Overexpression of the Cystic Fibrosis Transmembrane Conductance Regulator in NIH 3T3 Cells Lowers Membrane Potential and Intracellular pH and Confers a Multidrug Resistance Phenotype

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ABSTRACT Because of the similarities between the cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance (MDR) proteins, recent observations of decreased plasma membrane electrical potential ($\Delta\Psi$) in cells overexpressing either MDR protein or the CFTR, and the effects of $\Delta\Psi$ on passive diffusion of chemotherapeutic drugs, we have analyzed chemotherapeutic drug resistance for NIH 3T3 cells overexpressing different levels of functional CFTR. Three separate clones not previously exposed to chemotherapeutic drugs exhibit resistance to doxorubicin, vincristine, and colchicine that is similar to MDR transfectants not previously exposed to chemotherapeutic drugs. Two other clones expressing lower levels of CFTR are less resistant. As shown previously these clones exhibit decreased plasma membrane $\Delta\Psi$ similar to MDR transfectants, but four of five exhibit mildly acidified intracellular pH in contrast to MDR transfectants, which are in general alkaline. Thus the MDR protein and CFTR-mediated MDR phenotypes are distinctly different. Selection of two separate CFTR clones on either doxorubicin or vincristine substantially increases the observed MDR and leads to increased CFTR (but not measurable MDR or MRP) mRNA expression. CFTR overexpressors also exhibit a decreased rate of ^{3}H -vinblastine uptake. These data reveal a new and previously unrecognized consequence of CFTR expression, and are consistent with the hypothesis that membrane depolarization is an important determinant of tumor cell MDR.

INTRODUCTION

Tumor cells selected on one chemotherapeutic drug (e.g., actinomycin D) may develop resistance to other chemotherapeutics (e.g., anthracyclines, vinca alkaloids, epipodophyllotoxins) concomitant with resistance to the selecting agent (Biedler and Riehm, 1970). Multidrug resistance (MDR) is associated with decreased intracellular retention of chemotherapeutic drugs that is due to some combination of reduced rates of drug influx (Beck et al., 1983; Sirotnak et al., 1986; Ramu et al., 1989), altered intracellular binding (Beck et al., 1983), and postulated active efflux of the drugs (Danø, 1973; Hammond et al., 1989). Documented overexpression of P-glycoprotein (p-gp or MDR protein) in these cells and its relationship to resistance (Riordan and Ling, 1979) as well as the subsequent cloning of MDR genes encoding this protein and elucidation of transporter gene homology (Roninson et al., 1986; Scotto et al., 1986; Gros et al., 1986), led to the proposal that MDR protein directly catalyzes active drug efflux (Gerlach et al., 1986; Gros et al., 1986), and that this explains decreased cytoplasmic retention of chemotherapeutic drugs.

Upon cloning of the gene defective in cystic fibrosis patients (Riordan et al., 1989) it was immediately recognized that the MDR protein and the cystic fibrosis transmembrane conductance regulator (CFTR) are homologous members of an important family of transport proteins, the "ABC" family (Higgins et al., 1990). CFTR functions as a low conductance Cl^- channel that is dependent on cyclic adenosine monophosphate (Bear et al., 1992). The apparent dramatically different function of the MDR protein and the CFTR remains a curious and interesting topic of current membrane transport research. Recent data (Valverde et al., 1992; Gill et al., 1992; Altenberg et al., 1994; Bear, 1994; Luckie et al., 1994; Hardy et al., 1995) suggest that MDR protein may also be involved in modulating Cl^- conductance, directly or indirectly. (Hypotonic swelling of some cells overexpressing the MDR protein elicits increased whole-cell Cl^- conductance, or in some cases conductance that is "hypersensitive" to the hypotonic challenge (Valverde et al., 1992; Gill et al., 1992; Altenberg et al., 1994; Luckie et al., 1994). Recent work (Hardy et al., 1995) suggests protein kinase C may play an important role in regulating the anomalous conductance. Thus, MDR protein may either regulate endogenous Cl^- conductance in some cells, or perhaps transport Cl^- directly. Other workers (Ehring, 1994; Dong et al., 1994) have not observed increases in Cl^- conductance for cells overexpressing MDR protein upon hypotonic challenge. Since all electrophysiological study of Cl^- conductance in MDR cells has been with model systems grown for relatively long periods of

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Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; MDR, multidrug resistance; $\Delta\Psi$, plasma membrane electrical potential; pH_i, intracellular pH; MRP, MDR-related protein; ABC, ATP-binding cassette; BCECF, 2′,7′-bis(carboxy ethyl)-5,6-carboxy fluorescein; BCECF-AM, acetoxymethylester form of BCECF; di-4-ANEPPS, (dialkylamino)naphthalene pyridinium styryl.

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time in the presence of potent chemotherapeutics, since these selection procedures may induce altered expression/regulation of additional ion transport proteins (Luz et al., 1994), and since hypotonic shock is likely an indirect mechanism of "gating" the conductance mediated directly or indirectly by the MDR protein, it is not surprising that some variability has been noted. We believe more work with true transfectants not previously exposed to chemotherapeutic drugs (e.g., Valverde et al., 1992) may prove informative.)

A model for MDR protein activity that reconciles ion transport function with drug transport function remains elusive, although one suggestion is that ion transport anomalies in MDR cells perturb plasma membrane electrical potential and pH_i and thus indirectly influence drug retention via several different mechanisms (Roepe et al., 1993), and another is that MDR protein "alternates" between Cl^- channel (or channel regulator) and drug pump conformations (Gill et al., 1992).

The most direct support for the active drug pump model for MDR protein is provided by recent drug uptake studies with secretory vesicles isolated from *Saccharomyces cerevisiae* (Ruetz and Gros, 1994). Other studies have questioned the drug pump model (Roepe, 1992; Roepe et al., 1993; Simon et al., 1994; Bornmann and Roepe, 1994; Luz et al., 1994; Roepe, 1994), principally because of the fact that MDR cells exhibit resistance to and/or decreased retention of a tremendous variety of compounds, including actinomycin D, anthracyclines, colchicine, epipodophyllotoxins, ethidium bromide, gramicidin, mitomycin C, morphine, nigericin, peptides, puromycin, reserpine, rhodamine, technetium-sestamibi, topotecan, tetraphenylphosphonium, Tween 20, valinomycin, verapamil, vinca alkaloids, and yohimbine. An explanation for how one transport protein could conceivably bind and actively transport this plethora of agents is currently unavailable.

Since all compounds to which MDR cells are resistant and/or exhibit decreased retention of are hydrophobic, and since they are either weakly basic or cationic, or bind to intracellular targets in a highly pH-dependent manner (or are ionophores), an alternative possibility is that altered compartmental or cytoplasmic pH and/or electrical membrane potential promotes altered passive diffusion and partitioning of these compounds in such a way that availability to "target" is decreased (Roepe et al., 1993), thus leading to decreased retention and resistance. A unifying thesis that attempts to explain both anomalous pH_i and $\Delta\Psi$ regulation in MDR cells is that altered Cl^- translocation leads to decreased plasma membrane electrical potential by increasing Cl^- permeability and thus decreasing the dominance of $\Delta\Psi$ by K^+ conductance, and leads to altered pH_i regulation by disrupting normal $Cl^-/^-HCO_3$ exchange processes (Roepe et al., 1993; Luz et al., 1994; Roepe et al., 1994). Precisely how both perturbations are accomplished via overexpression of one ion channel or channel regulator remains to be determined.

An important prediction of this second model is that other mechanisms that decrease $\Delta\Psi$ or alter pH_i might lead to MDR. This would have extremely important implications for understanding chemotherapeutic drug bioavailability at the cellular level. Since it has recently been demonstrated that overexpression of the CFTR leads to decreased $\Delta\Psi$ (Stutts et al., 1993), presumably via increasing Cl^- conductance, we tested whether 3T3 cells overexpressing constitutively active CFTR exhibit an MDR phenotype. We have also recently tested whether a CFTR-mediated MDR phenotype exhibits any similarities to the MDR protein-mediated phenotype with regard to drug transport and retention (L. J. Robinson and P. D. Roepe, submitted for publication).

