cross-resistance pattern of the MCF-7 AdVp1000 cells. These cells are 600-, 667-, and 105,000-fold resistant to doxorubicin, daunorubicin, and mitoxantrone, respectively. Comparatively small degrees of resistance are observed for melphalan, vinblastine, and paclitaxel. This resistance is not due to P-glycoprotein or MRP overexpression, as demonstrated by immunoblot analysis (data not shown). Measurement of *MDR*-1 expression by quantitative PCR demonstrates very low levels requiring 35 cycles of amplification for detection, with no increase in the resistant sublines compared to parental cells. Quantitative PCR analysis also confirmed that no overexpression of MRP occurred in these cells. The PCR results are shown in Table II, along with other results described below. All levels are normalized to the level of expression in a control cell line, SW620, which is an unselected cell line that expresses low, but stable levels of *MDR*-1 and MRP. For comparison, the level of MRP by this quantitative PCR assay in VP-16 selected UMCC cells is 186, relative to a wild-type level of 8.3 in UMCC cells [Doyle et al., 1995b].

TABLE I. Cross-resistance Pattern of MCF-7 AdVp1000 Cells

Agent	MCF-7 IC_{50} (ng/ml)	MCF-7 AdVp IC_{50} (ng/ml)	Relative resistance
Doxorubicin	20.0	12,000	600
Daunorubicin	3	2,000	667
Mitoxantrone	0.4	42,000	105,000
Melphalan	4000	50,000	12.5
Vinblastine	0.46	1.2	2.6
Taxol	0.2	1.4	7.0
Verapamil	33	53	1.6

A partial explanation for the drug resistance found in MCF-7 AdVp cells is reduced growth rate. Classical cell biology experiments demonstrated that cells with lower growth rates exhibit doxorubicin resistance [Hug et al., 1986; Baral and Auer, 1990; Pelletier et al., 1990]. We recently confirmed in our laboratory that reducing the growth rate in MCF-7 cells by decreasing the concentration of fetal calf serum in the medium to 0.2% increases resistance by more than 100-fold for doxorubicin [Wosikowski et al., submitted]. A marked reduction in growth rate was observed in the MCF-7 AdVp cells. Concomitant with the increasing resistance found in the cells selected in higher concentrations of doxorubicin is a decrease in growth rate, shown in Figure 1. The doubling time increases from 45 h in parental cells to 57, 90, and 95 h in the AdVp100, 1000, and 3000 sublines, respectively, whereas resistance increases from 200- to 600- to 1,000-fold in the sublines, respectively.

The drug resistance cannot be explained by reduced topoisomerase IIα expression. One mechanism frequently invoked for resistance to anthracyclines is alteration in topoisomerase IIα expression or function. Figure 2 presents the results of RNase protection analysis of mRNA expression (A) and immunoblot analysis of protein expression (B) for topoisomerase IIα. Figure 2C demonstrates the results of a decatenation assay for topoisomerase activity. The presence of topoisomerase activity is indicated

TABLE II. Quantitative PCR[a] for *MDR-1*, MRP, and Na^+ Channel (α subunit)

	SW620[a]	MCF-7 parent	MCF-7 AdVp 100	MCF-7 AdVp 1000	MCF-7 AdVp 3000
MDR-1/28S[b]	10	0.09 (1.0)[c]	0.2 (2.4)	0.03 (0.3)	0.08 (0.9)
MRP/28S[b]	10	3.7 (1.0)[c]	4.4 (1.2)	3.2 (0.9)	7.8 (2.1)
Na^+ channel/28S[b]	10	0.62 (1.0)[c]	n.d.	2.6 (4.3)	5.0 (8.0)

[a]In previous studies, *MDR-1* mRNA levels in SW620 cells were arbitrarily assigned a value of 10, and the results for all other RNA samples were made relative to SW620, which was assayed in each reaction [Kang et al., 1995]. A value of 10 is also assigned to the level of expression found in SW620 for MRP and for the alpha subunit of the Na^+ channel.
[b]Quantitative PCR was carried out as previously described [Murphy et al., 1990]. However, β_2-microglobulin levels could not be used for normalization, since levels declined in the AdVp cells with advancing resistance. We thus separated 1 µg total RNA by gel electrophoresis, stained it with ethidium, and then determined the densitometric values for the 28S ribosomal band using a Fotoeclipse densitometer [Fotodyne].
[c]For ease of interpretation, the mRNA expression levels in parentheses are relative to the level found in the parental MCF-7 cell line.
n.d. = PCR quantitation not performed.

