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Functional Expression of P-Glycoprotein in *Saccharomyces cerevisiae* Confers Cellular Resistance to the Immunosuppressive and Antifungal Agent FK520

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We have recently reported that expression in yeast cells of P-glycoprotein (P-gp) encoded by the mouse multidrug resistance *mdr3* gene (Mdr3) can complement a null *ste6* mutation (M. Raymond, P. Gros, M. Whiteway, and D. Y. Thomas, Science 256:232–234, 1992). Here we show that Mdr3 behaves as a fully functional drug transporter in this heterologous expression system. Photolabelling experiments indicate that Mdr3 synthesized in yeast cells binds the drug analog [^{125}I]iodoaryl azidoprazosin, this binding being competed for by vinblastine and tetraphenylphosphonium bromide, two known multidrug resistance drugs. Spheroplasts expressing wild-type Mdr3 (Ser-939) exhibit an ATP-dependent and verapamil-sensitive decreased accumulation of [^{3}H]vinblastine as compared with spheroplasts expressing a mutant form of Mdr3 with impaired transport activity (Phe-939). Expression of Mdr3 in yeast cells can confer resistance to growth inhibition by the antifungal and immunosuppressive agent FK520, suggesting that this compound is a substrate for P-gp in yeast cells. Replacement of Ser-939 in Mdr3 by a series of amino acid substitutions is shown to modulate both the level of cellular resistance to FK520 and the mating efficiency of yeast *mdr3* transformants. The effects of these mutations on the function of Mdr3 in yeast cells are similar to those observed in mammalian cells with respect to drug resistance and transport, indicating that transport of a-factor and FK520 in yeast cells is mechanistically similar to drug transport in mammalian cells. The ability of P-gp to confer cellular resistance to FK520 in yeast cells establishes a dominant phenotype that can be assayed for the positive selection of intragenic revertants of P-gp inactive mutants, an important tool for the structure-function analysis of mammalian P-gp in yeast cells.

The development of multidrug resistance (MDR) is a major obstacle in cancer treatment by chemotherapy. Typical MDR displayed by tumor cells in vivo and cultured cells in vitro is characterized by their cross-resistance to a broad spectrum of structurally and functionally unrelated natural product cytotoxic agents, including vinca alkaloids, anthracyclines, and actinomycin D (13). In cultured cells, the emergence of MDR is caused by the overexpression of a small group of highly conserved membrane phosphoglycoproteins named P-glycoproteins (P-gps) (13). P-gps are encoded by a small family of genes, designated *mdr* or *pgp*, for which full-length cDNA clones have been isolated (8, 10, 14, 18, 20, 53). Analysis of the predicted amino acid sequence indicates that P-gps are integral membrane proteins containing 12 predicted transmembrane (TM) domains and two intracellular loops with consensus ATP-binding motifs (8, 18). P-gps have been shown to bind ATP and drug analogs (3) and to have ATPase activity (21), suggesting that they function in resistant cells as energy-dependent efflux pumps of cytotoxic drugs.

A number of lipophilic compounds have been identified as capable of reversing the MDR phenotype in drug-resistant cells. These P-gp modulators, which include calcium channel blockers such as verapamil and azidopine, calmodulin inhibitors, indole alkaloids, progesterone, and cyclosporins, are capable of increasing the intracellular concentration and cytotoxicity of chemotherapeutic agents in MDR cells (2, 57). Although the molecular mechanism of MDR reversal by P-gp modulators is not yet fully understood, they can be classified into two types: those which can bind to but are not transported by P-gp (e.g., progesterone) (52) and those which not only bind to but also are efficiently transported by P-gp (e.g., verapamil [VRP]) (56). The use of a number of P-gp modulators in cancer treatment is currently under clinical investigation (9, 47).

Cyclosporin A (CsA), FK506, and rapamycin are natural products with potent immunosuppressive and antifungal activities (24). These compounds suppress the immune system by blocking T-cell activation, by interfering with signal transduction (24, 43). While CsA is widely used clinically to prevent the rejection of transplanted organs and bone marrow, clinical trials with FK506 are in progress (49). These compounds are also attracting attention as effective MDR modulators. CsA has been shown to efficiently reverse vincristine and doxorubicin resistance in MDR-cultured cell lines, shares a common binding site on P-gp with vinca alkaloids and VRP, and is being tested clinically for its P-gp-inhibitory function (51). Although structurally distinct from CsA, FK506 and rapamycin also behave as P-gp modulators, increasing intracellular accumulation of adriamycin and daunomycin in MDR cells and inhibiting binding of the drug analog [^{125}I]iodoaryl azidoprazosin ([^{125}I]IAAP) to P-gp (1, 36). Recently, it was reported that human P-gp can transport CsA and FK506 (41). These findings have

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revealed a previously unidentified potential clinical application for these compounds.

P-gps are members of a superfamily of evolutionarily conserved transport proteins termed the ABC (ATP-binding cassette) superfamily of proteins, involved in the transmembrane transport of structurally heterogeneous substrates (26). The best-studied eukaryotic members of the ABC superfamily include the *pfmdr1* gene product of *Plasmodium falciparum*, associated with chloroquine resistance (15), the cystic fibrosis transmembrane conductance regulator protein, mutations in which lead to cystic fibrosis in humans (39), and the yeast *Saccharomyces cerevisiae STE6* gene product (30, 34), which mediates the export of **a**-factor pheromone, a farnesylated dodecapeptide required for mating (35). Yeast Ste6 and mammalian P-gps are over 50% homologous and have very similar predicted secondary structures and proposed membrane topologies (30, 34). Recently, we have shown that the structural homology between the yeast and mammalian transporters translates into functional homology, since a cDNA for the mouse *mdr3* gene can complement a null allele in a *ste6* deletion strain, by restoring the ability of the cells to export **a**-factor and to mate (38).

We have now analyzed the phenotypic consequences of *mdr3* expression in yeast cells on drug resistance and drug transport and show that in this heterologous system, P-gp behaves as a fully functional drug transporter. P-gp expressed in yeast cells binds the photoactivable drug analog [^{125}I]IAAP, transports vinblastine (VBL) in an ATP-dependent and VRP-sensitive manner, and confers resistance to growth inhibition by the immunosuppressive and antifungal agent FK520. Our results demonstrate that yeast provides an excellent system with which to genetically dissect the mechanism of action of P-gp, with respect to both peptide and drug transport.

MATERIALS AND METHODS

Yeast strains and culture media. *S. cerevisiae* JPY201, used throughout this study, was a gift from A. Varshavsky (California Institute of Technology, Pasadena) and has been previously described (34). Its relevant genotype is *MAT***a** *ste6*Δ *ura3*. Mating tester strain DC17 (*MAT*α *his1*) was a gift from M. Whiteway (Biotechnology Research Institute, Montreal, Quebec, Canada). Standard rich medium (YPD), synthetic medium lacking uracil (SD-Ura), and minimal medium were prepared as described elsewhere (45).

