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Functional Complementation of Yeast *ste6* by a Mammalian Multidrug Resistance *mdr* Gene

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Multidrug resistance in mammalian tumor cells is associated with the overexpression of *mdr* genes encoding P-glycoproteins, which function as drug efflux pumps. A yeast homolog of *mdr*, *STE6*, mediates export of a-factor mating peptide. Yeast *MAT*a cells carrying a *ste6* deletion produce no extracellular a-factor and therefore are defective in mating. Expression of a complementary DNA for the mouse *mdr3* gene in a yeast *ste6* deletion strain restored ability to export a-factor and to mate. A mutation (a serine to phenylalanine substitution at amino acid 939) known to affect the activity of the *mdr3* gene product abolished its ability to complement the yeast *ste6* deletion. Thus, functions of P-glycoproteins in normal mammalian cells may include the transmembrane export of endogenous peptides.

Multidrug resistance (MDR) in cultured cells in vitro and in tumor cells in vivo is associated with the overexpression of members of a small gene family, designated *mdr* or *pgp*, that codes for membrane P-glycoproteins (Pgps) (*1*). Pgps are composed of two homologous halves; each half consists of six predicted transmembrane domains and an intracellular loop with a consensus adenosine triphosphate (ATP) binding motif (*1*). Pgps apparently function as energy-dependent drug efflux pumps that reduce the intracellular accumulation of a wide variety of chemotherapeutic drugs in resistant cells (*2*). In addition, Pgps display a specific pattern of expression in normal tissues, implicating Pgps in normal transport of endogenous cellular substrates (*3*). If so, these normal substrates have yet to be identified.

Pgps are members of a superfamily of evolutionarily conserved transport proteins (*4*). This superfamily includes the *pfmdr1* gene product of *Plasmodium falciparum* associated with chloroquine resistance (*5*), the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein, mutations in which lead to CF in humans (*6*), and the yeast *Saccharomyces cerevisiae STE6* gene product (*7, 8*), which mediates the export of the a-factor pheromone, a farnesylated dodecapeptide required for mating (*9, 10*). The yeast *STE6* gene product and mammalian Pgps are up to 57% homologous, including conservative amino acid substitutions and have similar predicted secondary structure and proposed membrane topology (*7, 8*). To determine if the structural similarity between the yeast and the mammalian transporters translates into a functional homology, we asked if the mouse *mdr3* gene (*11, 12*) could complement the function of the *STE6* gene in a yeast *ste6* mutant strain.

A genomic DNA fragment containing the yeast *STE6* gene and a cDNA for the mouse wild-type *mdr3* gene were placed under the control of the promoter for the yeast alcohol dehydrogenase gene on the high copy vector pVT, yielding plasmids pVT-STE6 and pVT-MDR3S, respectively (*13*). In addition, a mutant *mdr3* derivative with a serine to phenylalanine substitution at amino acid position 939 was subcloned into pVT and the resulting construct designated pVT-MDR3F (*13*). The Ser^{939} to Phe^{939} substitution within predicted transmembrane domain 11 (TM-11) of the *mdr3* gene product decreases the activity and modifies the substrate specificity of this transporter for drugs of the MDR spectrum (*12*). These three constructs, along with plasmid pVT as negative control, were transformed into yeast JPY201 *MAT*a Δ*ste6*::*HIS3* cells, in which 70% of the *STE6* gene was deleted and replaced with the yeast *HIS3* gene (*7*). Thus, JPY201 cells do not export a-factor and are sterile (*7*).

The activity of the *STE6* or *mdr3* gene products was measured by the ability of transformed JPY201 cells to arrest growth of *MAT*α cells and to mate, functions dependent on extracellular a-factor pheromone production (*14*). In a growth arrest assay (Fig. 1), production of pheromone by a patch of cells inhibits growth of a lawn of cells of the opposite mating type, resulting in a clear zone or halo surrounding the patch (*15*). Control JPY201 cells transformed with pVT [JPY201(pVT)] were defective in the production of extracellular a-factor (*7*), but JPY201 cells transformed with pVT-STE6 [JPY201(pVT-STE6)] or pVT-MDR3S [JPY201(pVT-MDR3S)] produced a zone of growth inhibition of significant size, indicating their ability to export substantial amount of a-factor pheromone (*16*). In contrast, cells transformed with pVT-MDR3F [JPY201(pVT-MDR3F)] produced no discernible halo.