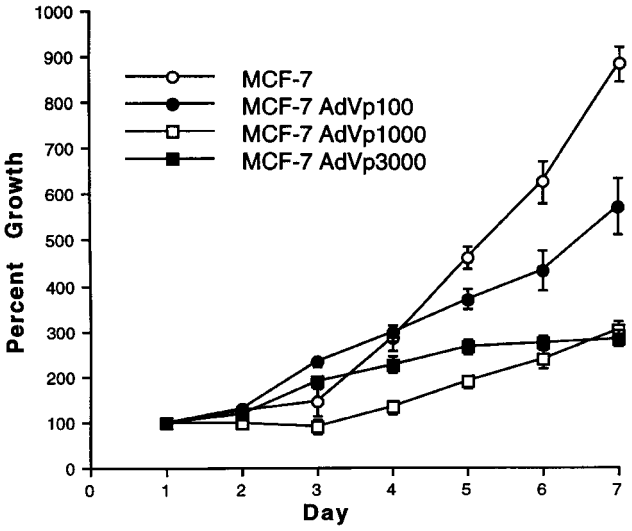

Fig. 1. Growth curves of resistant sublines. Cells were plated at 750 cells/well for MCF-7 parental cells, and 1,500 cells/well for resistant cells in 96-well dishes in 200 µl complete medium. One plate was harvested each day, and the quantity of cells assayed using sulforhodamine.

by conversion of kinetoplasts to minicircles. Increasing volumes of nuclear extract were added to 0.1 µg kinetoplast DNA. Identical results are observed in parental and both AdVp100 and AdVp1000 sublines, suggesting that alterations in topoisomerase II do not account for the resistance in these cells.

A decrease in daunomycin accumulation is observed in AdVp cells, with no change in subcellular distribution and no increase in accumulation in vesicles. Although early studies with radiolabeled doxorubicin suggested that accumulation was not decreased, continued selection of the MCF-7 AdVp cells resulted in a phenotype characterized by decreased daunomycin accumulation, as observed by Doyle and co-workers [Chen et al., 1990; Doyle et al., 1995c]. It has been claimed that accumulation of chemotherapeutic drugs in intracellular vesicles could contribute to multidrug resistance [Barrand et al., 1993; Sognier et al., 1994]. To explore a possible role of alterations in subcellular drug distribution in the mechanism of drug resistance in AdVp cells, we determined the distribution of daunomycin fluorescence by confocal microscopy. Since daunomycin distribution is affected by the level of drug accumulation, we evaluated subcellular daunomycin distribution at similar fluorescence levels by loading MCF-7 and MCF-7 AdVp1000 cells with different concentrations of daunomycin. Figure 3 shows that at low fluorescence levels, both

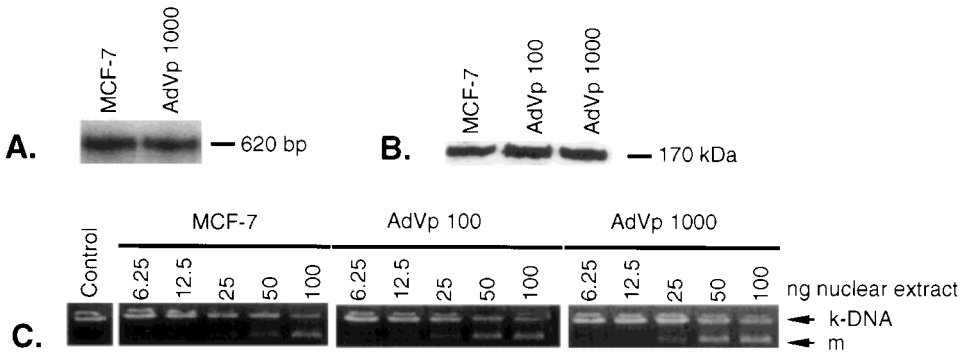

Fig. 2. Topoisomerase IIα expression and activity in MCF-7 AdVp cells. RNA was isolated from MCF-7 and MCF-7 AdVp1000 cells and analyzed by RNase protection assay. Protection of a 620 bp fragment representing topoisomerase IIα is shown in **A.** The immunoblot analysis in **B** demonstrates expression of the 170 kDa topoisomerase IIα in 100 µg total protein. Assay of topoisomerase IIα activity is shown in **C.** For this study, crude nuclear extracts were prepared and decatenation of catenated kinetoplast DNA (kDNA) to minicircles (m) was analyzed by gel electrophoresis.

cell lines showed fluorescent vesicles (compare A and B). At higher fluorescence levels, vesicles were less prominent, and the subcellular daunomycin distribution remained comparable in both cell lines (compare C and D, Fig. 3).

