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Analysis of Random Recombination between Human MDR1 and Mouse Mdr1a cDNA in a pHaMDR-Dihydrofolate Reductase Bicistronic Expression System

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ABSTRACT

Human P-glycoprotein (Pgp) confers multidrug resistance (MDR) to otherwise sensitive cells. The homologous mouse Pgps, which are encoded by mouse *mdr*1a (also known as *mdr*3) and *mdr*1b (also known as *mdr*1), confer different degrees of resistance to the same MDR drugs and inhibitors. To create recombinants for the study of sequences responsible for these differences in drug-resistance, chimeric cDNA libraries can be constructed by homologous recombination of pools of related sequences. This mutagenesis approach is called DNA shuffling. To select for chimeric Pgp with an altered resistance profile, DNA shuffling between the homologous but not identical drug interacting transmembrane domains 5 and 6 of human *MDR*1 and mouse *mdr*1a was used. The chimeric proteins were expressed in human KB-3-1 cells. One recombinant Pgp (clone 3-4) with a novel phenotype was analyzed in detail. Inhibitors of Pgp, including verapamil and cyclosporin A, were less effective in reversing resistance of the chimeric Pgp compared with wild-type Pgp, for certain drugs. However, [^{125}I]iodoarylazidoprazosin photoaffinity labeling of the chimeric Pgp and its binding competition with cyclosporin A, showed that cyclosporin A competed for the photoaffinity labeling. The chimeric Pgp cells stained less well with human-specific anti-Pgp mAb MRK16 than wild-type Pgp, despite having the described epitopes for MRK16. Staining with human-specific mAb UIC2 was increased when the chimeric protein was compared with wild-type Pgp. These results suggest an alteration in exposure of human Pgp specific epitopes in this chimeric Pgp, as well as a change in the interaction of reversing agents with the chimeric protein.

The development of drug resistance in tumor cells is a major obstacle to clinical response in cancer chemotherapy. Pgp, a 170-kDa plasma membrane protein, is one of the most widely studied of the proteins that are responsible for the phenomenon of multidrug resistance in mammalian cells. The protein acts as an energy-dependent pump that extrudes a broad range of hydrophobic cytotoxic drugs from the cell (reviewed by Gottesman and Pastan, 1993). Human Pgp, encoded by the *MDR*1 gene, consists of 1280 amino acids organized in two tandem repeats of 610 amino acids, joined by a linker region of 60 amino acids. Each repeat consists of an amino-terminal hydrophobic domain containing six potential TM domains, followed by a hydrophilic domain containing a nucleotide-binding site. Transmembrane domains 5/6 and 11/12 have been demonstrated previously by photoaffinity labeling with substrate analogs to be substrate interaction sites (Bruggemann *et al.*, 1992; Greenberger, 1993; Morris *et al.*, 1994). Mutagenesis studies further reveal that changes of several different amino acids throughout Pgp (especially in the predicted transmembrane domains 5/6 and 11/12 of Pgp) change its drug resistance pattern (Germann *et al.*, 1993; Loo and Clarke, 1993, 1994). Pgp, which is encoded by mouse *mdr*1a (*mdr*3), confers different degrees of resistance to known MDR drugs (Tang-Wai *et al.*, 1995). At similar levels of protein expression, the mouse *mdr*1a isoform seemed to be a more efficient drug efflux pump and showed levels of resistance that were superior to *mdr*1b or *MDR*1 for all drugs tested.

The reversal of MDR by inhibitors or modulators of Pgp may improve the outcome of cancer chemotherapy (Dalton *et al.*, 1989; Solary *et al.*, 1992; Yahanda *et al.*, 1992). Cyclosporin A, its analog PSC 833, verapamil, and other MDR modulators have been shown to increase cellular drug accumulation and reverse MDR through competitive binding to Pgp. Mouse *mdr*1a shows different sensitivity to the reversal

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ABBREVIATIONS: Pgp, P-glycoprotein; MDR, multidrug resistance; PCR, polymerase chain reaction; TM, transmembrane; MTX, methotrexate; IRES, internal ribosome entry site; DHFR, dihydrofolate reductase; FACS, fluorescence-activated cell-sorting; IAAP, iodoarylazidoprazosin; AM, acetoxymethyl ester.

effect of human *MDR*1 inhibitors (Yang *et al.*, 1990; Tang-Wai *et al.*, 1995). The mouse *mdr*1a was much less sensitive to modulators than the human *MDR*1.

We have initiated studies to select for Pgp mutants with altered drug resistance profiles as a potential tool in cancer gene therapy because those mutants could be used to protect bone marrow from the systemic toxicities of chemotherapy. Moreover, the localization of specific segments and amino acids implicated in substrate specificity and response to modulators in chimeric and mutant proteins, would facilitate the structure/function analysis of Pgp.

Chimeric cDNA libraries can be constructed by homologous recombination of pools of related sequences. This mutagenesis approach is called DNA shuffling or sexual PCR (Stemmer, 1994a; Lorimer and Pastan, 1995). Because of fragment exchange and error-prone PCR, overall sexual PCR is an effective method to create recombinant molecules which resemble the process of molecular evolution.