MATERIALS AND METHODS

Materials

BCECF, di-4-ANEPPS, nigericin, and valinomycin were purchased from Molecular Probes (Eugene, OR) and used without further purification. Doxorubicin was obtained as a 2 mg/ml solution in saline (Adria Laboratories, Columbus, OH), colchicine in powder form was from Sigma Chemical Co. (St. Louis, MO), and vincristine sulfate was obtained as a 1 mg/ml solution in saline (Lilly, Indianapolis, IN). Vinblastine sulfate was a kind gift of Lilly Research Laboratories. [^{3}H]vinblastine sulfate (16 Ci/mmol) and [^{3}H]azidopine (52 Ci/mmol) were from Amersham International (Arlington Heights, IL); purity of [^{3}H]vinblastine was >95% as assayed by high performance liquid chromatography with a Hypersil monolayer dimethyloctysilyl column. The integrity of commercial 5% CO_2 (balance air) mixtures used in this work was checked using standard $^-HCO_3$ solutions and application of the Henderson-Hasselbach relation. All other chemicals were reagent grade or better, purchased from commercial sources, and used without further purification or analysis.

Tissue culture

Construction of the cell lines used in this work has been described previously (Stutts et al., 1993). In brief, NIH 3T3 cells were seeded at 3×10^5 cells/30 cm^2, transfected with a murine amphotropic retrovirus vector harboring full length wild-type human CFTR cDNA and a neo^r marker, and selected on 0.25 mg/ml active G418. CFTR was under control of the viral long terminal repeat, and neo^r was under control of the simian virus 40 promoter. Surviving colonies were screened for CFTR overexpression by Western blot (Stutts et al., 1993), and several clones (including C3, C5, and C10 used in this work) were found to exhibit substantial Cl^- conductance and plasma membrane depolarization via electrophysiological methods (see also Results). In some cases colonies were further selected on 100 nM doxorubicin or 50 nM vincristine (see Results). A partial analysis of MDR, MRP, CFTR, and anion exchanger protein and mRNA expression in these lines is reported on here, and a more comprehensive analysis of these and related cell lines will be reported elsewhere.

Cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's minimum essential medium (DMEM) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Since we have documented that cell lines transfected with the CFTR homologue MDR 1 may exhibit significant alterations in the level of MDR protein expression even under continued "selective pressure" with G418 or chemotherapeutics (Luz et al., 1994), we have adopted the practice of discarding cell lines after six passages. Thus, unless lines are recharacterized after six passages and evidence provided that levels of expression of CFTR have not been altered, we consider the line unsuitable for additional study.

For single-cell photometry analysis of pH_i, cells were grown as above on glass coverslips (Corning Glassworks, 18 mm^2/0.11 mm thick) that were immobilized in standard tissue culture plates with a dab of autoclaved silicon vacuum grease (Dow-Corning). They were kept in media at 37°C and 5% CO_2 until immediately before mounting in a homebuilt perfusion

chamber (Luz et al., 1994; Roepe et al., 1994). After mounting, cells were immediately perfused with Hanks' balanced salt solution (HBSS) (118 mM NaCl/24.2 mM $NaHCO_3$/1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/0.6 mM Na_2HPO_4/0.5 mM KH_2PO_4/10 mM glucose) that had been equilibrated with 5% CO_2 and to 37°C (see single-cell photometry, below).

Single-cell photometry and measurement of pH_i

We have constructed a single-cell photometry apparatus by interfacing a Nikon diaphot epifluorescence microscope and associated optics to a Photon Technologies Inc. alphascan fluorometer (Luz et al., 1994; Roepe et al., 1994; see Fig. 1). Signals from photon multiplier tubes connected in T-format to the side port of the microscope were transferred to a Dell 433/L computer and analyzed with PTI software (Photon Technologies Inc., New Brunswick, NJ).

Cells were grown on sterile glass coverslips as described above and used >1.5 but <4 days after plating, i.e., before confluency but after several cell divisions. Coverslips were incubated with 5 μM BCECF-AM for 30 min before mounting on the microscope stage, and they were then continuously perfused at a constant rate (~6 ml/min) with HBSS buffer equilibrated with 5% CO_2 and to 37°C. Uniform BCECF staining was verified visually and by monitoring the intensity of 490 nm excitation and was found to be very similar for the different cells. Buffers harboring $^-HCO_3$ were continuously purged with 5% CO_2, and a fine jet of 5% CO_2 was directed over the mounted coverslip. Buffer pH was monitored with a microelectrode. Several control experiments verified that leak of the esterified BCECF-AM was minimal in the time required to make a measurement, and not any different for CFTR versus control transfectants. BCECF calibration curves obtained as described (Luz et al., 1994) for these cell lines verify pH_i-dependent behavior of BCECF is nearly identical for the different cell lines. Exposure to excitation light was limited to the time of data collection to limit photobleaching.

To calculate steady state pH_i, excitation ratios were collected for 20–25 individual cells perfused with HBSS as described. Then, using the same coverslip, calibration curves were obtained using the K^+/nigericin titration approach of Thomas et al. (1979) as described previously (Roepe, 1992; Roepe et al., 1993), but in a "single-cell mode" wherein buffer harboring nigericin was continuously flowed over the cells (Luz et al., 1994). Calibration curves harbored six points between 6.60 and 8.00 (±0.03 pH units; each point was the average of 12–15 cells (see Luz et al., 1994), and were well fit by an exponential ($R^2 > 0.95$). pH_i was calculated in this way for each cell line three to four times, using three to four separate coverslips, and the data averaged.

Cell survivability

We assayed drug resistance by colony formation assays. Cells were harvested by trypsinization and resuspended in DMEM pre-equilibrated to 37°C and 5% CO_2. They were plated at 500 cells/well using standard six-well plates. Chemotherapeutic drug was added, and cells were allowed to grow for 7 days. The plate was then stained with methylene blue, and the colonies scored visually. For each assay, survivability at a given drug concentration was determined in duplicate and the data averaged. Data shown in Results are the averages of three separate assays performed on different days.

We also checked resistance by growth inhibition assays. Cells were subcultured in 96-well plates and incubated overnight at 5% CO_2. Drug at various concentrations was added the following day and cells were grown for 3 days more at 5% CO_2. Cell growth was assayed by SRB staining as described (Skehan et al., 1990). We verified that staining was a linear function of both cell number and total cell-associated protein for these cell lines grown under the different conditions.

Northern blotting

RNA was isolated by the method of Chomczynski and Sacchi (1987), and Northern blots were performed under high stringency as described (Roepe et al., 1993). 0.7 kb *NdeI*/*Hind*III, 1.1 kb *Eco*RI and 0.8 kb *Pst*1 fragments from human MDR 1 cDNA, human β-actin cDNA, and acidic ribosomal phosphoprotein (rPO) cDNA, respectively (kindly provided by Drs. P. Borst and K. Scotto), were used to probe for human MDR 1, β-actin, and rPO. MRP cDNA was kindly provided by Dr. S. Cole (Cole et al., 1992). Probes were labeled by the random priming method. Blots were visualized by autoradiography (exposure times varied from 4–6 h for β-actin and rPO and up to 72 h for MDR and MRP; we used Kodak X-OMAT film)