Increasing drug resistance is associated with increasing rhodamine efflux. Rhodamine is a mitochondrial dye that has been shown to be a substrate for P-glycoprotein [Summerhayes et al., 1982; Lampidis et al., 1983; Efferth et al., 1989; Ludescher et al., 1992; Lee et al., 1994]. There is evidence that it is also a substrate for MRP [Twentyman et al., 1994; Gekeler et al., 1995], although in our hands, MRP overexpressing cells do not efflux a significant amount of rhodamine during a 60-min efflux period (data not shown). Figure 4 presents a FACS analysis of rhodamine accumulation and efflux in the AdVp100 and 1000 cells. We found decreased accumulation (Control) of rhodamine in AdVp100 cells by FACS analysis and a greater decrease in AdVp1000 cells. Efflux of rhodamine following a 60-min incubation of the cells in rhodamine-free medium results in a further decrease in accumulation (Efflux). The accumulation defect and efflux are partially reversed by cyclosporine A (CsA and CsA/Efflux, respectively).

Both rhodamine and daunomycin efflux are ATP-dependent. Rhodamine and daunomycin accumulation and efflux studies were carried out in parental and MCF-7 AdVp1000 cells in conditions designed to deplete the cells of energy. Parental and resistant cells were exposed to either standard medium or to glucose-free medium containing sodium azide and deoxyglucose for 15 min prior to incubation in rhodamine and then continued in ATP depleting conditions during the 30-min rhodamine accumu lation period. As shown in Figure 5, FACS analysis demonstrated that ATP depletion had no effect on parental levels of rhodamine accumulation. Cells were then incubated an additional 30 min in either complete or in ATP depleting medium in the absence of rhodamine to allow an efflux period. In complete medium, low levels of fluorescence are observed in AdVp1000 cells following the efflux period (AdVp). However, ATP depletion fully reversed the drug efflux (AdVp ATP−). Cells that were ATP depleted during the accumulation period were also placed in complete medium for the efflux period. These cells demonstrated restoration of efflux (AdVp ATP−/ATP+). These results demonstrate that rhodamine efflux is ATP-dependent in the AdVp cells.

Similar results were obtained with ^{3}H-daunomycin efflux studies. Figure 6A demonstrates the ^{3}H-daunomycin efflux present in the AdVp1000 cells, compared to the efflux present in MCF-7 parental cells. Relative to the amount of ^{3}H-daunomycin present following loading, the proportion of ^{3}H-daunomycin remaining after 30 min of efflux in the resistant cells was 26%, compared to 81% in parental cells. Like the ATP-dependent rhodamine efflux found in these cells, daunomycin efflux was inhibited by ATP depletion in the MCF-7 AdVp1000 cells (Fig. 6B). The daunomycin efflux in these cells is comparable to that shown in a well-characterized multidrug resistant colon cancer subline