Construction of *mdr3* yeast expression plasmids. Plasmid pVT101-U (pVT) is a high-copy-number plasmid used for expression in yeast cells of the mouse *mdr3* gene from the alcohol dehydrogenase promoter (54). Plasmids pVT-MDR3S and pVT-MDR3F, which contain the mouse wild-type *mdr3* gene and a mutant *mdr3* derivative with a Ser-939-to-Phe substitution, respectively, have been described elsewhere (38). To allow the construction of other expression plasmids with *mdr3* bearing different residues at amino acid position 939, two modifications of plasmid pVT were performed. First, a single *Nru*I site in the vector backbone was destroyed by digesting pVT with *Nru*I and inserting a *Kpn*I linker, yielding plasmid pVTΔ*Nru*I. Second, a portion of the multiple cloning site was deleted by digesting pVTΔ*Nru*I with *Pst*I and *Bam*HI, blunt ending with T4 DNA polymerase, and religating to create plasmid pVTΔ*Nru*IΔMCS. A novel restriction site (*Nru*I at position 2726) was introduced in *mdr3* by site-directed mutagenesis, without alteration of the corresponding coding sequence. This modified *mdr3* sequence was excised from plasmid pGEM-7Zf as a 4.1-kb fragment by digestion with *Sph*I and *Cla*I, blunt ended with T4 DNA polymerase, and cloned into the *Pvu*II site of pVTΔ*Nru*IΔMCS to create pVT-MDR3S(3.4). Replacement of Ser-939 by Ala, Cys, Thr, Tyr, Trp, or Asp was carried out by standard site-directed mutagenesis using single-stranded M13 DNA templates as described elsewhere (12). Reconstruction of these mutations into pVT-MDR3S(3.4) was performed by exchanging a wild-type *Nru*I-*Pst*I 785-bp fragment that overlaps amino acid position 939 by the corresponding mutagenized fragment, creating plasmids pVT-MDR3A, pVT-MDR3C, pVT-MDR3T, pVT-MDR3Y, pVT-MDR3W, and pVT-MDR3D, respectively. Presence of the desired mutation in the pVT-MDR3 plasmids was verified by DNA sequencing (42), and integrity of restriction enzyme sites used for cloning was verified by fragmentation with the corresponding endonucleases. JPY201 cells were transformed with the various pVT derivatives by the lithium acetate method (27). Yeast cells were grown routinely at 30°C.

Preparation of membranes. Exponentially growing cells in selective SD-Ura medium (A_{600} = 1.0 to 2.0) were harvested by centrifugation and rinsed twice in TNE (10 mM Tris [pH 7.0], 150 mM NaCl, 1 mM EDTA). Cells resuspended in TNE were disrupted by two successive passages through a French pressure cell at 20,000 lb/in^2. Unlysed cells were removed by centrifugation (1,000 × *g*, 10 min), and total membranes were concentrated by ultracentrifugation (100,000 × *g*, 30 min). Membranes were then resuspended at 5 mg/ml in TNE containing 30% glycerol and stored frozen at −80°C. All experimental steps were carried out at 4°C in the presence of protease inhibitors (phenylmethylsulfonyl fluoride at 1 mM; leupeptin, pepstatin A, and aprotinin, each at 5 μg/ml).

Detection of P-gp by immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed by standard protocols (32). Membranes were mixed with Laemmli sample buffer, incubated at room temperature for 20 min, and loaded on an SDS–7.5% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, and P-gps were identified by using the mouse monoclonal anti-P-gp antibody C219 (Centocor Corp., Philadelphia, Pa.) diluted at 1 μg/ml. Immune complexes were revealed by incubation with goat anti-mouse immunoglobulin G antibodies coupled to alkaline phosphatase (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). The blot was developed with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride substrates as recommended by the manufacturer (Bethesda Research Laboratories Life Technology, Inc., Burlington, Ontario, Canada).

Photoaffinity labelling. Membranes (25 to 100 μg) from yeast transformants were incubated with the drug analog [^{125}I]IAAP (2,200 Ci/mmol; Du Pont-New England Nuclear) at a final concentration of 60 nM in a reaction buffer (50 mM Tris [pH 7.5], protease inhibitors) for 1 h at 25°C in the dark. Membrane fractions were mixed with an appropriate amount of membranes from pVT transformants to reach a final amount of 100 μg in each tube. For competition experiments, membranes (80 μg) were similarly incubated with [^{125}I]IAAP but in the absence or presence of a 100-, 1,000-, or 10,000-fold molar excess of tetraphenylphosphonium bromide (TPP; Aldrich Chemical Co., Milwaukee, Wis.) or a 10-, 100-, or 1,000-fold molar excess of VBL (Sigma, St. Louis, Mo.). Membranes were then irradiated with UV for 5 min at 0°C as previously described (28) and immunoprecip-

itated with anti-P-gp monoclonal antibody C219 (10 μg/ml). The immunoprecipitation products were electrophoresed on SDS–7.5% polyacrylamide gels. Gels were fixed, dried, and exposed to X-ray films with one intensifying screen for 7 days at −80°C.

VBL accumulation in spheroplasts. Exponentially growing cells in selective SD-Ura medium (A_{600} = 1.0 to 2.0) were harvested by centrifugation and rinsed twice with water and once with SM1 buffer (1.2 M sorbitol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]/HCl [pH 7.0], 0.2 mM $CaCl_2$). Cells were resuspended in SM1 buffer and converted to spheroplasts during a 45-min incubation at 37°C in presence of zymolyase 100-T (ICN Biomedicals Canada Ltd., Mississauga, Ontario, Canada) at 1 mg/g (wet weight) of cells. The spheroplasts were washed twice and resuspended in SM1 buffer at 15 to 20 mg/ml. An equal volume of SM2 (0.85 mM sorbitol, 20 mM HEPES/HCl [pH 7.0], 0.2 mM $CaCl_2$, 10 mM $MgSO_4$) containing either 0.3 M glucose or 2-deoxyglucose was added. Cells were incubated 60 min at 25°C. Transport was initiated by addition of VBL to a final concentration of 0.1 μM plus 0.25 μCi of [^{3}H]VBL (Amersham, Oakville, Ontario, Canada) per ml followed by incubation at 30°C. In certain experiments, VRP or FK506 was added (final concentration of 20 or 3.3 μM, respectively) prior to initiation of transport. Aliquots of 50 μl were removed at the indicated time intervals, diluted into 2 ml of ice-cold SM1 buffer, and filtered through a glass fiber filter (Gelman type A/E) presoaked in SM1 supplemented with 10 μM VBL. Filters were washed twice, and radioactivity was determined by scintillation counting. Protein content was determined by the Bradford method (Bio-Rad) in the presence of 5% formic acid, using immunoglobulin G as a standard.