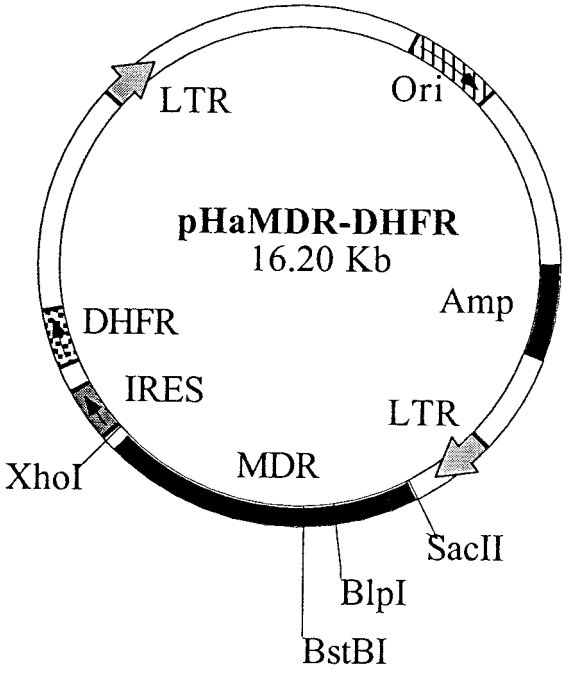

Fig. 1. The bicistronic retroviral expression vector pHaMDR-DHFR containing the Harvey virus long terminal repeat (*LTR*) followed by the human *MDR*1 gene, the IRES from encephalomyocarditis virus, and a mutant form of the mouse *DHFR* gene (dihydrofolate reductase) (Zhang *et al.* 1998). Unique restriction sites flanking *MDR*1 and *DHFR* are labeled.

An advantage of DNA shuffling is that it can optimize the function of genes without first determining which gene product is rate limiting. This process has been shown to yield functional optimization of different genes (Stemmer, 1994b; Crameri *et al.*, 1996, 1997).

In this study, we describe DNA shuffling between TM domains 5 and 6 of human *MDR*1 and mouse *mdr*1a. We analyze here one novel chimeric Pgp (clone 3-4) with an altered spectrum of cross-resistance to cytotoxins and specific insensitivity to modulators.

Materials and Methods

Substrates preparation for DNA shuffling. The substrates for the shuffling reaction were 300-bp double-strand DNA PCR products derived from pFRCMV-mdr1a and pSXLC-MDR with the primer sequences 5′-GAAGAAGCTAAGCGAATTGG-3′ and 5′-GTGC-CCACTCTTCGAATAGC-3′. The *MDR*1 and *mdr*1a sequences used here have 86% DNA sequence identity. The primers contain BlpI and BstBI restriction sites for subcloning into the pHaMDR-DHFR vector (Zhang *et al.*, 1998) (Fig. 1). PCR products were purified with Wizard PCR (Promega, Madison, WI).

DNaseI digestion. PCR product (4 μg) was digested with 0.15 units of DNase I (Sigma, St. Louis, MO) in 100 μl of 50 mM Tris·HCl, pH 7.4, 10 mM $MnCl_2$ for 1.5 min at 15° and terminated by heating at 90° for 10 min. The digestion products were passed through Centri-Sep Columns (Princeton Separations, Adelphia, NJ).

PCR without primers. The purified fragments (10 μl from *mdr*1a digestion plus 10 μl from *MDR*1 digestion) were added to the PCR mixture (0.4 mM each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 2.5 units of Perkin Elmer AmpliTaq DNA polymerase per 100 μl of reaction mixture). A PCR program of 94°, 3 min; 94°, 1 min; 55°, 1 min; 72°, 1 min (40 cycles); and 72° for 7 min was used.

PCR with primers. After 1:25 dilution of this primerless PCR product into the PCR mixture with the primers described above and 25 additional cycles of PCR, a single product of the correct size was obtained (Fig. 2E).

Cloning and sequencing. After digestion of the PCR product with terminal restriction enzymes (BlpI and BstBI) and gel purification, the reassembled fragments were ligated into the pHaMDR-DHFR bicistronic expression vector digested with BlpI and BstBI. Ten randomly chosen plasmids containing inserts were purified and sequenced on a 373A DNA Sequencing System (Applied Biosystems, Foster City, CA) using the *Taq* Dyedeoxy Cycle Sequencing kit. Sequences were analyzed using the SeqEd 675 Sequence Editor software supplied with this system.

Bacterial transformation library. DNA from the above ligation was electroporated into D10B competent cells (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Bacterial transformants (5×10^5) were collected from plates containing ampicillin.

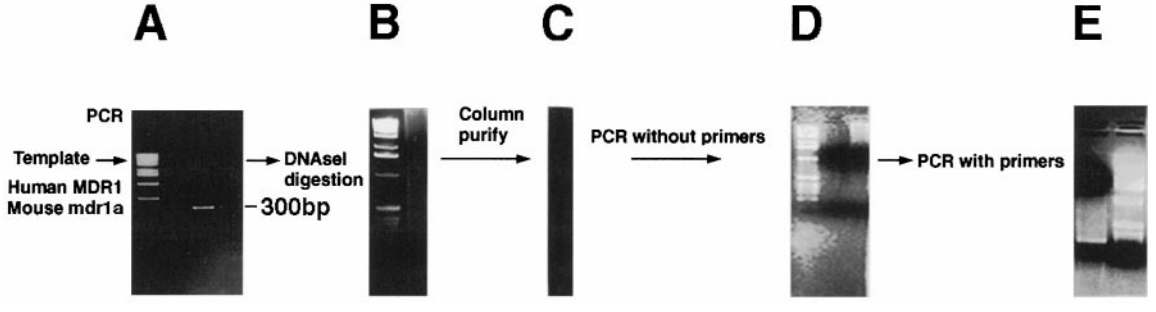

Fig. 2. Reassembly of 300 bp PCR product from domains 5 and 6 of human *MDR*1 and mouse *mdr*1a. A, PCR products before digestion. B, DNA after 1.5-min digestion with DNase I. C, Fragments after purification on spin column. D, Reassembly of fragments in the absence of primers. E, A single PCR product of the correct size after addition of primers and additional cycles of PCR.