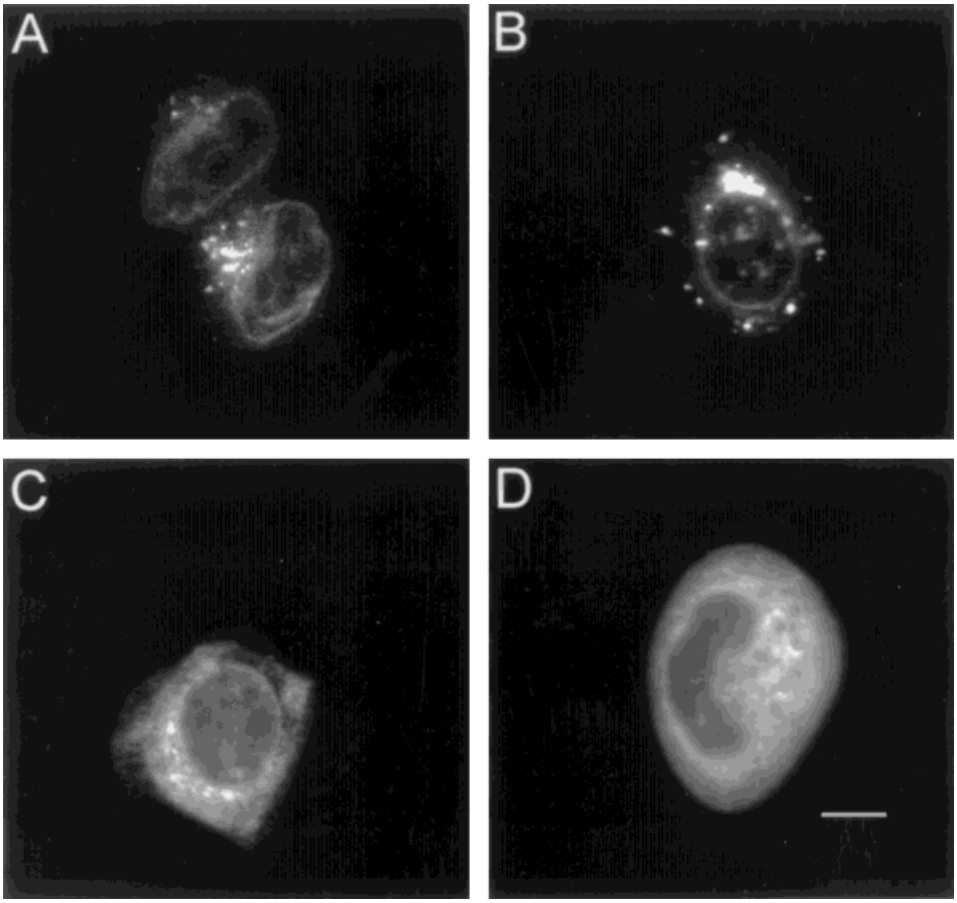

Fig. 3. Subcellular distribution of daunomycin fluorescence in MCF-7 AdVp cells. MCF-7 and MCF-7 AdVp1000 cells were plated on glass coverslips and loaded for 2 h at 37°C in control Ringer's with different concentrations of daunorubicin. Drug concentrations were varied to obtain comparable low or high fluorescence levels in both cell lines. MCF-7 cells were loaded with 0.1 μg/ml **(A)** or 10 μg/ml **(C)** daunorubicin. MCF-7 AdVp1000 cells were loaded with 10 μg/ml **(B)** or 100 μg/ml **(D).**

(Fig. 6C) expressing high levels of *MDR*-1/P-glycoprotein, SW620 Ad300 [Lai et al., 1991]. Both sublines demonstrate decreased daunomycin efflux following ATP depletion (ATP−). In both sublines, addition of complete medium at the initiation of the efflux period following energy depletion in the accumulation period results in rapid efflux in both the AdVp1000 cells, and the *MDR*-1 expressing SW620 Ad300 cells (ATP−/ATP+).

Molecular cloning experiments identify the sodium channel as overexpressed in MCF-7 AdVp cells. Using the differential display technique originally reported by Liang and others [Liang and Pardee, 1992; Liang et al., 1993], a clone with increased expression was found in the AdVp1000 cells. As shown in Figure 7, left panel, a PCR product with higher levels of expression was observed in the AdVp cells. This product was subcloned, sequenced, and determined to have 100% homology with the alpha subunit of the amiloride-inhibitable sodium channel. Northern blot analysis (right panel) confirmed overexpression of the sodium channel mRNA.

Quantitative PCR demonstrates increased sodium channel expression with increasing levels of resistance. Table II presents quantitative PCR measurements for MDR-1, MRP, and the sodium channel in parental MCF-7 cells and the resistant sublines. Quantitative PCR results were normalized to RNA quantities obtained in a parallel assay by densitometry of the ethidium-stained 28S ribosomal band following electrophoresis of 1 μg total RNA in a formaldehyde gel. An eightfold overexpression of the

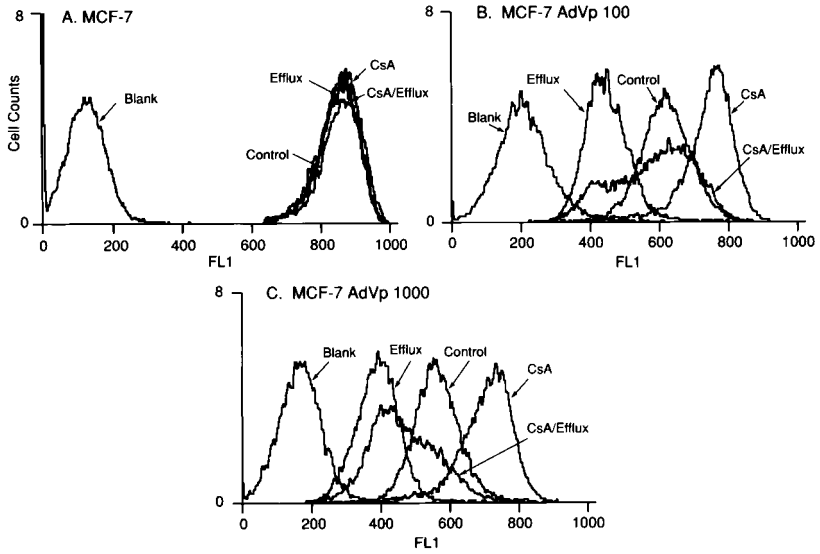

Fig. 4. FACS analysis demonstrating rhodamine efflux in MCF-7 AdVp cells. Rhodamine accumulation was performed in the presence (CsA) and absence (Control) of cyclosporine A for 30 min. Subsequently, cells were washed and placed in rhodamine-free medium, continuing in the presence (CsA/efflux) or absence (efflux) of cyclosporine A for another 30 mins. All cells were then washed and FACS analysis performed. The autofluorescence of cells without rhodamine accumulation is shown as the blank.