Quantitative mating assays. Mating efficiency was quantified by filter assays (48). Overnight cultures of JPY201 transformants in SD-Ura medium were diluted (A_{600} = 0.7) and allowed to grow for 3 h at 30°C, after which time aliquots were taken for viable cell counts. Aliquots of the cultures were then mixed with 0.5 volume of a saturated overnight culture of tester strain DC17, and filtered on a nitrocellulose filter (0.45 μm); the filters were incubated onto YPD agar plates for 4 h at 30°C to allow mating. Cells were then eluted from the filters with sterile water, and aliquots were plated onto minimal synthetic dextrose plates for the selection of diploids. Mating efficiency was calculated as the ratio of the number of diploids formed on selective medium to the number of haploid JPY201 cells introduced in the assay.

Antifungal antibiotics. FK520 (L-683,590) was kindly provided by Merck Sharp & Dohme Research Laboratories (Rahway, N.J.). FK520 is an FK506 analog containing an ethyl substituent replacing the allyl moiety at position C-21 of the macrolide ring (5). Stock solutions of the antibiotics were prepared at 10 mg/ml in methanol for liquid assays (5) or ethanol–10% Tween 20 as described previously (25) for plate assays.

Growth inhibition assays. FK520 resistance was tested by spotting portions (3 μl) of serially (10-fold) diluted overnight yeast cultures (grown in SD-Ura) onto YPD plates containing 100 μg of FK520 per ml. Control plates lacking FK520 contained ethanol and Tween 20 at the same concentrations as YPD plates containing FK520. Growth inhibition assays were routinely performed in YPD medium but could also be performed in selective medium (SD-Ura) without affecting the results. FK520 resistance was quantified by conventional microtitration techniques essentially as described previously (5). Assays were performed in flat-bottom 96-well microtiter plates (Corning, New York, N.Y.), with wells containing equal volumes (50 μl) of YPD medium with or without FK520. Yeast transformants grown to stationary phase in SD-Ura were rinsed in YPD medium and diluted in YPD (A_{590} = 0.5; 7 × 10^6 cells per ml). Equal volumes of these diluted cultures (50 μl) were added to each well and incubated at 30°C for 10 to 12 h. Cell growth was measured optically at 590 nm at approximately 2-h intervals (growth rate) or after 11 h of incubation (dose dependence) on a microtiter plate reader (Dynatech MR5000; Baxter Diagnostics Corp., Canlab Division). For controls, cell growth was monitored in the absence of FK520 in the presence of solvent. Solvent had no effect on growth of the cells at the concentration used in the assay.

Metabolic labelling and immunoprecipitation. Yeast transformants were grown to mid-logarithmic phase (A_{600} = 0.6 to 0.8) in SD-Ura. For labelling, 5 OD_{600} units of cells was harvested and incubated in a total volume of 0.5 ml of minimal medium supplemented with appropriate nutrients and containing 150 μCi of Tran^{35}S-label (1,079 Ci/mmol; ICN Biomedicals) for 60 min at 30°C with constant shaking. Labelled cells were harvested by centrifugation (1,000 × *g*, 10 min), washed once with H_2O, and resuspended in 100 μl of 1% SDS. This and all subsequent steps were performed in the presence of protease inhibitors. Cells were disrupted with glass beads by being vortexed six times with 30-s bursts and then heated for 10 min at 50°C. Cell extract was removed from the beads by centrifugation after dilution with 0.9 ml of immunoprecipitation buffer (final concentrations in the sample: 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, and 50 mM Tris [pH 7.5]). For immunoprecipitation, 0.5 ml of this cell extract was incubated overnight at 4°C with the polyclonal antibody anti-Mdr3 2037 (6) at a 1:100 dilution. Immune complexes were recovered by incubation for 2 h at 4°C with protein A-Sepharose beads, resuspended in Laemmli sample buffer, and electrophoresed on an SDS–7.5% polyacrylamide gel. Fluorography of the gel was performed by using a commercially available amplifier (En^{3}Hance; Du Pont) as recommended by the manufacturer. The gel was dried and exposed for 24 h at −80°C.

RESULTS

Expression of mouse Mdr3 in yeast cells. Plasmids for expression of wild-type and mutant mouse *mdr3* genes in the yeast *S. cerevisiae* have been described previously (38). Briefly, a full-length cDNA for the mouse wild-type *mdr3* gene was cloned into the yeast expression vector pVT, to produce plasmid pVT-MDR3S. pVT contains the 2μm origin of replication for high copy number, the *URA3* gene as a selectable marker, and the strong yeast alcohol dehydrogenase promoter (54). A mutant *mdr3* cDNA, encoding a Ser-to-Phe substitution at amino acid position 939, was also cloned into pVT, yielding plasmid pVT-MDR3F. The Ser-939-to-Phe substitution within predicted TM domain 11 of Mdr3 has been shown previously to decrease the activity and to modify the substrate specificity of this transporter for drugs of the MDR spectrum when overexpressed in cultured CHO cells (19, 28). Plasmids pVT-MDR3S, pVT-MDR3F, and pVT were introduced into *S. cerevisiae* JPY201 (*MAT***a** *ste6*Δ::*HIS3*) (34). Total membranes were prepared from the different transformants and analyzed by immunoblotting for the presence of immunoreactive wild-type and mutant Mdr3 proteins, using the mouse anti-P-gp monoclonal antibody C219 (Fig. 1A). This antibody recognized P-gps with an apparent molecular mass of 125 kDa in membranes from