Mammalian cell transfection library. DNA isolated from the bacterial library was electroporated into KB-3-1 cells (Akiyama *et al.*, 1985) with an electroporator (Bio-Rad, Hercules, CA). For a 0.4-cm cuvette, 10^7 cells and 10 μg of DNA resuspended in 0.8 ml of phosphate-buffered saline. The transfection conditions were 350 V and 960 mF.

Selection for methotrexate resistance. Twenty-four hours after electroporation, cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed bovine calf serum and selected in 30 ng/ml MTX. Three thousand methotrexate-resistance colonies were pooled 3 weeks later. The mammalian library was maintained in MTX.

Selection and counting of drug-resistant colonies. The dose-response survival curves of the mammalian library, the normal pHaMDR1-DHFR transfected KB-3-1 cells, and the selected colonies were determined by plating 1000 exponentially growing cells per 100-mm dish in the absence of drug. After a 16-hr incubation at 37°, the appropriate concentrations of stock solutions of colchicine, daunorubicin, taxol, vinblastine, and vincristine dissolved in dimethylsulfoxide (<0.5% of total volume/dish) were added to each cell line. Either cyclosporin A or verapamil at concentrations of 0.5 or 1 μg/ml was added to each cell line immediately after the addition of the cytotoxic drug. After incubating the cells at 37° for 10 days, colonies from the library that were found to be resistant to high drug concentrations were isolated and were grown in methotrexate-containing medium for further characterization. For counting, colonies were stained with 0.5% methylene blue in 50% ethanol and counted with a Manostal colony counter.

Immunofluorescence analysis. The level of cell-surface Pgp was determined by FACS analysis using MRK-16 (Hamada and Tsuruo, 1986), and UIC2 (Mechetner and Roninson, 1992), which react with external surface domains of Pgp. The staining was performed as described previously (Germann *et al.*, 1996).

Drug accumulation assays. Cells were harvested by trypsinization, washed, and resuspended in Iscove's modified minimal essential medium supplemented with 5% fetal bovine serum. For accumulation measurements, 500,000 cells were incubated at 37° in 5 ml of Iscove's modified minimal essential medium containing 5% fetal bovine serum and the fluorescent substrates 0.5 μg/ml rhodamine 123, 0.5 μM calcein AM, 5 μM daunorubicin, 0.5 μM bodipy-verapamil or bodipy-Taxol with or without 20 μM verapamil, 5 μM cyclosporin A, or 2.5 μM PSC 833. After 40 min, cells were pelleted and resuspended in 300 μl of phosphate-buffered saline and immediately analyzed by FACS, using a Becton-Dickinson instrument equipped with CellQuest software.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis were performed as described previously (Germann *et al.*, 1996). Photoaffinity labeling with [^{125}I]IAAP was carried out as described previously (Dey *et al.*, 1997).

Results

DNA shuffling between transmembrane domains 5 and 6 of human MDR1 and mouse mdr1a. Fig. 2 shows a DNase I digest of 300-bp PCR products from domains 5 and 6 of human *MDR*1 and mouse *mdr*1a. The column purified fragments were reassembled (initially without primers) to a single PCR product of the correct size.

To analyze the function of mutant DNAs generated using the DNA shuffling procedure, the PCR products were subcloned into the pHaMDR-DHFR vector (Fig. 1). This bicis-

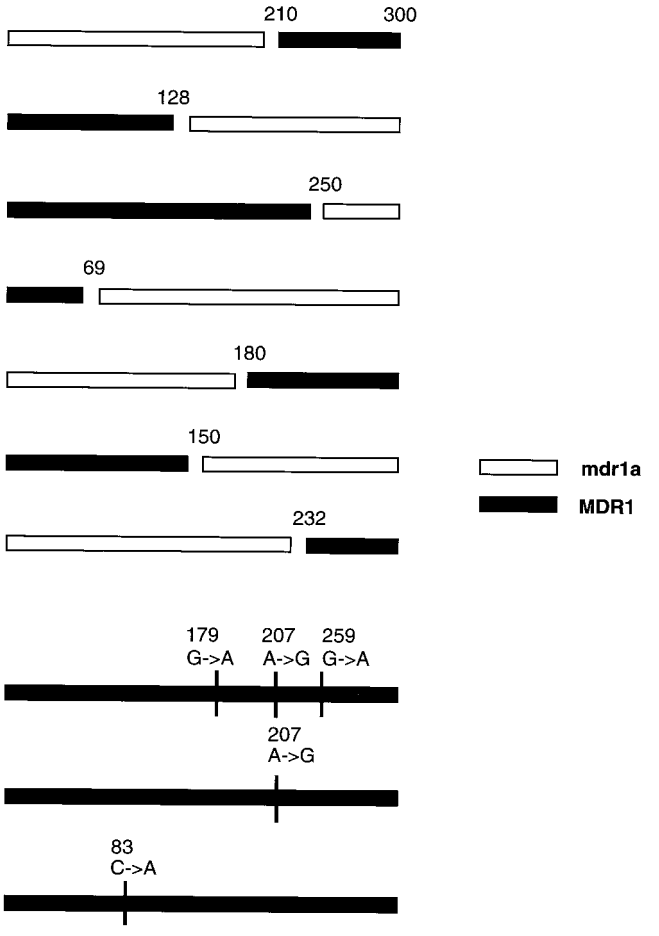

Fig. 3. Recombination in 10 randomly selected plasmids containing inserts. Clones resulting from reassembly of a mixture of human *MDR*1 and mouse *mdr*1a fragments were sequenced. DNA sequences are shown schematically. *Black*, human *MDR*1 sequences; *white*, mouse *mdr*1a sequences. Crossovers are arbitrarily shown to be midway between characteristic bases of the two sequences. Point mutations are labeled in their position.