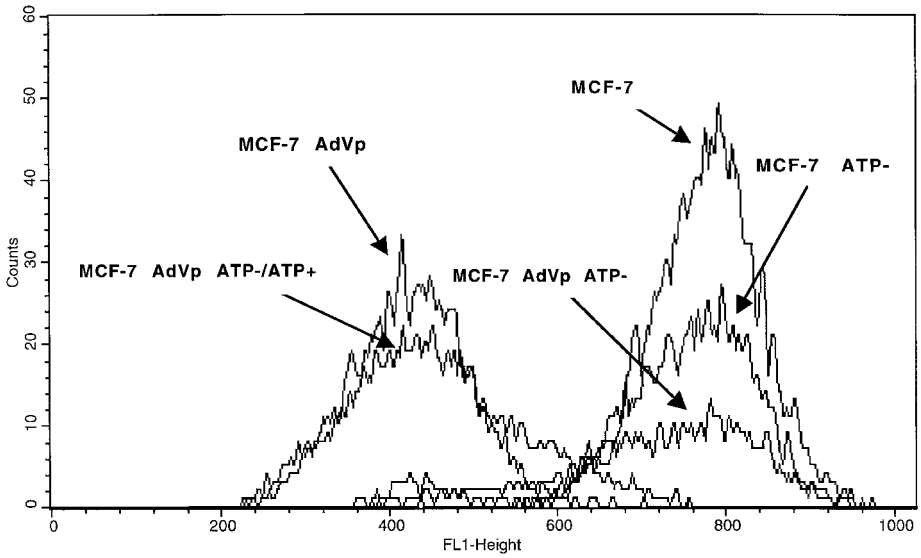

Fig. 5. FACS analysis demonstrating ATP-dependence of rhodamine transport in MCF-7 AdVp cells. Cells were placed in 50 mM deoxyglucose, 15 mM Na Azide for 15 min to achieve ATP depletion prior to incubation in rhodamine for 30 min and a subsequent 30-min efflux period. The results are compared to results obtained with parental MCF-7 cells without (MCF-7) or with ATP depletion (MCF-7 ATP−). MCF-7 AdVp cells were incubated either in the presence (MCF-7 AdVp ATP−) or the absence (MCF-7 AdVp) of treatment with ATP depleting conditions; and with readdition of standard assay medium to previously depleted cells to allow recovery of ATP-dependent processes (MCF-7 AdVp ATP−/ATP+).

alpha subunit of the Na+ channel is present in the AdVp subline with the highest level of resistance. Differences as high as 20-fold in αhENaC were observed in repeat experiments; no increase in expression of the βhENaC and the γhENaC subunits of the amiloride-sensitive Na+ channel could be detected by polymerase chain reaction (data not shown).

DISCUSSION

The multidrug resistant phenotype was initially described by Beck et al., [1979], Riordan