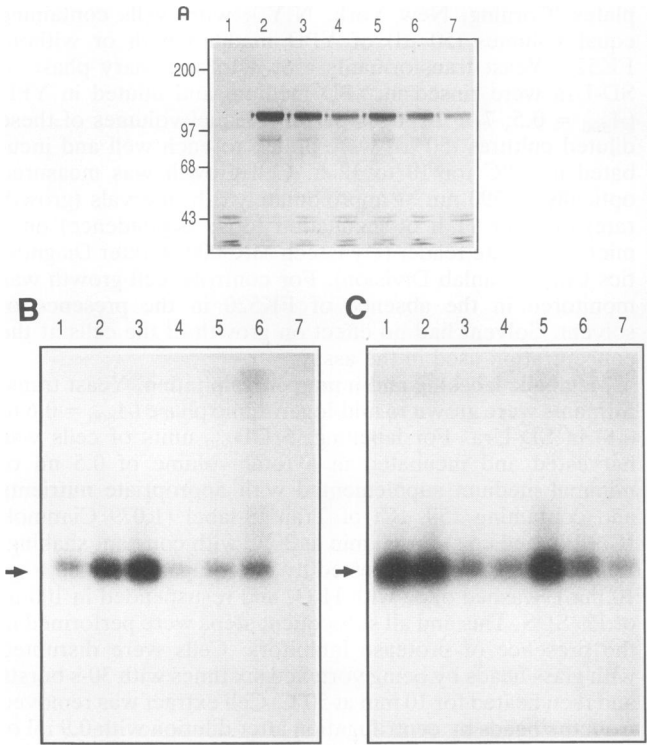

FIG. 1. Photoaffinity labelling of Mdr3 expressed in yeast cells. (A) Membrane proteins from pVT (lane 1), pVT-MDR3S (lanes 2 to 4), and pVT-MDR3F (lanes 5 to 7) transformants were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed with anti-P-gp monoclonal antibody C219. Lanes 1, 2, and 5, 25 μg; lanes 3 and 6, 12.5 μg; lanes 4 and 7, 6.25 μg. The positions of molecular mass standards (in kilodaltons) are indicated at the left. (B) Photoaffinity labelling with [^{125}I]IAAP. Membranes from pVT-MDR3S (lanes 1 to 3), pVT-MDR3F (lanes 4 to 6), and pVT (lane 7) transformants were incubated with [^{125}I]IAAP (60 nM) and cross-linked with UV. Lanes 1 and 4, 25 μg; lanes 2 and 5, 50 μg; lanes 3, 6, and 7, 100 μg. (C) Competition of [^{125}I]IAAP binding to membranes from pVT-MDR3S transformants. Membranes (80 μg) were incubated with [^{125}I]IAAP (60 nM) in the absence (lane 1) or presence of 100-, 1,000-, and 10,000-fold molar excesses of TPP (lanes 2 to 4, respectively) or 10-, 100-, and 1,000-fold molar excesses of VBL (lanes 5 to 7, respectively). The arrow indicates the position of the photolabelled Mdr3 protein expressed in yeast cells.

cells transformed with pVT-MDR3S (lanes 2 to 4) or pVT-MDR3F (lanes 5 to 7) but not in membranes from pVT control cells (lane 1). The amounts of wild-type Mdr3S and mutant Mdr3F proteins expressed in these membranes are similar and do not vary more than twofold (compare lanes 4 and 6). These results show that wild-type and mutant P-gps are expressed at high levels in the membrane fraction of transformed yeast cells.

Photolabelling of Mdr3 expressed in *S. cerevisiae* with [^{125}I]IAAP. Previous studies have demonstrated that P-gp expressed in MDR cell lines binds photoactivable drug analogs such as IAAP and azidopine and that this binding can be competed for by MDR drugs (3). Recent studies from our group have shown that binding of drug analogs to P-gp is impaired by the Ser-939-to-Phe mutation in TM domain 11 (28). We wished to determine whether Mdr3S and Mdr3F expressed in yeast cells could bind [^{125}I]IAAP in cross-linking experiments. Membranes from yeast cells transformed with plasmid pVT, pVT-MDR3S, or pVT-MDR3F were incubated with [^{125}I]IAAP in the absence or presence of competitive inhibitors and cross-linked under UV, and labelled P-gps were recovered by immunoprecipitation with monoclonal antibody C219. Figure 1B shows that wild-type Mdr3S expressed in yeast cells can bind the drug analog IAAP (lanes 1 to 3) and that binding to the Mdr3F mutant is significantly reduced (lanes 4 to 6). No labelled product was detected in control membranes (lane 7). TPP and VBL, two P-gp substrates in mammalian cells, significantly inhibited IAAP binding to Mdr3S. At 1,000-fold molar excess of cold ligand, photolabelling of Mdr3S was inhibited by 64 and 88% for TPP and VBL, respectively (Fig. 1C, lanes 3 and 7), in agreement with previously reported values for similar experiments carried out on P-gp expressed in mammalian or *Escherichia coli* cells (4, 17). These results indicate that P-gp expressed in yeast membranes is capable of binding drug analogs and that the characteristics of drug binding of wild-type and mutant P-gps established in mammalian cells are retained when these proteins are expressed in membranes of yeast cells.

VBL accumulation in spheroplasts expressing Mdr3. Mammalian cells overexpressing P-gp exhibit a reduced intracellular accumulation of a variety of chemotherapeutic drugs, a phenotype dependent on the presence of cellular ATP (13). To evaluate the phenotypic consequences of P-gp expression in yeast cells, we measured the accumulation of [^{3}H]VBL in spheroplasts prepared from yeast cells transformed with plasmid pVT-MDR3S, pVT-MDR3F, or pVT (Fig. 2). Spheroplasts were incubated with [^{3}H]VBL under either normal metabolic conditions (Fig. 2A, +ATP) or conditions of ATP depletion (Fig. 2A, −ATP), and drug accumulation was monitored over 60 min. Under normal metabolic conditions, Mdr3S spheroplasts accumulated little if any [^{3}H]VBL, while Mdr3F spheroplasts accumulated the drug at levels undistinguishable from those observed in control spheroplasts (Fig. 2A, +ATP). The maximum accumulation of [^{3}H]VBL by control or Mdr3F spheroplasts was almost four times that determined for spheroplasts expressing wild-type Mdr3S. The difference in [^{3}H]VBL accumulation between Mdr3S and control spheroplasts was ATP dependent and completely abolished by incubation of the spheroplasts with deoxyglucose prior to initiation of the transport reaction (Fig. 2A, −ATP). Under conditions of ATP depletion, intracellular [^{3}H]VBL accumulation was similar in control and Mdr3-expressing spheroplasts, seven times (Mdr3S) and two times (Mdr3F, pVT) higher than that measured under normal conditions.

The ability of VRP, a known P-gp modulator, to affect [^{3}H]VBL accumulation in Mdr3-expressing spheroplasts was tested (Fig. 2B). Addition of VRP to the transport reaction causes a dramatic increase in [^{3}H]VBL accumulation into Mdr3S spheroplasts, with intracellular levels of [^{3}H]VBL similar to those measured in control pVT and Mdr3F spheroplasts under the same experimental conditions (Fig. 2B, +VRP). VRP did not enhance [^{3}H]VBL accumulation in Mdr3F and pVT control spheroplasts. Therefore, expression of Mdr3S in yeast spheroplasts causes the appearance of an ATP-dependent, VRP-sensitive reduction in drug accumulation, demonstrating functional P-gp expression in yeast cells.

Cellular resistance of Mdr3-expressing yeast cells to growth inhibition by the antifungal agent FK520. A fundamental property of mammalian cells overexpressing P-gp is that they exhibit extensive cross-resistance to a series of cytotoxic drugs (13). Since Mdr3 expressed in yeast cells is capable of drug binding and transport, it was of interest to