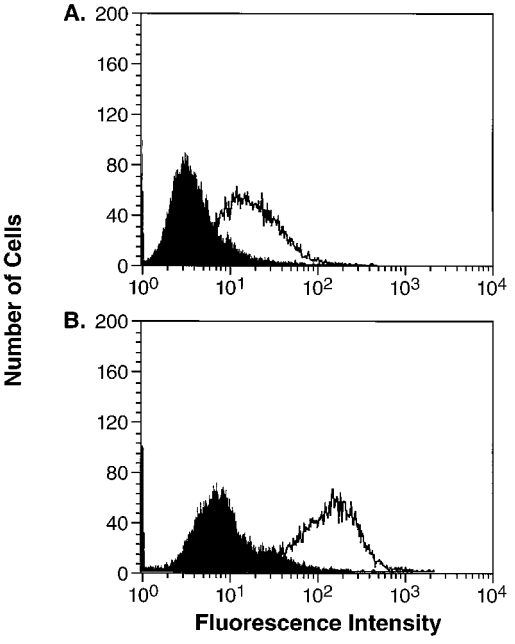

Fig. 4. Analysis of the mammalian library cell surface expression of Pgp. KB-3-1 cells stably transfected with pooled DNA isolated from the bacterial library (A) or the wild-type pHaMDR1-DHFR (B) were subjected to FACS analysis after staining with human Pgp external surface epitope-specific monoclonal antibody MRK-16 (*unshaded profiles*) and control mouse IgG2a (*shaded profiles*), as described in Materials and Methods.

tronic mammalian expression vector contains the *MDR*1 gene, an IRES, and the mutant murine DHFR gene, which confers methotrexate resistance, as a dominant selectable marker. Ten randomly chosen plasmids containing inserts were sequenced. Seven of the ten were chimeric for human *MDR*1 and mouse *mdr*1a sequences as the result of different recombination events (Fig. 3). Three had the human *MDR*1 sequence with one or more point mutations per sequence. Pooled DNA isolated from the bacterial library was electroporated into KB-3-1 cells.

After transfection, plasmid-containing cells were selected in methotrexate for the expression of the second gene (DHFR) in the vector to avoid secondary selective pressure on the *MDR*1 gene on the plasmid or the host. The majority of the MTX-resistant cells expressed a substantial level of Pgp, as evidenced by the recognition of Pgp by the monoclonal antibody MRK-16 and determined by FACS analysis (Fig. 4).

Isolation of clones with altered drug resistance pattern. The mammalian library and the wild-type pHaMDR1-DHFR transfected KB-3-1 cells were selected with different concentrations of unrelated antitumor agents including the microtubule-destabilizing agents colchicine and taxol; the anthracycline daunorubicin; and the Vinca alkaloid vincristine. The mammalian library showed a decrease in relative resistance to all the antitumor agents compared with the relative resistance of pHaMDR1-DHFR transfected KB-3-1 cells.

Ten highly resistant colonies for each drug were isolated and were grown in methotrexate selection for genomic DNA purification and PCR sequence analysis. In contrast to the bacterial library clones, the majority of the mammalian library clones picked from selective medium had the wild-type human *MDR*1 sequence. One taxol-resistant clone was chimeric for mouse *mdr*1a and human *MDR*1 as the result of recombination. Of the amino acid residues in TM domains 5 and 6 of this clone, 70% were from mouse *mdr*1a and 30% were from human *MDR*1, which resulted in five amino acid changes in *MDR*1: Ile299Met, Thr319Ser, Leu322Ile, Gly324Lys, and Ser351Asn.

Expression of the wild-type and the recombinant Pgp. Fluorescence intensity by FACS analysis with anti-Pgp MoAbs MRK16 and UIC2 of the recombinant clone cells was compared with fluorescence intensity of the wild-type pHaMDR1-DHFR transfected KB-3-1. As shown in Fig. 5, A and B, the recombinant clone cells stained less strongly with MRK16 than the wild-type pHaMDR-DHFR cells. In contrast, UIC2 fluorescence intensity was higher than in the wild-type Pgp (Fig. 5, C and D), but the fluorescence distribution was biphasic and some cells expressed very low P-gp when detected with UIC2. Whole cell lysates from both cell lines were prepared and analyzed for their level of Pgp expression by Western blotting using the anti-P-glycoprotein monoclonal antibody C219. The levels of total cellular expression in the recombinant clone cells detected with this mono-

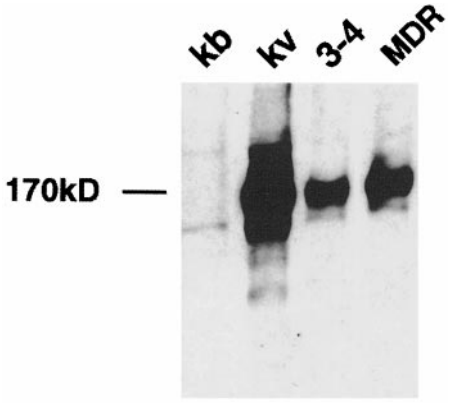

Fig. 6. Protein expression of Pgp. Parental KB-3-1 (*kb*), high Pgp expressing *MDR*1 cells KB-V1 (*kv*), the recombinant 3-4 clone cells (*3-4*), and the wild-type pHaMDR-DHFR (*MDR*) cells were analyzed for protein expression. Cells were lysed, and total cell lysates (10 μg of protein/lane) were subjected to immunoblot analysis with anti-Pgp monoclonal antibody C219.