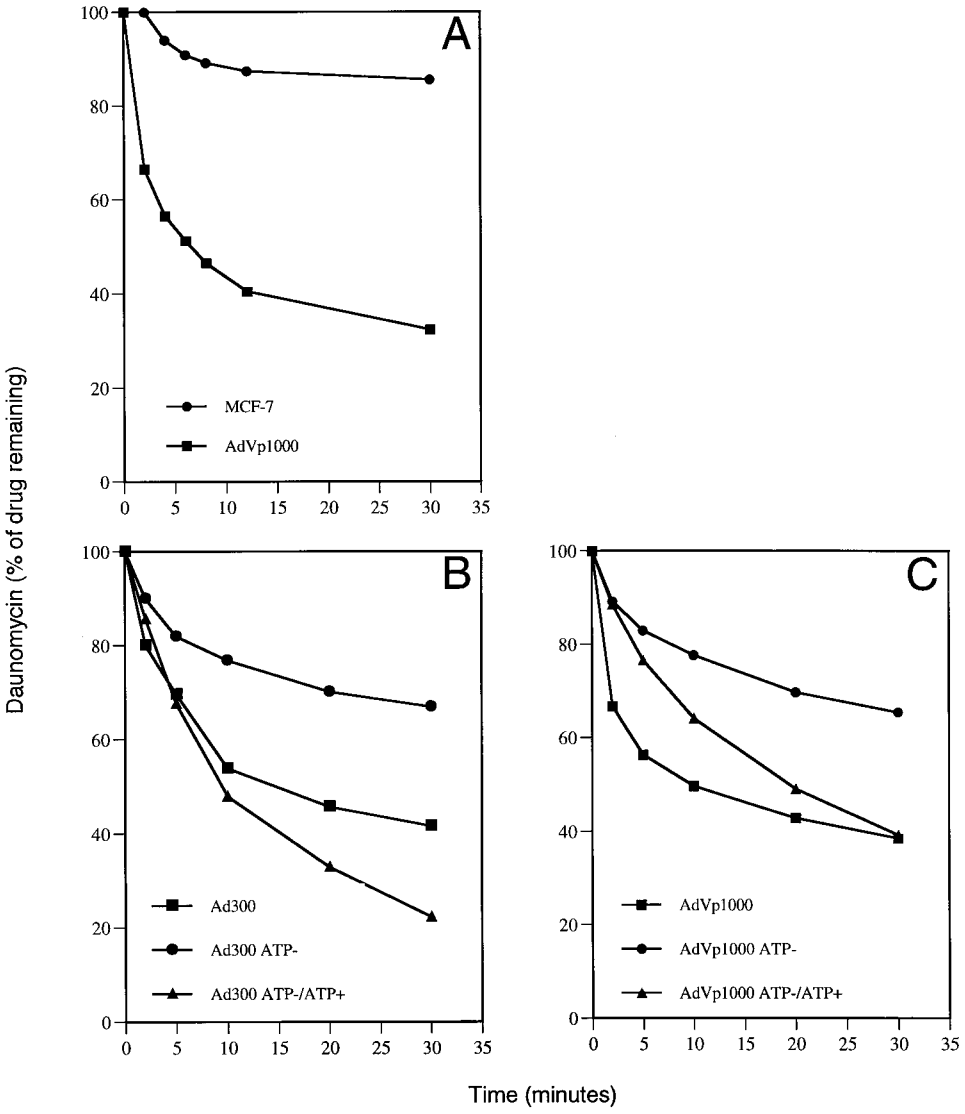

Fig. 6. Daunorubicin efflux in MCF-7 AdVp1000 cells and SW 620 Ad300 cells. Cells were plated in 6-well dishes 24 h before the assay, then incubated in 14 nM ^{3}H-daunorubicin for 60 min prior to the drug efflux assay. The plates were washed in ice-cold PBS to remove daunorubicin and then the media was exchanged at the indicated time points. Data are expressed as the percentage of daunorubicin remaining at each time point, relative to the amount that was present in the cells before the efflux began. **A** Efflux in MCF-7 AdVp1000 is compared to efflux in parental MCF-7. **B** and **C** Cells were incubated without or with 50 mM deoxyglucose and 15 mM Na Azide for 15 min to achieve ATP depletion prior to the addition of 14 nM ^{3}H-daunorubicin for 60 min. Efflux was initiated, continuing in ATP depleted conditions (ATP−) with deoxyglucose and Na Azide. The notation ATP−/ATP+, as in Figure 5, indicates that the accumulation was carried out in ATP depleting conditions, but that the efflux was carried out in standard assay medium to allow recovery of ATP-dependent processes. In B, results with P-glycoprotein overexpressing SW620 Ad300 cells are presented, whereas in C, results in MCF-7 AdVp1000 cells are shown.

and Ling [1979], and Biedler and Peterson [1981]. In these early studies, it was recognized that resistance to structurally unrelated natural products and decreased drug accumulation were the hallmarks of the phenotype that we now associate with overexpression of MDR-1. Approximately 20 years later, a second ATP-dependent transporter, MRP (the multidrug resistance-associated protein), was linked with multidrug resistance [Cole et al., 1993]. MRP overexpressing cells are primarily resistant to VP-16 and doxorubicin. Whereas efflux of multiple agents is clearly observed with *MDR*-1 expressing cell lines, it has been more difficult to determine a specific role for MRP in the transport of chemotherapeutic agents. In fact,