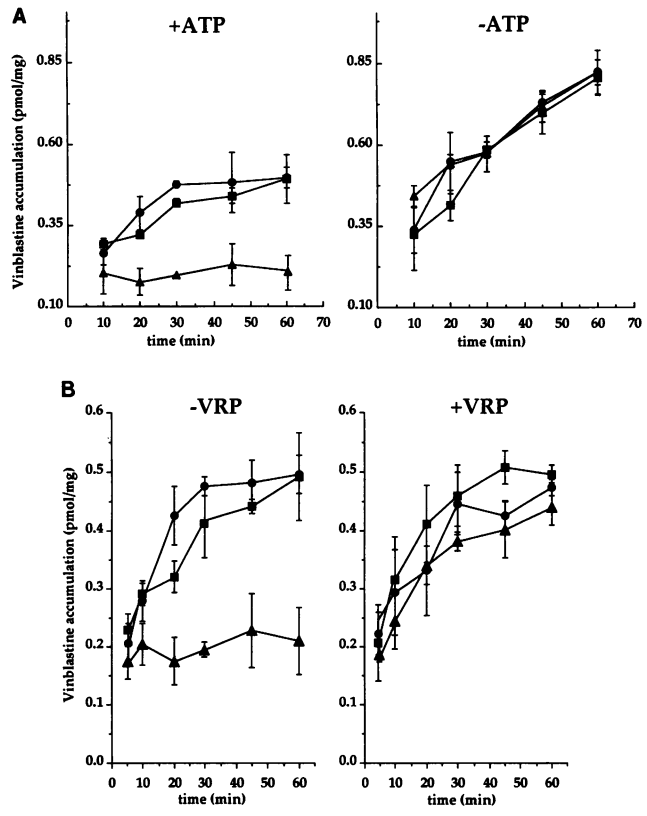

FIG. 2. VBL accumulation in spheroplasts. Yeast cells transformed with plasmid pVT (circles), pVT-MDR3S (triangles), or pVT-MDR3F (squares) were converted to spheroplasts and incubated for 1 h at 25°C in the presence of 0.3 M glucose (A, +ATP; B) or 2-deoxyglucose to achieve ATP depletion (A, −ATP). Transport was initiated by the addition of [^{3}H]VBL at 0.2 μCi/ml. Aliquots were removed at the indicated time points, and cell-associated radioactivity was determined by rapid filtration and scintillation counting. (B) In a competition experiment, VRP (20 μM) was added prior to the addition of VBL. Results are expressed as picomoles of VBL per milligram of total proteins. The data represent duplicate determinations of three independent experiments.

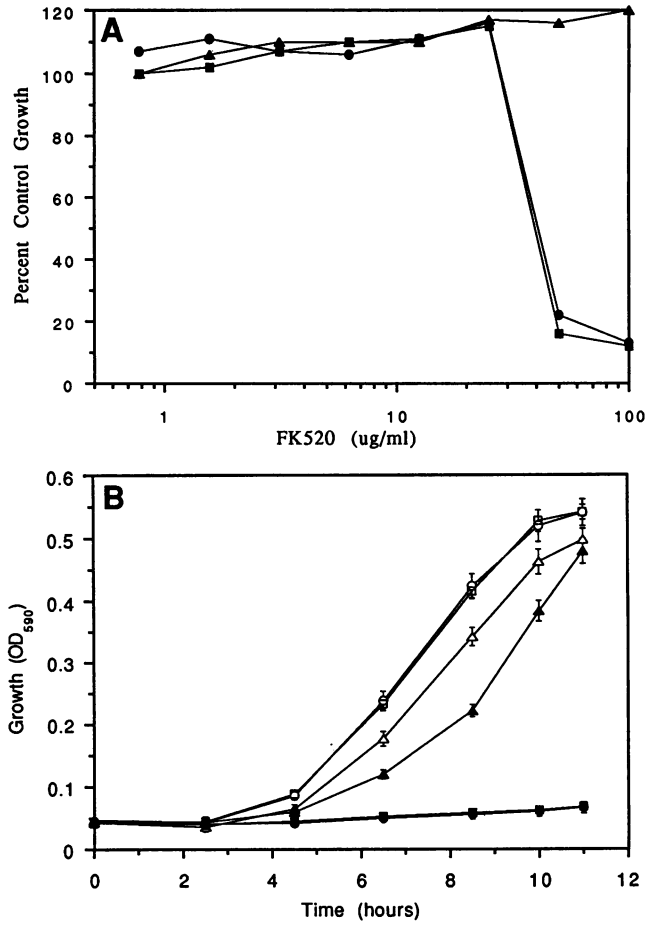

FIG. 3. Effects of FK520 on growth of Mdr3S- and Mdr3F-expressing yeast cells. The antifungal effects of FK520 on growth of yeast cells transformed with plasmid pVT (circles), pVT-MDR3S (triangles), or pVT-MDR3F (squares) were determined in liquid culture assay. (A) Yeast transformants were grown for 11 h in increasing concentrations of FK520, and growth was determined by optical density. Results are presented as the percentage of control growth for each transformant in the same medium lacking FK520. Values reported represent averages of two experiments. (B) Growth of yeast transformants was determined in YPD medium in the absence of FK520 (open symbols) and in the presence of FK520 at 100 μg/ml (filled symbols). Values reported represent means ± standard deviations of three experiments performed in duplicate. OD_{590}, optical density at 590 nm.

determine whether it could also confer drug resistance in yeast cells at the cellular level, as measured in cytotoxicity assays. Unfortunately, these efforts were unsuccessful since *S. cerevisiae* cells proved to be intrinsically resistant to common MDR drugs, including colchicine, VBL, adriamycin, and actinomycin D (data not shown). Recent reports have shown that the immunosuppressive agent FK506 is capable of functioning as an MDR reversal agent and substrate for P-gp (1, 36, 41). The observation that FK506 and structural analogs also possess antifungal activity (5, 25) prompted us to investigate the interesting possibility that P-gp could confer cellular resistance in yeast cells to growth inhibition by this class of compounds.

For this purpose, a growth inhibition assay in liquid cultures was used to determine the level of sensitivity of JPY201 transformants to FK520, an FK506 analog with similar immunosuppressive and antifungal properties (5). Figure 3A demonstrates that FK520 inhibits growth of pVT and Mdr3F transformants in a dose-dependent manner, exhibiting a 50% inhibitory concentration of approximately 40 μg/ml. In contrast, Mdr3S transformants were totally resistant to concentrations of FK520 as high as 100 μg/ml. It was not possible to determine the 50% inhibitory concentration for Mdr3S transformants in this type of assay, since 100 μg/ml represents the limit of solubility for FK520 in YPD culture medium. The effect of FK520 (at 100 μg/ml) on the growth rate of yeast transformants was also monitored over a period of 11 h in liquid cultures. Figure 3B shows that growth of pVT and Mdr3F cells was completely inhibited by FK520 at that concentration. However, growth of Mdr3S cells was only minimally affected by FK520, reaching cellular densities similar to those of Mdr3S cells grown in the absence of FK520 after an 11-h incubation. Taken together, these results demonstrate that functional expression of P-gp encoded by *mdr3* in yeast cells is sufficient to confer resistance to growth inhibition by the immunosuppressive and