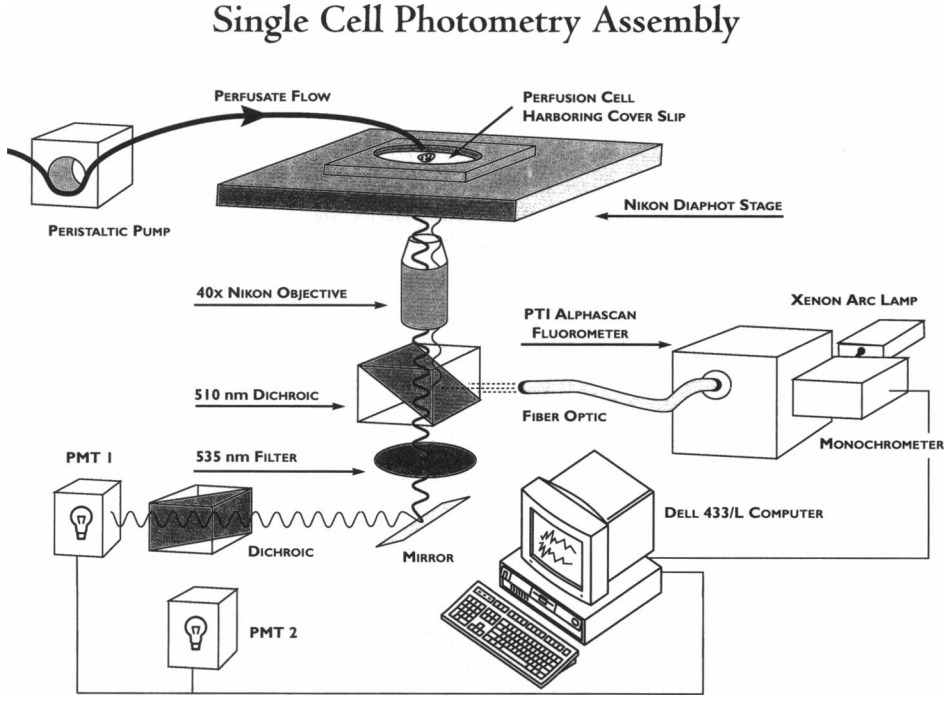


FIGURE 1 Schematic of the single-cell photometry apparatus used to measure intracellular pH (pH_i). A fiber optic was used to direct excitation to the epifluorescence microscope and a 510 nm dichroic reflected 439/490 nm light but passed intracellularly trapped BCECF emission centered at 535 nm. See Materials and Methods and Luz et al. (1994) for additional detail.

(Eastman Kodak, Rochester, NY), and levels of mRNA were quantitated by imaging β-radiation with a Betascope 603 blot analyzer (Betagen), or by autoradiography followed by densitometry using a Stratagene stratascan 7000 interfaced to an AST personal computer. The intensities of bands of interest were normalized relative to background emission from the blot, and CFTR expression was ratioed versus that for β-actin.

Reverse transcriptase polymerase chain reaction (RT-PCR)

In an attempt to detect trace MDR and MRP mRNA in various cell lines, and to construct a convenient 550 bp cDNA probe for CFTR mRNA, we used RT-PCR essentially as described (Bremer et al., 1992; Noonan et al., 1990) with several modifications. A master mix containing magnesium, diethylpyrocarbonate-treated deionized water, deoxynucleotides, RNase inhibitor, and reverse transcriptase was prepared and then 20 μl aliquoted into individual reaction tubes. Alternatively, if a large-scale reaction was carried out, the reagents were added individually to the tubes in the order listed above. The large-scale reaction had a total volume of 80 μl. Appropriate primers (typically 10 pmol each) and template (typically 1 μg total RNA) were then added to the tubes. The final concentrations of reagents were 5 mM $MgCl_2$, 1× Perkin-Elmer PCR Buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3; Perkin-Elmer Cetus, Norwalk, CT), 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 1 U/μl RNase inhibitor, 2.5 U/μl murine leukemia virus reverse transcriptase, 2.5 μM random hexamer primers (Perkin-Elmer Cetus), and 0.88–4.17 μg RNA.

The reaction mixture was incubated at room temperature for 20–40 min, and then the tubes were placed in a Perkin-Elmer Gene Amp PCR System 9600 and cycled once through the following program: reverse transcription (15 min at 42°C), denaturation (5 min at 99°C), cooling (5 min at 5°C). The resultant cDNA template was amplified by PCR: β-microglobulin primers were included with the target primers, and reactions with the two sets of primers were also carried out in separate tubes and the data compared. The tubes were cycled as follows: hold (5′ at 95°C) 40 cycles (30 s at 95°C/30 s at 55°C/75 s at 72°C); hold 10 min at 72°C. Standard quantitation curves were also generated with known amounts of RNA and plasmid DNA.

The PCR fragments were electrophoresed on 0.8% agarose and stained with ethidium bromide. Gels under ultraviolet (UV) illumination were photographed with a Polaroid camera. The photographs were scanned with a LaCie scanner interfaced to a Macintosh Quadra computer, and analyzed with photoshop software. The method works very well for low levels of anion exchanger, MDR, MRP, and CFTR expression, and will be described in more detail elsewhere (M. M. Hoffman and P. D. Roepe, manuscript in preparation). We very easily detect CFTR mRNA in the C3, C5, and C10 clones (not shown, see below) but not MDR mRNA, even though we can very easily detect it in other cell lines known to express the message (M. M. Hoffman, L. Y. Wei, and P. D. Roepe, unpublished data).

A 550 bp RT-PCR product was isolated in high yield from the CFTR-expressing C10 clone (see Results). This fragment corresponds to the 5′ region of the published cDNA sequence (Riordan et al., 1989) and is defined by the primers 5′ CCC GAG AGC TCA ACA TGC AGA GGT CGC CT 3′ and 5′ TAC TTA TTT TAT CTA GAA CAC GGC 3′. After PCR, the fragment was analyzed by restriction analysis using either *AluI* or *NciI*. Predicted patterns based on the published cDNA sequence (Riordan et al., 1989) were verified by agarose gel electrophoresis. This probe was also random-primed and used in Northern blot analysis as described above.

Western blotting

Western blotting was performed as described (Luz et al., 1994; Wei and Roepe, 1994) using the monoclonal antibody C219 and the ECL detection method (Amersham International).

Drug uptake

Uptake of ^{3}H-vinblastine was as described (Wei and Roepe, 1994). Briefly, cells were subcultured in six-well plates at 5×10^5 cells/well and then incubated overnight at 5% CO_2 before performing the assay. Each well was incubated with 0.5 μM [^{3}H]vinblastine (200 mCi/mM) at 5% CO_2 for variable time. The wells were then washed with cold media to remove uninternalized vinblastine. Radioactivity in the cells, incubation media, and wash media was assayed by liquid scintillation spectrometry. Total cell-associated protein was quantitated by an amido black assay. We verified that the average protein content per cell was similar for the different cells. Zero uptake was determined by incubation in buffer at 4°C.

Measurement of cell volume

Total cell volume was calculated after determining the mean particle size of cell suspensions by the single threshold Coulter method (Kachel, 1990) performed with a ZM Coulter counter (Coulter Scientific Instruments, Hialeah, FL), as described in detail previously (Roepe et al., 1993), using polystyrene beads of various sizes to calibrate. We have previously determined that size/voltage calibration curves obtained by this method allow for extremely reproducible (±0.5 μm) mean particle size determination from suspensions of $3–4 \times 10^4$ cells, using three plots of counts versus voltage (see Roepe et al., 1993; Kachel, 1990), with each count performed in triplicate.

For some cell lines, we also measured intracellular water volume by ratioing ^{14}C-inulin versus ^3H_2O disintegrations per minute (dpm) as described (Rottenberg, 1979). 1×10^6 cells in 1 ml of HBSS/10 mM HEPES, pH 7.3, were incubated with 1 μCi of ^3H_2O water and 15 μCi of ^{14}C-inulin at 37°C, and the cells were then isolated by centrifugation. Radioactivity in the supernatant and the cell pellet was quantitated with a dual channel $^{14}C/^3H$ dpm program using a Beckman LS 5801 scintillation counter. ^{14}C and ^{3}H quench curves were calculated using known dpm in the same buffer and scintillation fluid volumes that were used in individual experiments (see also Roepe et al., 1993).