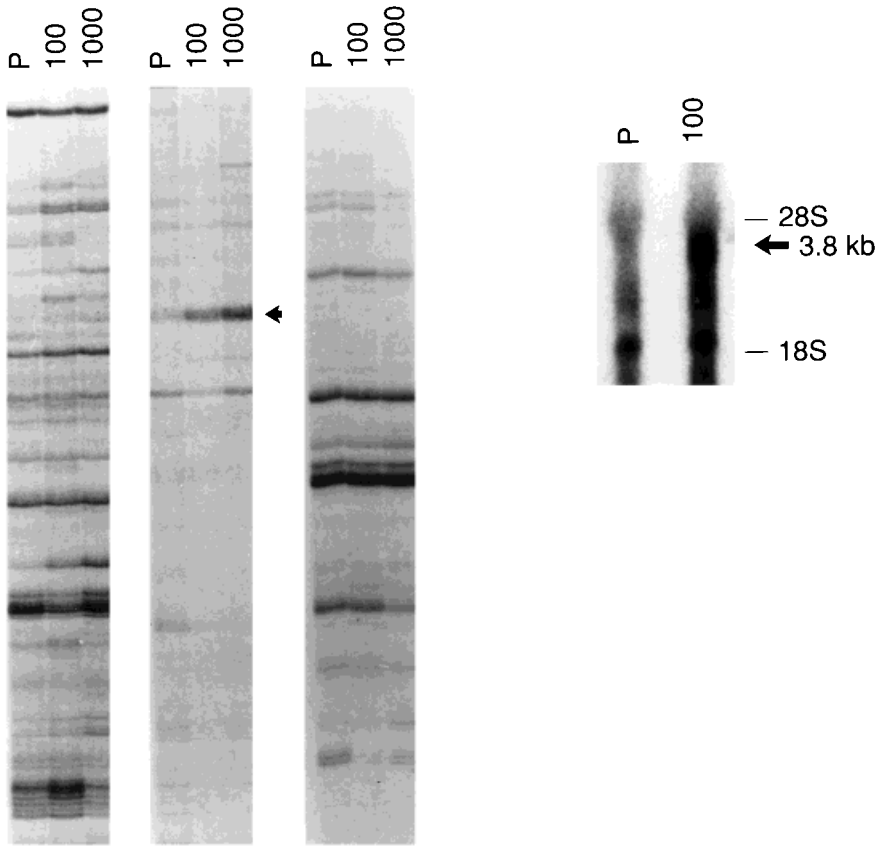

Fig. 7. Molecular cloning of the human alpha subunit of the Na+ channel. The left panel shows the results of the differential display technique. The arrow identifies the band that was excised and sequenced. On the right is a Northern blot demonstrating increased expression of the alpha subunit of the amiloride-sensitive Na+ channel in the MCF-7 AdVp1000 cells. Abbreviations used: P: MCF-7; 100: MCF-7AdVp100; 1000: MCF-7AdVp1000.

the possibility has been raised that MRP is a glutathione conjugate transporter and that compounds must be either glutathione conjugated or glucuronidated prior to transport [Jedlitschky et al., 1996]. Thus two transporters of entirely different phenotypes can confer multidrug resistance. Increasing evidence, as in the studies presented here, suggests the presence of at least a third transporter.

Cell lines displaying a non-Pgp multidrug resistant phenotype were catalogued by Center [1993]. Although many overexpress MRP, the resistance of some cannot be explained by MRP [Futscher et al., 1994; Yang et al., 1995]. Various groups have identified malignant human cell lines selected in mitoxantrone, displaying comparable phenotypes: mitoxantrone resistance, and to a lesser extent, doxorubicin resistance, with little or no cross resistance to vinblastine or vincristine. These include sublines derived from HL60 leukemia cells, WiDr colon cancer cells, EPG85-257 gastric cancer cells, and MCF-7 breast cancer cells [Dalton et al., 1988; Harker et al., 1989; Dietel et al., 1990; Taylor et al., 1991; Nakagawa et al., 1992]. Like the MCF-7 AdVp cells, these sublines typically do not overexpress *MDR*-1 or MRP, are resistant to multiple anthracyclines, with mitoxantrone resistance being the most prominent feature, and display efflux of mitoxantrone. The repeated isolation of an identical phenotype implies the presence of a distinctive drug efflux mechanism akin to that mediated by P-glycoprotein.

We studied MCF-7 AdVp cells, which were originally reported because of increased expression of a 95 kDa protein that correlated with increasing resistance, and decreased expression in drug-sensitive revertants [Chen et al., 1990]. The protein recognized by the antibody against P95 has been shown to be an N-linked sialoglycoprotein with a 35kDa polypeptide core [Doyle et al., 1995a]. Extensive efforts have been made to determine whether this protein,

termed P95, is directly related to the drug resistance phenotype. P95 has been detected in human leukemia samples; samples with higher levels were correlated with in vitro resistance to daunorubicin and with decreased daunorubicin accumulation [Doyle et al., 1995c]. However, we observed overexpression of this protein in four unselected cell lines (A549, EKVX, HOP-18, OVCAR-5) without evidence of rhodamine efflux, suggesting that the 95 kDa protein was not directly mediating drug efflux (data not shown). Recently, Doyle et al [1996] reported overexpression of the *H19* gene, identified by screening an expressed cDNA library with the P95 antisera. The authors concluded that it was unlikely that *H19* encoded P95, but hypothesized that *H19* could induce P95 overexpression.