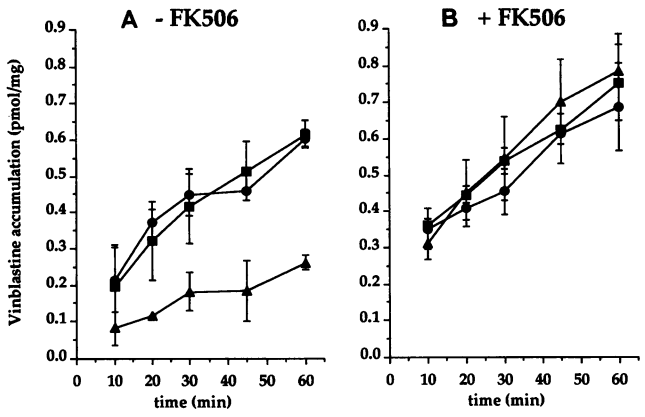

FIG. 4. Modulatory effect of FK506 on VBL accumulation in spheroplasts. Yeast cells transformed with plasmid pVT (circles), pVT-MDR3S (triangles), or pVT-MDR3F (squares) were converted to spheroplasts, and [^{3}H]VBL accumulation was measured as described in the legend to Fig. 2. The modulatory effect of FK506 on [^{3}H]VBL accumulation was tested by adding FK506 (3.3 μM) prior to initiation of [^{3}H]VBL transport. The data shown represent means and standard errors of two experiments carried out in duplicate.

antifungal agent FK520. The failure of Mdr3F protein to confer FK520 resistance provides additional genetic evidence that the capacity of Mdr3S transformants to grow in the presence of FK520 is caused by functional expression of P-gp.

It was recently established that FK506 is actively transported by P-gp encoded by human *MDR1* (41). We wished to determine whether the appearance of cellular resistance to FK520 in yeast cells expressing wild-type mouse *mdr3* was the result of active transport of this compound by P-gp in these cells. Since neither FK506 nor FK520 radiolabelled analogs can be commercially obtained, we addressed this issue by testing the capacity of unlabelled FK506 to compete for the transport of a known P-gp substrate, [^{3}H]VBL (Fig. 4). For this purpose, [^{3}H]VBL accumulation in yeast spheroplasts expressing either wild-type Mdr3S or mutant Mdr3F was monitored in the absence (Fig. 4A) or presence (Fig. 4B) of unlabelled FK506. As previously shown for VRP (Fig. 2B), addition of FK506 was found to completely abrogate the Mdr3S-mediated reduction of [^{3}H]VBL accumulation detected in these cells, resulting in intracellular levels of [^{3}H]VBL similar to those measured in Mdr3F and control pVT spheroplasts. These results are indicative of a direct interaction between VBL and FK506 at the site of transport on P-gp and strongly suggest that cellular resistance to FK520 displayed by yeast cells expressing wild-type *mdr3* is indeed the result of active transport of this compound by P-gp in these cells.

Effects of Ser-939 mutations on FK520 resistance. To better understand the role of Ser-939 in the interaction of FK520 with P-gp and to identify the structural requirements involved in this interaction, we constructed Mdr3 expression vectors with this serine residue replaced by alanine, cysteine, threonine, tyrosine, tryptophan, and aspartic acid. We then tested the effects of these mutations on the ability of Mdr3 to confer FK520 resistance to yeast cells. Briefly, mutations were introduced by site-directed mutagenesis in *mdr3* subfragments, and full-length mutant *mdr3* clones were reconstructed by exchanging a wild-type *mdr3* fragment from pVT-MDR3S with the corresponding mutagenized fragment, creating plasmids pVT-MDR3A, pVT-MDR3C, pVT-MDR3T, pVT-MDR3Y, pVT-MDR3W, and pVT-MDR3D. These plasmids, along with plasmids pVT-MDR3S, pVT-MDR3F, and pVT, were then transformed into strain JPY201, and mass populations of the resulting transformants were examined for their levels of resistance to FK520 by a spot test growth inhibition plate assay (Fig. 5). Substitutions of Ser-939 were found to differentially modulate the ability of Mdr3 to confer cellular resistance to FK520, as yeast cells transformed with the different plasmids displayed either high levels of resistance (pVT-MDR3S, pVT-MDR3A, and pVT-MDR3C), a low level of resistance (pVT-MDR3T), or almost complete sensitivity (pVT-MDR3Y, pVT-MDR3W, pVT-MDR3F, and pVT-MDR3D) similar to that of pVT control cells.

We further quantitated the effects of the different mutations on the ability of Mdr3 to confer resistance to growth inhibition by FK520, using a liquid culture assay (Fig. 6). Conservative substitution of Ser-939 by small nonpolar residues such as Ala or Cys had only a moderate effect on resistance, resulting in an approximately twofold decrease in growth of yeast cells in presence of FK520. The introduction of Thr produced a mutant with altered activity, which showed a sixfold reduction in FK520 resistance. Finally, the nonconservative replacement of Ser-939 by larger residues with bulkier side chains such as Tyr, Trp, or Phe or a charged residue such as Asp had a drastic effect and completely abolished the capacity of Mdr3 to confer resistance to FK520. When tested in mammalian cells, conservative substitutions at Ser-939 such as Ala and Cys had little effect on the activity of Mdr3 toward MDR drugs, while nonconservative substitutions such as Tyr, Trp, and Asp significantly reduced the activity of Mdr3 toward these drugs (12). Taken together, these results indicate that the modulatory effect of independent Ser-939 mutations on Mdr3-mediated FK520 resistance in yeast cells is similar to the effect of these mutations on Mdr3-mediated drug resistance in mammalian cells. To verify that the biologically inactive Mdr3 mutants were properly expressed, yeast transformants were metabolically labelled, and labelled proteins were immunoprecipitated with an isoform-specific anti-Mdr3 polyclonal antibody (2037) described previously (6). Figure 7 shows that the different transformants express similar amounts of wild-type or mutant Mdr3 proteins (lanes 2 to 9), absent from control pVT transformants (lane 1). Taken together, these results confirm that FK520 resistance detected in pVT-MDR3S cells (Fig. 3) is caused by functional P-gp expression and show that Ser-939 plays an important role in the interaction of FK520 with P-gp.

Effects of Ser-939 mutations on mating activity. We have shown that expression of mouse Mdr3 in a yeast *ste6* deletion strain could functionally substitute for the yeast Ste6 transporter, by restoring the ability of the cells to export **a**-factor and to mate (38). The introduction of the Ser-939-to-Phe substitution completely abolished the capacity of Mdr3 to complement the biological activity of Ste6, thereby indicating that Ser-939 is also an important determinant for **a**-factor transport. To determine whether the variations in levels of FK520 resistance observed between the different Mdr3 Ser-939 mutants were reflected by parallel modulation of **a**-factor transport activity, the mating efficiency of the transformants expressing the mutant Mdr3 proteins was measured by a quantitative mating assay (Fig. 5). First, replacement of Ser-939 by Ala (pVT-MDR3A) had only a moderate effect on mating, resulting in a twofold decrease in mating efficiency compared with transformants