Photolabeling with [^{3}H]azidopine

We followed the protocol of Bruggemann et al. (1989) with some modifications. According to the General Electric Co. (Wendell Phelps, personal communication), the UV lamp used in Bruggemann et al. (1989) emits light centered at ~365 nm. In brief, 1×10^6 cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in 100 μl PBS, and placed in wells of a 96-well polystyrene plate. They were incubated with 1 μCi of ^{3}H-azidopine (52 μCi/nmol) for 60 min at room temperature in the dark, and then photolabeled under a UV lamp (General Electric, 3020-BLB) at 4°C. This lamp emits light centered at 366 nm (bandwidth ~80 nm). Applied and emitted power values are 8.0 and 0.8 W, respectively. The distance between the lamp and the samples was varied in different experiments (as was the time of illumination; see Results) but was typically ≤2.5 cm. Labeling was also performed with a higher energy lamp (UVP, San Gabriel, CA) with emission centered at 255 nm. Labeled cells were collected and lysed in cracking buffer (50 mM Tris, pH 6.8/10% glycerol/10 mM DTT/2% SDS/0.1% bromophenol blue) and resolved by SDS/PAGE as described (Wei and Roepe, 1994). Gels were dried and autoradiography was carried out at −80°C. In some experiments, before drying, gels were soaked in Autofluor (National Diagnostics) essentially according to the manufacturer's instructions (several incubation times were shortened because we used thin gels), to increase the efficiency of ^{3}H detection.

RESULTS

MDR phenotype for CFTR clones not previously exposed to chemotherapeutics

Chemotherapeutic drug resistance was measured by colony formation in growth media harboring a range of chemother-

apeutic drug concentrations (Fig. 2). Several recently described clones isolated after transfecting NIH3T3 cells with CFTR cDNA (Stutts et al., 1993) showed resistance to several chemotherapeutics that was significantly greater than the control (Table 1). Notably, these clones were not previously exposed to chemotherapeutic drugs. Analogously, cells transfected with human MDR 1 (M. M. Hoffman and P. D. Roepe, submitted for publication) or MRP cDNA (Grant et al., 1994) that are not previously exposed to chemotherapeutic drugs before the drug resistance assay typically exhibit 2- to 10-fold levels of drug resistance, and non-drug-selected murine (mu) MDR 1 transfectants (Gros et al., 1991; Devault and Gros, 1990) have been reported to exhibit between 3- and 20-fold levels.

(The vast majority of cell lines used to study MDR protein function have been selected for long periods of time with potent chemotherapeutic drugs. Some protocols for creating MDR "transfectants" also include growth on chemotherapeutic drugs. It is important to recall that levels of drug resistance due solely to MDR protein overexpression (without any possible complications due to additional effects caused by chemotherapeutic drug selection) have only been examined in a few studies. Guild et al. (1988) infected NIH3T3 cells with mu MDR cDNA via retroviral-mediated transfer and screened 12 colonies for drug resistance. These clones were not selected with chemotherapeutic drug before analysis and were found to be ≤2.7-fold resistant to doxorubicin (mean 1.3-fold), vinblastine (mean 1.7-fold), and colchicine (mean 1.4-fold). Mass populations of true mu MDR 1/LR73 transfectants (Devault and Gros, 1990) exhibit similar resistance, whereas several well-expressing clones from this study were found to be more resistant (3- to 20-fold, depending on the drug and MDR protein isoform). Increased resistance for some of these mu MDR clones might be due in part to recently observed elevated pH_i (Luz et al., 1994), which is predicted to increase MDR (Keizer and Joenje, 1989; Roepe et al., 1993). Note also that levels of resistance for two true MRP clones (T-2 and T-5) not previously exposed to chemotherapeutics (Grant et al., 1994) are also similar to the levels seen for these CFTR transfectants.)

These CFTR clones were previously shown to exhibit significant Cl^- conductance and plasma membrane depolarization, as measured by voltage clamping (Stutts et al., 1993; see Table 1). This is due to overexpression (confirmed by Western blot; see Stutts et al., 1993) of the CFTR, which functions as a low conductance Cl^- channel (Bear et al., 1992). Thus, decreased $\Delta\Psi$ and increased Cl^- conductance verifies the expression of functional CFTR in these cells.

As the CFTR clones are grown in increasing concentrations of either doxorubicin, vincristine, or colchicine (Fig. 2), there is a marked increase in the number of surviving colonies of >50 cells, relative to control cells also selected on G418 (Stutts et al., 1993). Similarly, growth inhibition assays as performed for low-level MDR kidney carcinoma cells (not shown; see Wei and Roepe, 1994) reveal measurable resistance to doxorubicin, vincristine, and colchicine for the CFTR transfectants, but they are not resistant to methotrexate (not shown). This pattern of chemotherapeutic drug resistance is very similar to that exhibited by other cells that become MDR via overexpression of the MDR protein. However, interestingly, resistance does not appear to be related to relative alkalinization of the cytosol as seen for cells overexpressing MDR protein (Keizer and Joenje, 1989; Thiebaut et al., 1990; Roepe et al., 1993; Luz et al., 1994), since these CFTR transfectants do not exhibit elevated pH_i (Table 1). In fact, four of five clones exhibit mildly acidic pH_i, relative to the control transfectants. Perhaps of interest is the previous observation (see Stutts et al., 1993) that C5, the one clone that does not exhibit lower pH_i, is also the one CFTR clone that is apparently unresponsive to forskolin. We do not note significant alterations in total

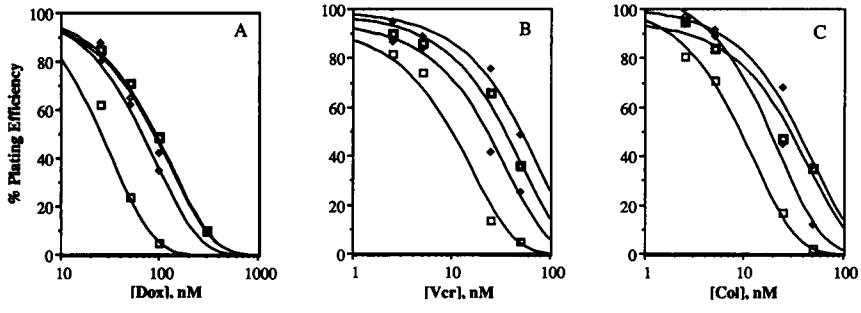


FIGURE 2 Resistance to (*A*) doxorubicin, (*B*) vincristine, (*C*) colchicine as measured by colony formation assays for several CFTR-overexpressing colonies (C3, □; C5, ◆; and C10, ◇) and control 3T3 transfectants (□) created as described (Stutts et al., 1993). Note there is 85% survivability for the control transfectants at 4 μM doxorubicin (not shown, but see Fig. 5 *A*). All cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum and 200 U/l each of penicillin and streptomycin and 0.25 mg/ml G418. Cells were harvested by trypsinization, and 500 cells were plated per well of a six-well plate and allowed to attach for several hours in the absence of G418. Cells were then grown at 37°C and 5% CO_2 in DMEM harboring increasing concentrations of the chemotherapeutics. After 7 days, the plates were stained with methylene blue, and the number of colonies was scored visually. The number of colonies in the wells harboring no chemotherapeutic drug was similar for all the cell lines, and averaged between 85 and 110 colonies/well. For each assay, % plating efficiency at a given drug concentration (relative to the cell line grown in the absence of drug) was analyzed in duplicate. Data shown are the average of three separate assays (SE < 5%). Each data set was fit by a function of the form $y = (a)10^{(-b)x}$, using five data points (including no drug); R^2 was > 0.95 in each case. Fold resistance (Table 1) was computed using the IC_{50} values calculated from these fits.