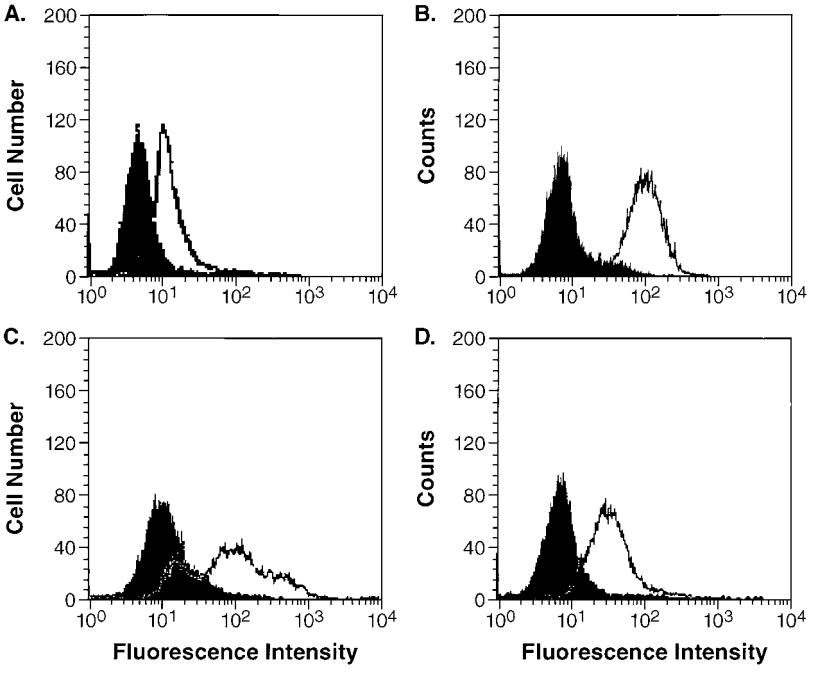

Fig. 5. Analysis of the fluorescence intensity of the recombinant clone cells by FACS using two anti-Pgp monoclonal antibodies. The recombinant clone cells (A and C) and the wild-type pHaMDR-DHFR cells (B and D) were subjected to FACS analysis after staining with MRK-16 (A and B, *unshaded profiles*) or UIC2 (C and D, *unshaded profiles*) monoclonal antibody, and control mouse IgG2a (*shaded profiles*) as described in Materials and Methods.

clonal antibody were somewhat less than in the wild-type *MDR*1 transfected cells (Fig. 6). Similar results were obtained with the anti-Pgp polyclonal antibody PEPG 13 (Bruggemann *et al.*, 1992) by Western blotting.

Drug resistance of recombinant clone 3-4. To assess the resistance of recombinant clone 3-4 to various cytotoxic drugs and reversing agents, growth of the recombinant clone cells, the wild-type *MDR*1 cells, and the nontransfected KB-3-1 cells were compared using clonogenic cell killing assays. Both recombinant clone cells and wild-type *MDR*1 transfected cells exhibited resistance to the selecting agent colchicine, Furthermore, in the presence of 1 μM of the reversing agent cyclosporin A, the growth of the wild-type *MDR*1-transfected cells was inhibited, whereas the growth of the recombinant clone cells was similar to growth without cyclosporin A. When both cells were selected with the natural drug product, taxol, the recombinant clone cells were somewhat less resistant to taxol overall compared with the wild-type *MDR*1-transfected cells. In the presence of 1 μM cyclosporin A, the growth of both cell lines was inhibited. These results are consistent with the initial ability of this clone to survive in taxol despite a reduced level of chimeric Pgp expression. To verify these results, the chimeric cDNA was recloned by PCR back into the bicistronic vector, and was retransfected into KB-3-1 cells. Similar results were obtained with the new clone (Fig. 7), showing that the phenotype was caused by the 3-4 mutations as opposed to variations in host cells.

Analysis of the drug transport function of the recombinant Pgp. The abilities of the wild-type and the recombinant Pgp expressed in KB-3-1 cells to transport different fluorescent substrates were determined. As shown in Fig. 8, both Pgp-expressing cells accumulated considerably less bodipy-verapamil, daunorubicin, and calcein AM than the drug-sensitive KB-3-1 cells. The recombinant clone cells accumulated more rhodamine 123 compared with the wild-type pHaMDR-DHFR transfected cells.

In the presence of reversing agents such as verapamil (20 μM), cyclosporin A (5 μM), or PSC 833 (2.5 μM), wild-type *MDR*1-expressing cells accumulated the different fluorescent substrates at levels comparable with that of the control cells because these agents inhibit Pgp dependent drug efflux. In contrast, there was no efflux inhibition in the recombinant clone cells by any of these agents (Fig. 9). Similar results were obtained with the new recloned clone.