PLASMID	MATING EFFICIENCY	FK 520 RESISTANCE 0 μg/ml	FK 520 RESISTANCE 100 μg/ml
pVT	$<3.0 \times 10^{-7}$ (<0.2%)		
pVT-MDR3S	2.0×10^{-4} (100%)		
pVT-MDR3A	1.0×10^{-4} (50%)		
pVT-MDR3C	1.6×10^{-5} (8%)		
pVT-MDR3T	2.3×10^{-6} (1.2%)		
pVT-MDR3Y	1.0×10^{-6} (0.5%)		
pVT-MDR3W	$<3.1 \times 10^{-7}$ (<0.2%)		
pVT-MDR3F	$<3.3 \times 10^{-7}$ (<0.2%)		
pVT-MDR3D	$<3.2 \times 10^{-7}$ (<0.2%)		

FIG. 5. Mating and drug resistance characteristics of yeast cells expressing wild-type and mutant Mdr3 proteins. Yeast cells were transformed with pVT plasmids expressing wild-type Mdr3 (pVT-MDR3S) and mutant Mdr3 in which Ser-939 has been replaced by Ala, Cys, Thr, Tyr, Trp, Phe, or Asp to give plasmid pVT-MDR3A, pVT-MDR3C, pVT-MDR3T, pVT-MDR3Y, pVT-MDR3W, pVT-MDR3F, or pVT-MDR3D, respectively. The capacity of JPY201 transformants to mate with haploid *MAT***a** cells was determined and is expressed as the percentage relative to the value for pVT-MDR3S transformants, which was set at 100%. Values are means of three experiments (relative errors on each value were less than 50%). Yeast transformants were tested for resistance to FK520 by a spot test growth inhibition assay. Equal numbers and serial 10-fold dilutions of each transformants were spotted on YPD plates containing FK520 at either 0 μg/ml or 100 μg/ml. Plates were photographed after incubation at 30°C for 48 h.

expressing wild-type Mdr3 (pVT-MDR3S). Second, introduction of Cys (pVT-MDR3C) had a more pronounced effect on mating, generating mating efficiencies only 8% of that of the wild type. Third, substitution of Ser-939 by Thr (pVT-MDR3T) significantly reduced the mating activity of the transformants, which showed a mating efficiency approximately 2 orders of magnitude lower than that of Mdr3S-expressing cells. Finally, nonconservative substitutions at position 939 dramatically decreased (pVT-MDR3Y) or completely abolished (pVT-MDR3W, pVT-MDR3F, and pVT-MDR3D) the ability of Mdr3 to restore **a**-factor transport in yeast cells, resulting in 200-fold (Tyr) or at least 500-fold reductions in mating frequency of the corresponding transformants with respect to wild-type Mdr3S-expressing cells. Thus, the functional consequences of Ser-939 substitutions on **a**-factor transport and mating are strikingly similar to those observed for FK520 resistance. The analysis of independent *mdr3* mutants suggests that Mdr3 expressed in yeast cells can transport both **a**-factor and FK520 and that the transport of these two substrates is by way of similar mechanisms.

DISCUSSION

Genetic analysis in prokaryotic (*E. coli*) or lower eukaryotic (yeast) cells provides a powerful approach to identify structure-function relationships in complex mammalian proteins. This technique is particularly useful for multispanning integral membrane proteins for which three-dimensional structure information is not available. While eukaryotic membrane proteins are often quite toxic to *E. coli*, resulting in no or only low levels of expression, this class of hydrophobic proteins appears to be particularly suited for expression in the yeast *S. cerevisiae* and *Schizosaccharomyces pombe* (see reference 55 and references therein). In an attempt to establish a simple system useful for the genetic and biochemical analysis of a mammalian P-gp, we have expressed a mouse *mdr3* cDNA in *S. cerevisiae*. In a previous report, we showed that when expressed in a yeast *ste6* deletion strain, P-gp encoded by *mdr3* could functionally substitute for the yeast **a**-factor transporter Ste6, by restoring the ability of the cells to export **a**-factor and to mate (38). In the present report, we demonstrate that in this

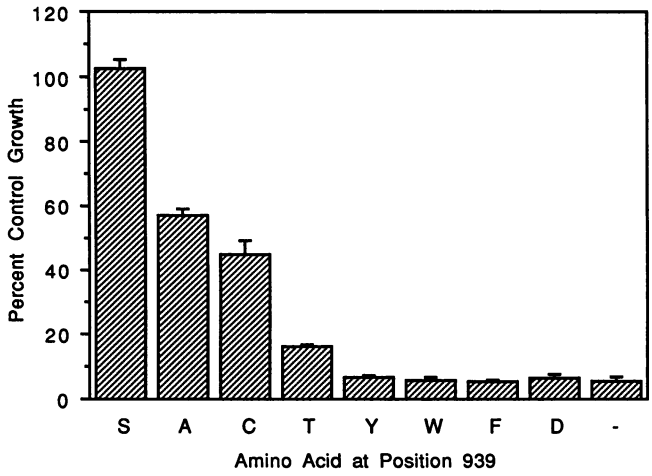

FIG. 6. Growth inhibition of FK520 on yeast transformants expressing wild-type and mutant Mdr3 proteins. Yeast transformants expressing either wild-type (S) or mutant (A, C, T, Y, W, F, and D) Mdr3, or pVT control cells (−), were grown for 11 h at 30°C in YPD medium containing FK520 at 100 μg/ml, and growth was determined by measuring optical density (A_{590}). Results are presented as the percentage of control growth for each transformant in the same medium lacking FK520. Values reported represent means ± standard deviations of three experiments performed in duplicate.

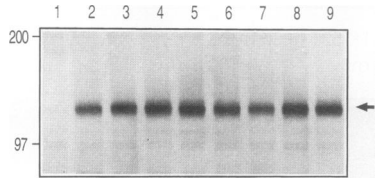

FIG. 7. Immunodetection of wild-type and mutant Mdr3 proteins expressed in yeast transformants. ^{35}S-labelled cell extracts from yeast cells transformed with plasmids pVT (lane 1), pVT-MDR3S (lane 2), pVT-MDR3A (lane 3), pVT-MDR3C (lane 4), pVT-MDR3T (lane 5), pVT-MDR3Y (lane 6), pVT-MDR3W (lane 7), pVT-MDR3F (lane 8), and pVT-MDR3D (lane 9) were incubated with an anti-Mdr3 polyclonal antibody (6). Immune complexes were isolated with protein A-Sepharose and separated by SDS-PAGE on a 7.5% gel. The gel was treated with En^{3}Hance, dried, and exposed for 24 h. Molecular mass standards shown on the left are myosin (200 kDa) and phosphorylase *b* (97 kDa). All transformants express similar amounts of the Mdr3 protein with an apparent molecular mass of 125 kDa (indicated by the arrow).

yeast expression system, P-gp encoded by *mdr3* behaves as a functional drug transporter: P-gp is localized in the membrane fraction of the cells, binds the photoactivable drug analog [^{125}I]IAAP, and transports VBL in an ATP-dependent and VRP-sensitive manner. Moreover, we find that expression of P-gp in yeast cells is able to confer cellular resistance to the immunosuppressive and antifungal agent FK520, a close analog of the recently described P-gp substrate FK506 (5). Furthermore, we demonstrate that FK506 can compete [^{3}H]VBL transport in yeast cells expressing P-gp (Fig. 4). From these results, we conclude that P-gp expressed in *S. cerevisiae* is fully functional and acts as both a peptide and a drug transporter. The human *MDR1* gene has also been successfully expressed in yeast cells, but the consequences of this expression on drug resistance and/or drug transport in these cells remain unclear, causing either increased (31) or decreased (40) cellular drug resistance.