TABLE 1

Cell line	ΔΨ		pH_i	Mean cell diameter (μM)	Fold resistance to		
	Voltage clamp	ANEPPS			Doxorubicin	Vincristine	Colchicine
3T3/cont	58	61	7.37 ± 0.05	15.3	1.0	1.0	1.0
C3	35	27	7.21 ± 0.04	15.8	4.2	4.1	3.5
C5	34	43	7.43 ± 0.05	16.1	3.2	6.0	4.5
C10	27	23	7.15 ± 0.04	15.5	4.2	2.8	2.3
C11	45	50	7.06 ± 0.04	14.7	1.4	1.3	1.1
C12	47	53	7.11 ± 0.04	16.3	1.3	1.5	1.2

Electrical membrane potential (ΔΨ), intracellular pH (pH_i), size, and fold chemotherapeutic drug resistance for control 3T3 transfectants and five clones expressing different levels of CFTR (Stutts et al., 1993). Values for ΔΨ determined by voltage clamp (±4 mV) are taken from Stutts et al. (1993). ΔΨ was also determined by the K^+/valinomycin null point titration method using di-4-ANEPPS as described (Roepe et al., 1993; Luz et al., 1994) in a double-blind fashion; values shown are the averages of three determinations (SE < 7.5%). pH_i was measured by single-cell photometry using BCECF as described (see Materials and Methods and Luz et al., 1994). Note that mild acidification of pH_i for one CFTR transfectant has also been noted in a previous study (Elgavish, 1991). Calibration of BCECF response was by the K^+/nigericin method at six different pH (see Materials and Methods); curves were well fit by an exponential ($R^2 > 0.95$ in each case) and there was no significant difference in BCECF responses for the different cells. Fold resistance to the chemotherapeutics was by ratioing calculated IC_{50} values from the curves presented in Fig. 2. Variability among different clones of control transfectants was <1.2-fold. In additional work vinblastine resistance for C3 was found to be 3.7-fold. Cell diameter was calculated via the Coulter method (see Materials and Methods); values shown are the average of three determinations based on three size versus voltage plots (variability ≤ 0.6 μm).

cell volume (mean cell size or average water volume, see Table 1) for the CFTR transfectants.

Importantly, the drug resistance exhibited by the CFTR transfectants is not due to expression of MDR protein or mRNA. In fact, similar to others (Currier et al., 1992) we cannot demonstrate expression of any significant MDR protein or mRNA for the parental 3T3 cells or the CFTR expressers via our sensitive Western and Northern procedures (Luz et al., 1994; Wei and Roepe, 1994), which we have previously demonstrated are capable of detecting very low levels of endogenous expression. We are also unable to measure increased MDR or MRP mRNA via overexposure of Northern blots or by RT-PCR (not shown; see Materials and Methods). In any case, based on previous analysis of low-level MDR cells (Luz et al., 1994; Wei and Roepe, 1994) or low-level MRP-expressing MDR cells (Grant et al., 1994), we can firmly conclude that any trace MDR protein or MRP expression that may avoid detection would not be sufficient to confer the measured drug resistance. These data indicate the measured MDR is not due to MDR protein or MRP overexpression.

In contrast to previous analysis of MDR cells created by selection with chemotherapeutic drugs, the calcium channel blocker verapamil does not appear to reverse the drug resistance exhibited by these cells in growth inhibition assays, at levels of verapamil that are not cytotoxic (data not shown). However, we are unaware of any studies that have shown verapamil reverses low-level MDR in MDR-protein overexpressing cells not previously exposed to chemotherapeutic drugs.

Photolabeling with [^{3}H]azidopine

Another yardstick of the MDR phenotype used by many laboratories is photolabeling of the MDR protein with various azido compounds, including [^{3}H]azidopine (Greenberger et al., 1990). In repeated attempts using various UV sources, concentrations of cells or azidopine, time of illumination (up to 90 min), and distance between illumination and sample (see Materials and Methods), we were unable to demonstrate efficient photolabeling of the CFTR protein above background (Fig. 3) even though we were able to efficiently photolabel mu MDR 1 protein in 1–1 cells (Fig. 3, *lane 6*), which express a very high level of the protein (Gros et al., 1991; Luz et al., 1994). However, we stress that the levels of CFTR protein in these transfectants are low, and no one to our knowledge has ever demonstrated efficient photolabeling of low levels of MDR protein above considerable background using MDR transfectants not previously exposed to chemotherapeutic drug. As shown in Fig. 3 we too are unable to demonstrate efficient photolabeling of mu MDR 1 protein in EX4N7 cells ("true" transfectants not previously exposed to chemotherapeutics; see Devault and Gros, 1990). This is also the case for a variety of other photoaffinity probes under a variety of conditions (Dr. L. M. Greenberger, personal communication). Thus, these negative data for CFTR do not necessarily indicate that photolabeling of high levels of CFTR with [^{3}H]azidopine (in analogy to previous work with MDR protein) is impossible.

Altered drug transport for CFTR clones

The MDR phenotype is associated with decreased intracellular retention of chemotherapeutic drugs, that is due to some combination of reduced rates of influx (Beck et al., 1983; Sirotnak et al., 1986; Ramu et al., 1989), altered intracellular binding (Beck et al., 1983; Roepe, 1992), and/or postulated active efflux (Gros et al., 1986; Gottesman and Pastan, 1988; Higgins and Gottesman, 1992).

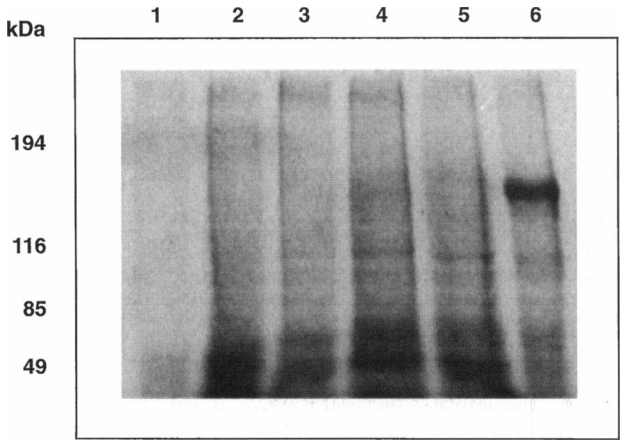


FIGURE 3 Photolabeling with [^{3}H]azidopine. 1×10^6 cells were prepared as described in Materials and Methods and photolabeled for 60 min. After SDS/PAGE the gel was soaked in autofluor, dried, and exposed for 48 h using Kodak X-OMAT film. (*Lane 1*) 3T3 control transfectants; (*lane 2*) C3; (*lane 3*) C5; (*lane 4*) LR73 CHO cells (parent for cells in lanes 5 and 6); (*lane 5*) EX4N7 cells (a "true" mu MDR 1 transfectant; see Devault and Gros, 1990); (*lane 6*) 1–1 cells, which are essentially the cells in lane 5 further selected on 50 ng/ml vinblastine for several months. 1–1 cells express at least 10-fold more MDR protein relative to EX4N7 (see Luz et al., 1994). The only band efficiently labeled is mu MDR 1 protein in the highly overexpressing 1–1 cell line. Note that further exposure, higher energy illumination, the use of 3×10^6 cells, or up to 5 μCi [^{3}H]azidopine per reaction does not reveal efficient photolabeling of the mu MDR 1 protein in EX4N7 or the CFTR protein in C3 or C5 (not shown). Upon further exposure, many dozens of additional bands are revealed (not shown), and it is thus impossible to distinguish any photolabeling of lower levels of the ABC transporters above background. We would thus argue that the photolabeling of MDR protein is not as specific as sometimes interpreted, but we stress that many other investigators argue differently.

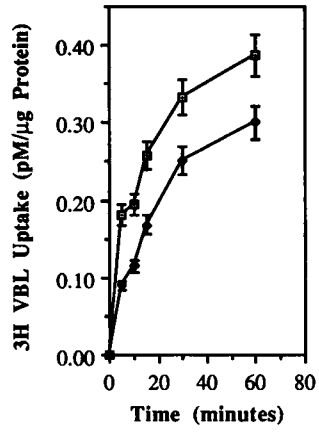


FIGURE 4 Accumulation of [^{3}H]vinblastine in control transfectants (⊡) and the C3 CFTR transfectant (◆). Cells were grown as described in Materials and Methods and exposed to 0.5 μM ^{3}H-vinblastine (200 μCi/μM), and the supernatant and cell pellets were collected at various times (Wei and Roepe, 1994). Accumulation at zero time was measured by incubating the cells with ^{3}H-vinblastine in media preequilibrated to 4°C (a non-permissive temperature for influx). In each assay, each time point was performed in duplicate and the data averaged. Curves shown are the average of three assays performed on three different days; SE < 10%.