Photoaffinity labeling of the recombinant Pgp. Photoaffinity labeling experiments with [^{125}I] IAAP in plasma membrane preparations were performed to determine the drug-binding properties of the recombinant Pgp. *cis*-(*Z*)-Flupentixol, an antipsychotic drug, has been shown to be a potent agent for reversing Pgp-mediated drug resistance and an enhancer of Pgp photoaffinity labeling with IAAP (Dey *et al.*, 1997). Before labeling with IAAP, membranes from the wild-type *MDR*1-transfected cells, and from the recombinant clone cells were incubated with *cis*-(*Z*)-flupentixol for 3 min at 21°. As shown in Fig. 10, both the wild-type and the recombinant Pgp membranes were labeled with IAAP, but labeling of recombinant clone 3-4 was reduced several-fold compared with wild-type Pgp. Despite its relative inability to reverse colchicine resistance of the recombinant clone cells (Fig. 7), cyclosporin A competed for the [^{125}I]IAAP photoaffinity labeling of both the wild-type and the recombinant Pgp.

Discussion

Characterization of a novel chimeric Pgp. In this study, we have found that random recombination between human *MDR1* and mouse *mdr*1a, which changes five amino acids in the segment, including TM5 and TM6 and the adjacent extracellular loops in Pgp, decreases resistance to colchicine and taxol. This recombinant is also relatively insensitive to the ability of known Pgp modulators, such as verapamil, cyclosporin A, and PSC 833, to inhibit Pgp pump-

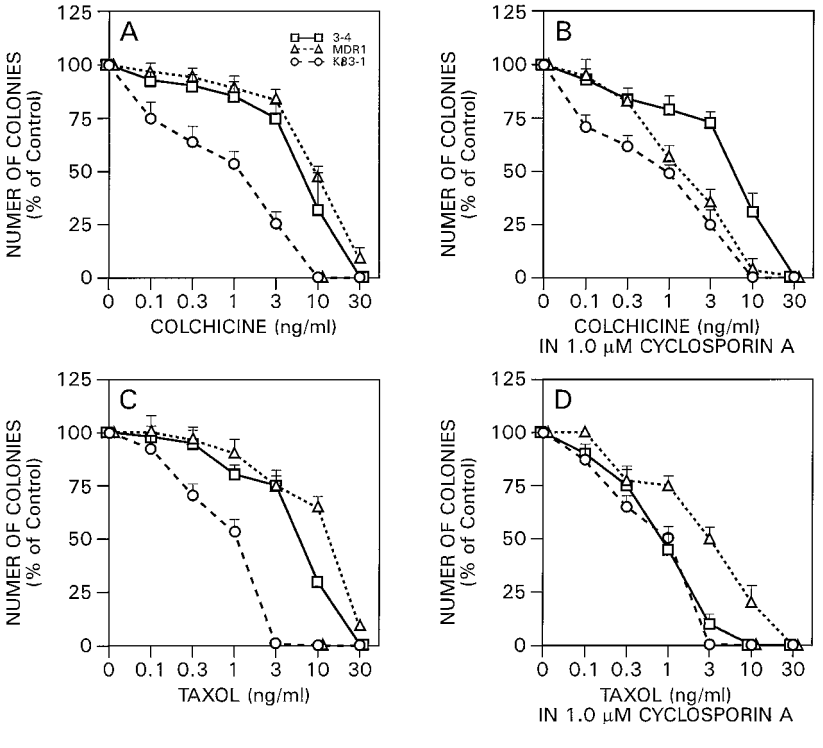

Fig. 7. Drug survival characteristics of the recombinant 3-4 clone cells, the wild type pHaMDR-DHFR cells, and the drug-sensitive KB-3-1 cells. Colony formation assays were performed as described in Materials and Methods to determine the drug sensitivity of the different cell lines. Drug survival was measured in increasing concentrations of colchicine (A), colchicine + 1 μM cyclosporin A (B), taxol (C), or taxol + 1 μM cyclosporin A (D). □, 3-4; △, *MDR*1; ○, KB-3-1. *Points*, average of three independent experiments.

ing of several fluorescent substrates and colchicine but not taxol. This new phenotype is unique to the recombinant and is not characteristic of either mouse *mdr*1a or human *MDR*1.

As reported previously, specific and distinguishable functional characteristics have been found between Pgps encoded by human *MDR*1 and mouse *mdr*1a with respect to drug resistance profiles and sensitivity to modulators. It has been shown that human *MDR*1 is more sensitive to modulators than its mouse counterparts (Tang-Wai *et al.*, 1995). This work identifies some of the amino acid residues responsible for differences in substrate and modulator specificity between human *MDR*1 and mouse *mdr*1a. All of these residues are highly conserved among Pgps. The major change among the five amino acid changes in *MDR*1 is Gly324Lys [(i.e.,