The mechanism by which P-gp can recognize a large number of structurally and functionally unrelated substrates remains unsolved. Several biochemical and genetic lines of evidence suggest that the membrane-associated domains of P-gp are involved in that process (7, 11, 16, 33, 37). We have recently documented that a single Ser-to-Phe substitution within TM domain 11 strongly modulates the activity of P-gps (19). Biochemical analyses have shown that this mutation affects the capacity of P-gps to reduce cellular drug accumulation, to mediate efflux of drugs, and to bind photoactivable drug analogs (28). In the present study, we demonstrate that when expressed in yeast cells, P-gp carrying the Ser-to-Phe mutation also displays a reduced capacity to bind [^{125}I]IAAP and fails to transport VBL and to confer FK520 resistance. Thus, the effects of the Ser-to-Phe mutation in TM domain 11 of P-gp expressed in yeast cells, namely, impaired drug binding, transport, and resistance, are comparable to those observed when the same proteins are expressed in mammalian cells (19, 28).

Replacement of Ser-939 by a series of amino acids (Ala, Cys, Thr, Tyr, Trp, and Asp) strongly modulates the capacity of P-gp encoded by *mdr3* to confer resistance to a number of cytotoxic drugs and to transport adriamycin in mammalian cells, the size of the lateral chain rather than the charge appearing to be the primary structural determinant at that site (12). Since our analysis of the Ser-to-Phe mutation in yeast cells indicated that Ser-939 is also important for the interaction of P-gp with **a**-factor and FK520, we have expressed these *mdr3* mutants in yeast cells and tested the effects of these mutations on FK520 resistance and on mating. We show that substitution of Ser-939 in Mdr3 by different amino acids similarly modulates the level of FK520 resistance and the level of mating, in a manner that parallels the activity of these mutants for cytotoxic drugs in mammalian cells (12). These results demonstrate that Ser-939 plays a key role in the interaction of P-gp not only with cytotoxic drugs such as colchicine, adriamycin, and actinomycin D but also with peptides such as **a**-factor and with immunosuppressive and fungicidal agents such as FK520. These results also clearly demonstrate that transport of both **a**-factor and FK520 in yeast cells is mechanistically similar to drug transport in mammalian cells. This further establishes the yeast system as a valid model for the structure-function analysis of P-gp.

The heterologous expression of mammalian P-gp in yeast cells provides two distinct phenotypes for mutational analysis of this transporter, namely, mating and FK520 resistance. These two phenotypes can be independently assayed for the functional screening of mutant P-gp molecules impaired in **a**-factor or FK520 transport and, most importantly, for the positive selection of second-site intragenic revertants of P-gp mutants inactive in each, or both, of these two processes. The rapid isolation and analysis of second-site intragenic revertants of nonfunctional P-gp mutants should provide important information about interactions between specific structural and functional domains in P-gp. The yeast system may allow us to genetically dissect the mechanism of action of P-gp with respect to peptide and drug transport, an experimental strategy difficult in mammalian cells.

Understanding the mechanism of P-gp mediated transport requires knowledge of the structure of the protein. As yet, structural models for P-gp has relied principally on predictions from computer-based algorithms (8, 18). However, recent studies have suggested a membrane organization for P-gp distinct from the generally accepted one predicted by hydropathy plot (46, 58). The functional expression of P-gp as a drug-binding protein and transporter in yeast cells, described in this report, indicates that P-gp is properly targeted and inserted in the plasma membrane of yeast cells. Therefore, in the absence of three-dimensional structure for P-gp, the yeast system provides a valid tool with which to examine the topological arrangement of this membrane transporter, using a target-reporter gene fusion system. By fusing a truncated version of the HIS4 protein containing the histidinol dehydrogenase domain to specific TM domains of P-gp, it will be possible to evaluate the topology of specific positions within the protein in vivo (44). A knowledge of the topology of the membrane-associated regions in P-gp should be important for future functional studies.

CsA, FK506, and rapamycin are natural products which possess potent immunosuppressive and antifungal properties (5, 24, 25). While CsA is a cyclic undecapeptide, FK506 and rapamycin both belong to the macrolide antibiotic family (24, 43). The major cellular targets for these drugs, collectively designated immunophilins, are cyclophilin for CsA and FKBP for FK506 and rapamycin (24, 43). Immunophilins are ubiquitous cytosolic proteins which possess peptidyl-propyl *cis-trans* isomerase activities and which are highly conserved phylogenetically (24, 43). Fungal homologs of human cyclophilin and FKBP have been identified and shown to mediate CsA, rapamycin, and FK506 cytotoxicity in yeast cells (23, 29, 50). The observation that three FK506 analogs exhibit parallel antifungal and immunosuppressive potencies

suggests that molecular recognition of FK506 in yeast and T cells may be related (5), although additional specific cellular targets in yeast cells appear to be required to mediate the antifungal activity of FK506 (23). Such an FK506-sensitive target has recently been identified from *S. cerevisiae* and shown to be involved in amino acid import (22).

In addition to their application as potent immunosuppressants, CsA, FK506, and rapamycin have been shown to function as effective P-gp modulators (1, 36). The recent demonstration that CsA and FK506 are substrates for P-gp (41) coupled with the observation that they also possess antifungal activity (5, 23) prompted us to investigate whether P-gp could confer resistance in yeast cells to growth inhibition by this class of compounds. Using plate and liquid culture assays, we were able to demonstrate that yeast cells expressing P-gp encoded by *mdr3* could grow in the presence of FK520, an FK506 analog with similar immunosuppressive and antifungal properties (5). Results similar to those presented here for FK520 were obtained when the original compound FK506 was tested, indicating that substitution of the allyl moiety by an ethyl substituent at position C-21 on the macrolide ring does not affect the interaction of these compounds with P-gp (data not shown).

The identification of FK520 as a substrate for P-gp in yeast cells together with the amenability of the FK506 structure to chemical modifications (43) should prove extremely useful for structure-activity studies. As previously demonstrated for CsA (51), the potent immunosuppressive properties of FK506 could be disadvantageous for its potential administration to cancer patients as a reversal agent. Consequently, the investigation of a series of chemically derived FK506 analogs in our yeast P-gp expression system should allow the identification of analogs with less immunosuppressive and better reversal activities for the treatment of MDR in vivo. The availability of a simple and fast microtiter assay will make it possible to investigate the activity of several chemical derivatives in combination with a large number of mutant P-gps. Such structure-activity relationship studies with a series of FK506 analogs should also prove useful for a better understanding of the structural requirement for efficient P-gp–FK506 interactions, allowing in turn the rational design of improved drugs and MDR modulators.

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