Our studies of the MCF-7 AdVp subline suggest the presence of an ATP-dependent efflux mechanism. Both daunomycin and rhodamine efflux were found to be ATP-dependent. When cells are cultured out of drug, reversion to a more sensitive phenotype occurs, with loss of rhodamine efflux (data not shown). Although reduced growth rate is a partial explanation for the resistance observed, the cells demonstrate no alterations in topoisomerase IIα expression or function. Cloning studies identified an eight-fold overexpression of the gene encoding the α subunit of the Na+ channel.

Overexpression of the Na+ channel is unlikely to directly result in the 100,000-fold mitoxantrone resistance observed in these cells, but it may be linked through its physiologic role in Na+ reabsorption, which is essential for homeostasis. Amiloride-sensitive sodium channels have been identified in many epithelia, including renal cortical collecting tubules, urinary bladder, descending colon, salivary and sweat ducts, and respiratory tract [Garty, 1994]. Amiloride blocks Na+ reabsorption through the sodium channel, which appears to be comprised of at least three subunits, α, β, and γ [McDonald et al., 1994; Canessa et al., 1994; Voilley et al., 1994]. Although transfection of all three subunits generates the largest Na+ current, transfection of the αhENaC subunit alone has also been shown to generate an amiloride-sensitive Na+ current in xenopus oocytes [McDonald et al., 1994]. Thus the lack of overexpression of the β and γ subunits in the resistant MCF-7 AdVp cells does not exclude the possibility that the α subunit alone could modulate Na+ reabsorption. Increased pHi and depolarization have been proposed to contribute to multidrug resistance [Roepe et al., 1993]. In principle, if the Na+ channel is active, overexpression could result in an elevation in intracellular [Na+] and/or membrane depolarization. Most mammalian cells express transporters that couple Na+ and H+ (or its equivalent, i.e., HCO_3^-), such as Na+/H+ exchangers and electrogenic $NaHCO_3$ cotransporters. Overexpression of the Na+ channel could result in an elevation in intracellular [Na+] (by reducing the driving force for the Na+/H+ exchanger) and/or depolarization (by increasing the influx of HCO_3^- via $NaHCO_3$ cotransport) leading to elevation of intracellular pH (pHi). However, pHi was not different in MCF-7 (7.11 ± 0.05, $n = 8$) and MCF-7 AdVp1000 cells (6.98 ± 0.06, $n = 6$). Although at this time we cannot rule out completely that overexpression of the Na+ channel produces a membrane depolarization, which in turn contributes to the multidrug resistance of AdVp cells, this seems unlikely because amiloride has no impact on drug efflux (data not shown). In summary, our results indicate that whereas the alpha subunit of the amiloride-sensitive Na+ channel is overexpressed in AdVp cells relative to the level in parental cells, it does not seem to account for the drug resistance phenotype.

Alternatively, regulation of Na+ channel expression may be linked with that of the as-yet undefined transporter. Interestingly, in cells with a cystic fibrosis mutation in the CFTR gene, the activity of the Na+ channel is increased two- to threefold. This underlies one component of the pathophysiology of cystic fibrosis, increased sodium reabsorption in the airways, which increases the viscosity of secretions [Noone and Knowles, 1993]. Transfection of the wild-type CFTR into CF cells results in suppression of the increased Na+ channel activity. One recently proposed model for ABC transporters suggested that a transporter could be coupled with an ion channel, regulating conductance in the channel [Al-Awgati, 1995; Higgins, 1995].

Whether or not the Na+ channel is ultimately shown to be of importance, or even linked, to the drug resistance mechanism in these cells, it is clear that the MCF-7 AdVp cells display a distinctive phenotype. This phenotype is characterized by a decrease in drug accumulation, comparable to that observed in

Pgp-expressing cells, which is due to ATP-dependent efflux of the drug. Since the substrates for this efflux appear to be primarily anthracyclines, isolation of this putative pump offers the prospect of identifying a mechanism of resistance that would be important in diseases treated with anthracyclines. These would include, but not be limited to, the acute leukemias and breast cancer.

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