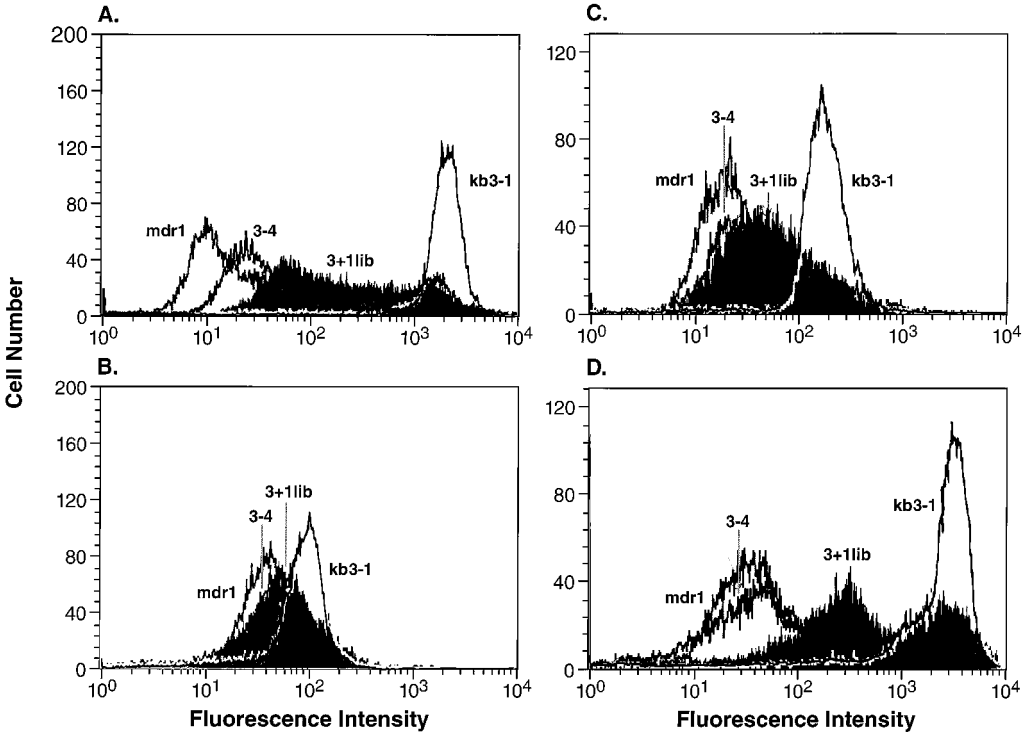

Fig. 8. Accumulation of fluorescent substrates in the recombinant 3-4 clone cells (*3-4*), the wild type pHaMDR-DHFR cells (*mdr1*), the mammalian 3+1 library (*3+1lib*), and the drug-sensitive parental KB-3-1 cells (*kb3-1*). Substrate accumulation was determined by FACS: Rhodamine (A), daunorubicin (B), bodipy-verapamil (C), and calcein AM (D).

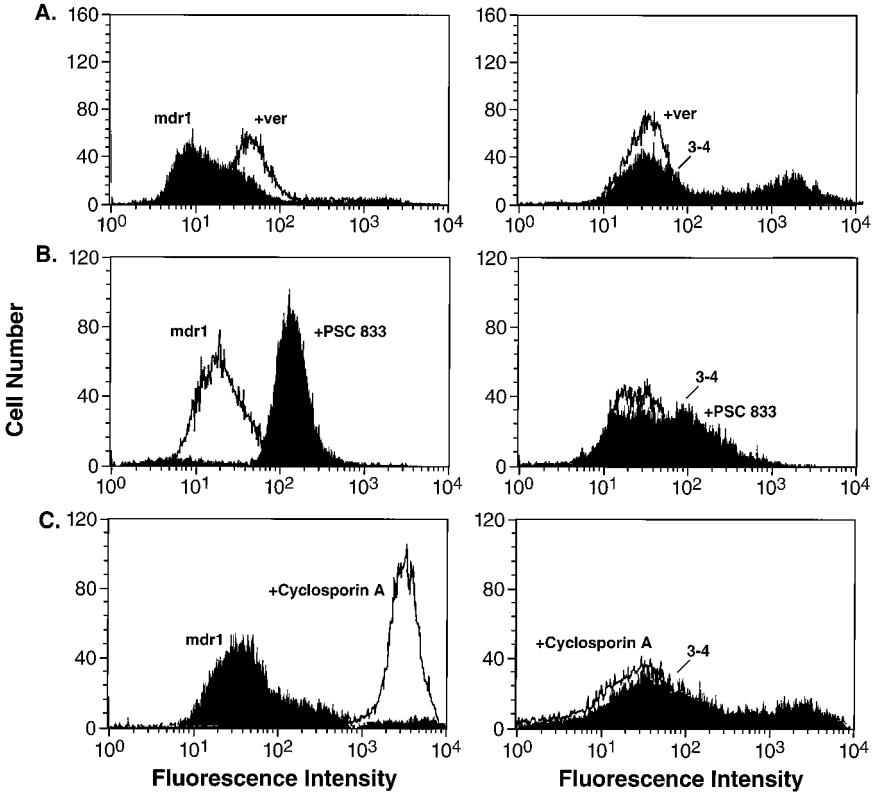

Fig. 9. Fluorescent substrate accumulation and the effect of reversing agents. A, Rhodamine 123 accumulation in the presence or absence of 20 μM verapamil in the wild-type pHaMDR-DHFR cells (*left*), or the recombinant 3-4 clone cells (*right*). B, Bodipy verapamil accumulation in the presence or absence of 2.5 μM PSC 833. C, Calcein AM accumulation in the presence or absence of 5 μM cyclosporin A.

exchanging a nonpolar amino acid (glycine) for a basic amino acid (lysine)].

Our study also demonstrates the differential effects of inhibitors on the chimeric and the wild-type transporters. In general, cyclosporin A is more effective in inhibiting transport by the chimeric transporter of taxol but not of colchicine, rhodamine 123, bodipy-verapamil, and daunorubicin, in contrast to the wild-type *MDR*1, which is reversed by cyclosporin A in the presence of all those drugs. It has been previously shown that inhibitors of *MDR*1 show differential effects on reversing resistance of different drugs depending on whether the Pgp mutant G185V or wild-type transporters are analyzed (Cardarelli *et al.*, 1995). This is not surprising, because it has been shown that Pgp-transported drugs differ from each other in steric interactions with Pgp (Boscoboinik *et al.*, 1990); also, there is evidence for the presence of more than one drug binding site on Pgp (Dey *et al.*, 1997). A study of the kinetics of interaction of substrates and inhibitors with Pgp suggests a model in which drugs and inhibitors interact with Pgp in either the inner aspect, the outer aspect, or both aspects of the plasma membrane bilayer (Stein *et al.*, 1994). Such a model also implies more than one site of interaction of substrates and inhibitors with the transporter. Our results demonstrated that cyclosporin A was able to compete for [^{125}I]IAAP binding but was unable to reverse colchicine resistance of the 3-4 clone; the existence of different sites of interaction of colchicine and [^{125}I]IAAP can explain those results. The data presented in this article together with the previous results mentioned above are consistent with this model.