Thus, in Fig. 4 we compare accumulation of ^{3}H-vinblastine for C3 and the control cells. Clearly, overexpression of functional CFTR lowers the rate of drug accumulation. Similar decreased rates of accumulation have been seen for MDR transfectants (Hammond et al., 1989) although in this study the MDR transfectants were selected on chemotherapeutic drug before drug transport, so the data are not directly comparable. In any case, uptake for C3 approaches equilibrium in 60–70 min, and intracellular levels are lower for C3 at 60 min. Conversely, we find no evidence for increased rates of drug efflux for the CFTR transfectants (L. J. Robinson and P. D. Roepe, submitted for publication). Similar reduced rates of accumulation for doxorubicin as measured with fluorometric methods (Ramu et al., 1989; Roepe, 1992; Bornmann and Roepe, 1994) are also observed for C3 and other CFTR clones (L. J. Robinson and P. D. Roepe, submitted for publication).

Phenotype of CFTR transfectants after low-level doxorubicin selection

With a few exceptions (e.g., Guild et al., 1988; Devault and Gros, 1990), resistance for MDR transfectants has always been measured after the transfectants were selected on chemotherapeutic drug. Quantitative comparison of the MDR exhibited by these CFTR clones and the majority of MDR transfectants that have been created is thus difficult due to different levels of CFTR versus MDR protein expression and other complications caused by the chemotherapeutic drug selection (including likely induction of additional, non-MDR protein-mediated resistance mechanisms in the drug-selected transfectants). Levels of resistance for true MDR or MRP transfectants (non-chemotherapeutic drug-selected) are comparable to those that we measure here (Fig. 2) for the CFTR transfectants (Guild et al., 1988; Devault and Gros, 1990; M. M. Hoffman, L. Y. Wei, and P. D. Roepe, unpublished; see earlier parenthetical note in the section "MDR phenotype for CFTR clones not previously exposed to chemotherapeutics").

As is the case for true mu MDR transfectants (Devault and Gros, 1990), after these CFTR clones are selected on either 100 nM doxorubicin or 50 nM vinblastine for 10–14 days, resistance to doxorubicin, vincristine, and colchicine is raised substantially (Fig. 5). This selection leads to severe growth arrest for the control transfectants and essentially complete cell death within an additional 7–10 days of selection, but allows for facile isolation of rapidly expanding resistant colonies for the CFTR overexpressers. The increased resistance is not due to increased MRP or MDR protein expression as measured by RT-PCR or Northern blot (see above). In fact, short-term, low-level selection with doxorubicin or vincristine appears to elevate CFTR mRNA levels several-fold for mass populations of C3 or C5 cells (see Fig. 6 *A*).

We further tested this result by selecting a single CFTR-expressing clone with 100 nM doxorubicin as above, and then expanding seven randomly picked individual drug-

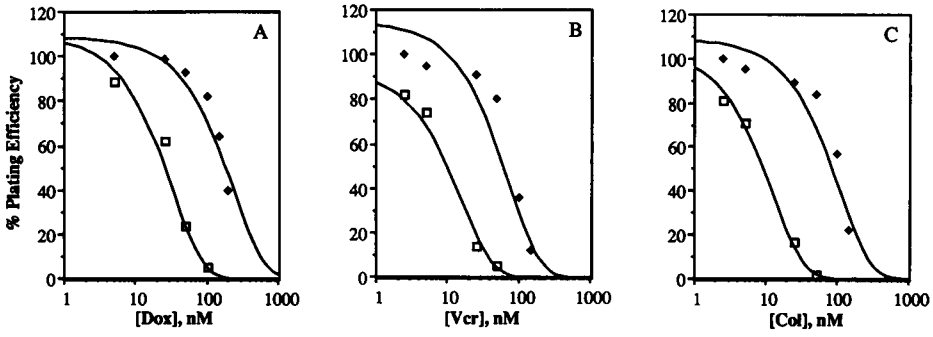


FIGURE 5 Resistance to (*A*) doxorubicin (dox), (*B*) vincristine (vcr), and (*C*) colchicine (col) as measured by colony formation assays for control 3T3 transfectants (□) and colony C3 selected for 2 weeks on 100 nM doxorubicin (C3D1; ♦). Fold resistance for C3D1 is ~8.9, 7.7, and 10.5 to doxorubicin, vincristine, and colchicine, respectively. Similar increased MDR is seen for other CFTR colonies selected for short periods of time on either 100 nM doxorubicin or 50 nM vincristine. Similar selection for the control transfectants yields no surviving cells. More detailed comparison between different drug selected colonies will be published elsewhere. For each assay, drug concentrations were analyzed in duplicate. Data shown are the average of two separate assays. Each data set was fit by a function of the form $y = (a)10^{(-b)x}$; R^2 was > 0.91 in each case.

resistant colonies for 3 weeks in the presence of doxorubicin. RNA was isolated, and Northern blot analysis (Fig. 6 *B*) revealed increased CFTR mRNA in all of the clones; no clones exhibiting similar (or lower) CFTR expression were found. Overexpression varied between 2.2- and 4.8-fold (mean 2.9-fold) among the different clones. This degree of variable CFTR expression is not seen in subclones of the original CFTR clones grown in the absence of doxorubicin. The results indicate that low-level, short-term selection with chemotherapeutics may elevate CFTR mRNA in some cells.

Relationships between resistance, CFTR expression, and $\Delta\Psi$ or $\Delta\mu$

The data described above point to interesting similarities between the CFTR and MDR protein-mediated MDR phenotypes. As a first attempt to analyze these, in Fig. 7 we plot the relationships between drug resistance and relative [CFTR] as determined previously (Fig. 7 *A*; Stutts et al., 1993); $\Delta\Psi$ for the five clones computed as the average between the voltage clamp and di-4-ANEPPS determinations (Fig. 7 *B*); and computed $\Delta\tilde{\mu}_H$, where the chemical gradient in H^+ (in mV) is added to $\Delta\Psi$ for the different cells (following the convention that alkaline inside ΔpH is negative) (Fig. 7 *C*). We are unaware of any similar plots for MDR cells that are MDR solely by virtue of overexpression of MDR protein or MRP.

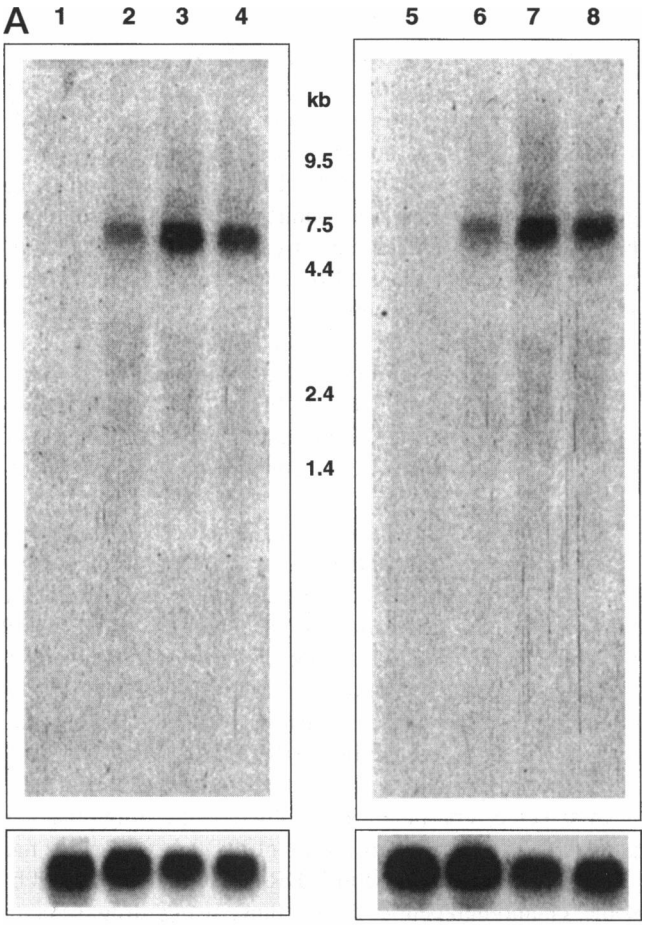
With the exception of particularly high antimitotic resistance for C5, there is in general a positive relationship between drug resistance and relative CFTR expression (Fig. 7 *A*). The relationship is smoothest for doxorubicin. We note that C5 is the sole CFTR clone previously characterized (Stutts et al., 1993) as unresponsive to forskolin stimulation. When resistance is plotted relative to the $\Delta\Psi$ (Fig. 7 *B*), an apparent decline in resistance to the antimitotics is seen at low $\Delta\Psi$, again because of the behavior of C5 and also the C10 clone, which is the most acidic of the significantly depolarized clones (Table 1). A strong positive relationship is again seen for doxorubicin, which apparently begins to plateau near −30 mV.