Recent studies also indicate that mutations in TM11 affected Pgp interaction with known modulators, such as verapamil and progesterone (Kajiji *et al.*, 1994). Another mutation in the TM6 region of Pgp, consisting of a single codon deletion (Phe335), is resistant to modulation of Pgp by cyclosporins (Chen *et al.*, 1997; Hrycyna C, Pastan I, Gottesman MM, unpublished observations). Furthermore, it has been shown that an Ala339Pro substitution lowered the sensitivity to cyclosporin A in hamster Pgp for some drugs (Ma *et al.*, 1997).

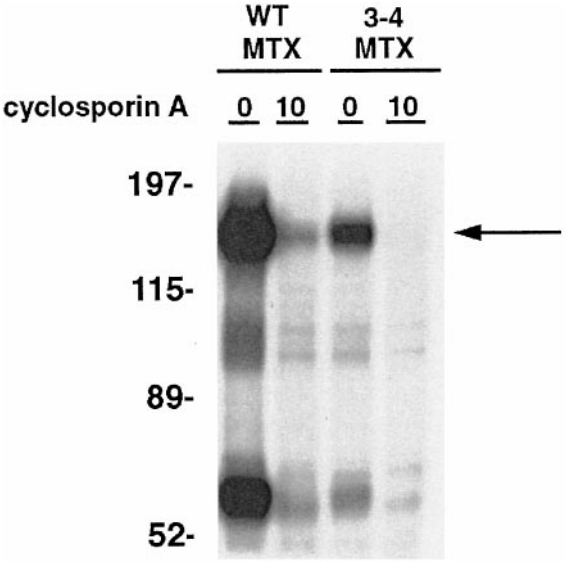

Fig. 10. Effect of 10 μM cyclosporin A on [^{125}I]IAAP labeling of the wild-type and recombinant Pgp cell membranes in the presence of *cis*-(*Z*)-flupentixol.

Despite high resistance to reversing agents, the recombinant clone 3-4 cells express less Pgp than the wild-type cells. Similar results have been shown recently with a human myeloma cell line that is resistant to inhibitors of Pgp (Abbaszadegan *et al.*, 1996). The recombinant clone cells stained less well with mAb MRK16 and better with UIC2 fluorescence intensity. These results suggest the existence of different Pgp conformers with different epitope exposure. Mouse mAb UIC2 is specific for the extracellular moiety of the human *MDR*1 Pgp (Mechetner and Roninson, 1992). The addition of UIC2 to tissue culture media decreases the activity of Pgp toward all the tested Pgp transport substrates (Mechetner and Roninson, 1992), which suggests that it may interact directly with a drug interaction site on Pgp. The conformational epitope that is recognized by UIC2 is distinct from the epitopes of the other monoclonal antibodies; only UIC2 fails to react with a mutant Pgp that carries a deletion in the first extracellular loop (Schinkel *et al.*, 1993). It has been shown recently that changes in UIC2 reactivity can be used as a sensitive assay to analyze conformational transitions associated with Pgp function (Mechetner *et al.*, 1997). Our results are consistent with the presence of two different conformers of chimeric Pgp, with each conformer being the only one expressed on different cell populations.

The introduction of *MDR*1 expressing vectors into the bone marrow of patients undergoing chemotherapy to allow dose intensification has been proposed. Mutant transporters like the chimeric protein reported here, which is resistant to MDR modulating agents, could be used in gene therapy to protect bone marrow, because it would be possible to reverse drug resistance of *MDR*1-expressing cancer without reversing resistance of the chimeric Pgp protecting the bone marrow.

Use of the bicistronic vector pHaMDR-DHFR. The use of bicistronic retroviral vectors containing a therapeutic gene, an intercistronic IRES element, and a selectable *MDR*1 gene has been developed for the gene therapy of several disorders (Aran *et al.*, 1994; Metz *et al.*, 1996). Our present effort uses the bicistronic vector as a stable expression system for Pgp. Our results demonstrate that the use of the bicistronic vector for stable transfection by methotrexate selection guarantees that the majority of the methotrexate resistance clones also express Pgp. As shown in Fig. 4, a library of chimeric Pgp molecules can be selected in methotrexate without concern about pleiotropic cellular effects caused by selection in MDR drugs.

Implications of MDR shuffling. Stemmer (1994b) has demonstrated that by applying repeated cycles of DNA shuffling and selection, he can create a library of chimeras from a pair of homologous genes from different species with improved activity and a new function. Our results demonstrate that the shuffling between transmembrane domains 5 and 6 of human *MDR*1 and mouse *mdr*1a generates a new phenotype that could be useful in selecting for MDR mutants with altered drug resistance and differential reversing effects as a potential tool for cancer gene therapy. Despite the large diversity in the bacterial library, after selection of the mammalian library with MDR drugs, the majority of the clones had the wild-type *MDR*1 sequence, which suggests that this region cannot tolerate most random recombination events. It

should be useful in the future to shuffle larger regions from human *MDR*1 and mouse *mdr*1a that are less conserved to select for more MDR mutants with an improved drug resistance profile.

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