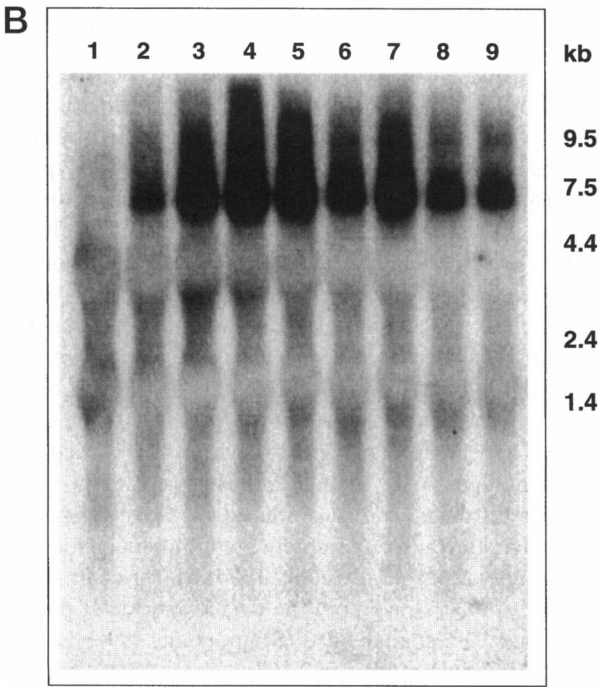
When the pH gradient is taken into consideration and resistance plotted versus computed electrochemical potential (Fig. 7 *C*), the data points cluster much nearer each other and the divergence in the trends for the antimitotics seen in Fig. 7 *B* becomes less pronounced. These data, along with previous work (Luz et al., 1994; Roepe et al., 1993) empirically suggest that there is a possible additive effect of decreased $\Delta\Psi$ and alkaline pH_i with regard to resistance, whereas acid pH_i and decreased $\Delta\Psi$ may be antagonistic, particularly with regard to resistance to the antimitotics. This could be due to a different pH dependency for antimitotic binding to tubulin in this pH_i range relative to anthracycline binding to nucleic acid (see Mukhopadhyay et al., 1990).

Without similar plots for true MDR transfectants, it is difficult to compare these trends to those that likely exist for true MDR transfectants. However, for drug-selected MDR cells that overexpress MDR protein, in general, a positive relationship is observed when relative [MDR protein] is plotted versus resistance. This relationship typically plateaus early in the second log of resistance. This also appears to be the case when decreased $\Delta\Psi$ or increased ΔpH is plotted versus resistance (see Roepe et al., 1993). The $\Delta\Psi$ versus doxorubicin resistance plot for a series of drug selected MDR myeloma cells analyzed previously (Roepe et al., 1993) appears to plateau near 50% membrane depolarization, as also appears to be the case for the series of CFTR transfectants. Thus, although the CFTR and MDR protein mediated MDR phenotypes are different (notably with regard to pH_i); they appear to share some important features.

DISCUSSION

Including the data in this paper, overexpression of three different eukaryotic ABC transporters has been associated with MDR phenomena (see also Devault and Gros, 1990;





Grant et al., 1994). Three theories invoked to explain MDR currently receive attention; thus we shall address the present data in the context of these models.

The first theory postulates that the MDR protein is a multisubstrate pump (Gros et al., 1986; Gerlach et al., 1986; Gottesman and Pastan, 1988; Ruetz and Gros, 1994) that is somehow able to bind and actively efflux a large variety of structurally divergent compounds including actinomycin D, anthracyclines, colchicine, cyclosporin A, morphine, valinomycin, various peptides, vinca alkaloids, and many others; i.e., MDR protein directly transports drugs. This model has recently been extended to suggest that the protein product of the MRP gene is also a multisubstrate drug pump (Zaman et al., 1994); however, more detailed thermodynamic and kinetic data (showing transport by MRP against a substrate concentration gradient at a rate faster than passive diffusion) are required to unequivocally support MRP drug pumping activity.

The pump model is widely accepted among researchers that study chemotherapeutic drug resistance, but remains controversial because of the difficulty in rationalizing the lack of specificity. Recent data have been obtained in support of this model for MDR protein (Ruetz and Gros, 1994; Sharom et al., 1993; Shapiro and Ling, 1995); however, estimated turnover from these studies (drug molecules translocated/MDR protein molecule/min) reveal a measured process that is extremely slow and 10^3–10^5-fold slower than is estimated to be required to explain altered drug accumulation and retention in MDR cells (Demant et al., 1990; see also Simon and Schindler, 1994; Bornmann and Roepe, 1994; Roepe, 1994, 1995). In addition, several recent kinetic studies of drug efflux are inconsistent with the active drug efflux model (Roepe, 1992; Bornmann and Roepe, 1994; L. J. Robinson and P. D. Roepe, submitted for publication). For these reasons and others we do not favor this interpretation for the CFTR-mediated MDR phenotype.

The second model (Roepe et al., 1993, 1994; Luz et al., 1994) theorizes that MDR protein overexpression alters

FIGURE 6 Northern blot analysis of relative CFTR expression for transfectants selected on low levels (100 nM) of doxorubicin for short periods of time (see Materials and Methods). In (*A*), RNA was isolated from mass populations of either C3 (*left*) or C5 (*right*) after selection, and probed with a 550 bp CFTR fragment (see Materials and Methods). Lanes 1 and 5 are 3T3/c control transfectants, lanes 2 and 6 are C3 and C5 before drug selection, lanes 3 and 7 are C3 and C5 selected on doxorubicin in the presence of G418, and lanes 4 and 8 are C3 and C5 selected on doxorubicin in the absence of G418. Fold overexpression varied between 2.1 and 3.5 in the different examples (see Materials and Methods); control β-actin hybridization is shown at the bottom. Similar data were obtained via selection with 50 nM vincristine (not shown). In (*B*), a single CFTR clone (*lane 2*) was similarly selected with doxorubicin, and seven individual resistant colonies that appeared within 10 days (*lanes 3–8*) were picked and propagated in 100 nM doxorubicin for an additional 2 weeks. Lane 1 is a control 3T3/c transfectant that does not express CFTR. CFTR overexpression in the drug selected clones varied between 2.2- and 4.8-fold (see Materials and Methods). Note that our CFTR probe is highly specific in these high stringency Northerns (see Materials and Methods) and that MDR and MRP mRNA are about 2.6 and 1.8 kB smaller than this CFTR mRNA, respectively. The blot is slightly overexposed to enhance the signal in lane 2.

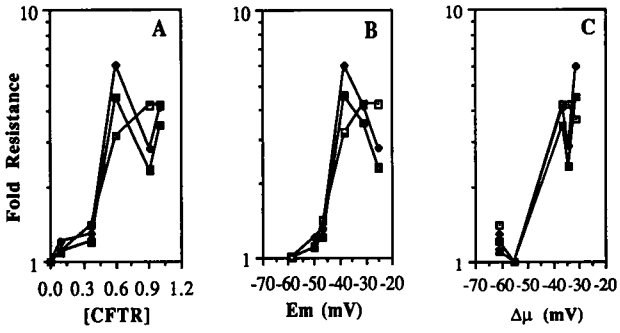


FIGURE 7 Plot of resistance to the different chemotherapeutics versus (*A*) relative expression of CFTR in the series; (*B*) relative $\Delta\Psi$; and (*C*) computed plasma membrane $\Delta\tilde{\mu}_{H}$, i.e., the sum of $\Delta\Psi$ and the computed chemical gradient in protons. (⊡) Doxorubicin resistance; (◆) vincristine resistance; and (■) colchicine resistance. See text for discussion.

plasma membrane electrical potential ($\Delta\Psi$) and/or pH_i (i.e., MDR protein overexpression alters the character and/or magnitude of plasma membrane electrochemical potential), which then affects passive diffusion and target binding efficiency and hence alters retention/partitioning of the drugs via several different mechanisms; i.e., MDR protein indirectly promotes decreased intracellular drug accumulation. This model can be extended to account for altered compartmentalization of drugs seen in resistant cells that do not express MDR protein (i.e., other mechanisms that perturb potential for plasma membrane or other compartmental membranes may contribute to drug resistance, such as overexpression of vacuolar H^+ ATPase; see Ma and Center, 1992) and also ties together a variety of other curious observations for MDR cells that currently elude interpretation via the first model, such as dramatic overexpression of Na^+/H^+ and $Cl^-/^-HCO_3$ exchanger mRNA in MDR cells (Roepe et al., 1993; Luz et al., 1994) and the provocative resistance that MDR cells exhibit to a variety of ionophores. It may also provide a satisfying explanation for why different cells expressing similar levels of MDR protein exhibit different levels of resistance, since maintenance of plasma membrane potential is different in various eukaryotic cell types. Importantly, it is currently not known whether elevated pH_i and decreased $\Delta\Psi$ are additive or synergistic with regard to chemotherapeutic drug resistance, or relatedly whether lower pH_i and decreased $\Delta\Psi$ are antagonistic (see Table 1), which may be particularly important to understanding the differences between the MDR protein and CFTR-mediated MDR phenotypes as well as patterns of gene expression in CFTR overexpressers selected for higher levels of drug resistance (L. Y. Wei and P. D. Roepe, manuscript in preparation). Along with the large volume of data pertaining to CFTR channel function, which is predicted by theory and verified by experiment (Stutts et al., 1993) to lead to altered plasma membrane potential when the channel is overexpressed, this model presents a logical framework for interpreting the data in this paper.

A third model, through identification of anomalous Cl^- conductance in MDR cells (Valverde et al., 1992; Gill et al., 1992), essentially marries the first two by suggesting that MDR protein functions as both a drug pump and a Cl^- channel or channel regulator (Hardy et al., 1995). It remains to be unequivocally proven whether MDR protein is a Cl^- channel, and in fact some recent data (Ehring et al., 1994) appear to contradict previous work (Valverde et al., 1992), while other recent data (Gill et al., 1992; Altenberg et al., 1994) apparently support it. Luckie et al. (1994) have suggested that anomalous ion conductance in MDR cells may be the result of a higher "sensitivity" of endogenous channels to hypotonicity caused by some combination of drug selection and MDR protein overexpression, and more recently Hardy et al. (1995) have determined that some complexity may be due to different levels of protein kinase C activity in different cell types. In any case, interestingly, overexpression of the MRP homologue has also been associated with anomalous Cl^- conductance (Jirsch et al., 1993).

It perhaps bears mentioning that many years of controversy accompanied elucidation of Cl^- channel function for the CFTR and controversy continues to this day with regard to understanding regulation of the channel. If the MDR protein does function as a channel it is unclear what gates its conductance, and if it modulates some other endogenous conductance, it is unclear what channels are being perturbed. To further complicate the picture, there is wide variability in the cell types studied to date and most have been selected with potent chemotherapeutic drugs that can perturb cells in many ways. Bear (1994) has confirmed that Cl^- conductance specific to MDR cells may be blocked by some chemotherapeutic drugs; thus, using drug selected cells in these experiments introduces complications. Also, the hypotonic shock used to induce anomalous conductance likely represents indirect "gating," and effects could be very different in different cell types. Cell lines of B-cell lineage used in Ehring et al. (1994) are unlikely to behave identically relative to fibroblasts used in other work. In any case, if the MDR protein does indeed function as some type of Cl^- transporter, and/or stimulates Cl^- channel activity, it would be predicted to have important effects on plasma membrane electrochemical potential. Thus, facets of the third model support the second model.

We suggest that membrane depolarization plays a key role in MDR, since decreased $\Delta\Psi$ has been measured for true mu MDR 1 transfectants (Luz et al., 1994) and correlated with resistance in a series of MDR cells (Roepe et al., 1993), and appears to play some role in the CFTR-mediated MDR phenotype. Also, model studies have clearly established that diffusion and partitioning of the hydrophobic cations vincristine and doxorubicin are highly dependent on the magnitude of $\Delta\Psi$ (Bally et al., 1985; Mayer et al., 1985; Praet et al., 1993). Since both the CFTR and MDR protein may increase Cl^- conductance (Stutts et al., 1993; Bear et al., 1992; Valverde et al., 1992; Gill et al., 1992; Hardy et al., 1995), which is predicted to lower $\Delta\Psi$, CFTR and MDR transfectants may share the property of drug resistance through membrane depolarization. Since doxorubicin and

vincristine are lipophilic cations, resistance to these is not totally unexpected in the CFTR transfectants. Importantly, $\Delta\Psi$ perturbations may indicate surface potential perturbations that may have important effects. However, if resistance in the CFTR clones is due to membrane depolarization, the observed colchicine resistance is provocative, since this hydrophobic drug does not harbor positive charge (see below).

The present data may be interpreted in the context of any of the models, but we favor the second model, because of the obvious specificity difficulties inherent in the first model, kinetic evidence that argues against active efflux of drugs in MDR cells and these CFTR transfectants (L. J. Robinson and P. D. Roepe, submitted for publication), and the scarcity of integral membrane proteins that function as both pumps and channels as suggested by the third model. However, based on previous hypotheses (Gill et al., 1992; Hardy et al., 1995) one could speculate that CFTR is another example of a channel or channel regulator that also pumps chemotherapeutics. This hypothesis initially appears unlikely because of the relative paucity of evidence for copious ATP hydrolysis by the CFTR (in analogy to MDR protein, energy for active drug transport would presumably be required in the form of ATP hydrolysis), unless energy for active transport is provided in the form of some ion gradient (e.g., H^+ or Cl^-). Further detailed drug transport measurements that examine the kinetic and thermodynamic predictions of such a model will be helpful in this regard. Alternatively, we suggest, via the second model, that the exhibited colchicine resistance is due to an indirect effect of membrane depolarization. Possibilities include 1) altered vesicle traffic; 2) perturbation in tubulin organization near the plasma membrane (i.e. altered availability of drug target; see Aszalos et al., 1986); 3) changes in membrane fluidity or leaflet distribution of lipid. All of these have previously been observed in MDR cells and could conceivably be perturbed by alterations in plasma membrane $\Delta\Psi$.

Our results demonstrate a new property for the CFTR (which can be added to the list already containing Cl^- channel, ion channel regulator, and water channel), namely, that overexpression may lead to chemotherapeutic drug resistance. Thus, a third member of the ABC transporter family shares this important property. The additional shared characteristics of anomalous Cl^- conductance and membrane depolarization in cells overexpressing the CFTR or MDR protein suggest that the ability of these proteins to influence passive drug translocation represents an important mechanism of drug resistance that does not necessarily require direct active drug transport. Finally, these data should be considered with regard to evolving therapy of CF (Collins, 1992), which may result in persistent overexpression of the CFTR.

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