The ability of JPY201 transformants to mate was also assayed (Fig. 2 and Table 1). Pheromone production triggers mating between haploid cells of opposite mating types, which leads ultimately to the formation of diploid cells (*14*). Thus, diploid formation provides a sensitive measure for the extracellular production of biologically active a-pheromone (*10*). The mating defect of JPY201(pVT) cells was corrected by expression of either the *STE6* or the wild-type *mdr3* gene but not by the mutant *mdr3* gene. The difference in mating efficiencies between JPY201(pVT-STE6) and JPY201-(pVT-MDR3S) cells may reflect their differential ability to transport extracellular a-factor, a likely consequence of the evolutionary distance between the yeast and the mammalian transporters expressed in these transformants. The construction and functional testing of chimeric molecules between *STE6* and *mdr3* should help to identify the protein domains responsible for optimal a-factor transport by STE6.

To verify that the MDR3(Phe^{939}) protein was appropriately expressed and targeted, we prepared cell membranes from JPY201 transformants and analyzed them by immunoblotting for the presence of the wild-type and mutant *mdr3* gene products (Fig. 3). JPY201(pVT-MDR3S) and JPY201(pVT-MDR3F) cells produce similar amounts of immunoreactive MDR3(Ser^{939}) and MDR3(Phe^{939}) proteins, which are not detected in control cells transformed with plasmid pVT. The mouse *mdr3* gene products expressed in yeast cells display a faster

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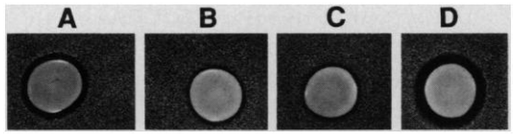

Fig. 1. Determination of a-factor production by growth arrest assay. JPY201 cells transformed with plasmid pVT-MDR3S (**A**), pVT-MDR3F (**B**), pVT (**C**), or pVT-STE6 (**D**) were grown to stationary phase in SD-URA. Cells (10^6) were spotted directly onto a lawn of yeast strain M323-2A (*MATα ssl1-7 sst2::URA3 leu2-3, 112 trp1 ura3-52*), which carries the *ssl1 sst2* mutations rendering it hypersensitive to growth inhibition by a-factor (*22*). Plates were photographed after incubation at 30°C for 24 hours.

Table 1. Mating efficiency of JPY201 transformants. Fresh cultures of JPY201 transformants (in SD-URA medium) and of yeast strain DC17 (in YPD medium) were mixed in microfuge tubes, briefly pelleted, incubated at 30°C for 3 hours, and serial dilutions were plated onto minimal synthetic dextose medium for the selection of diploids. The frequency of mating was calculated as the ratio of the number of diploids formed on selective medium to the number of input JPY201 cells used in the assay. The reported values are the mean of three independent experiments performed in duplicate. The standard deviations for the measurements were 3.0×10^{-2} for pVT-STE6 and 4.2×10^{-4} for pVT-MDR3S. The frequency $<5.0 \times 10^{-6}$ represents the lowest mating efficiency detectable in this assay (*23*).

JPY201 transformants	Mating frequency
pVT-STE6	6.7×10^{-2}
pVT-MDR3S	7.1×10^{-4}
pVT-MDR3F	$<5.0 \times 10^{-6}$
pVT	$<5.0 \times 10^{-6}$

electrophoretic mobility than predicted from their calculated molecular mass of 140 kD (*11*), the reason for this discrepancy being unknown. The failure of a properly expressed MDR3(Phe^{939}) mutant protein to complement the *ste6* mutation provides additional and direct genetic evidence that the extracellular production of a-factor by JPY201(pVT-MDR3S) transformants is due to the expression of a functionally active MDR3(Ser^{939}) protein.

This suggests that Pgp encoded by the mouse *mdr3* gene transports a-factor in yeast cells by mechanisms similar to those involved in drug transport in mammalian cells. Drugs transported by Pgps share a common hydrophobic nature, which has been proposed to allow them to partition within the plasma membrane, from which they are eventually expelled (*17*). Similarly, a-factor is a hydrophobic lipopeptide, which contains a number of aromatic residues and which is modified by the attachment of a methyl group and a farnesyl moiety to the COOH-terminal cysteine residue (*9*). Because isoprenylation of a-factor appears to be necessary for export of this peptide by yeast cells (*18*), it is possible that this modification is also required for transport of a-factor by Pgp.

In *P. falciparum*, mutations in TM-11 of the *pfmdr1* gene product have been found associated with chloroquine resistance, including a serine to cysteine substitution at amino acid position 1034 (*19*). Although no significant sequence homology can be detected between the TM-11 domains of the *mdr* and the *pfmdr* gene products, both segments have the potential to form amphipathic helices, with Ser^{939} of *mdr3* and Ser^{1034} of *pfmdr1* mapping near the boundary of the hydrophobic side of the predicted helices (*12*). A serine residue maps at the equivalent position in TM-11 of the *STE6* gene product (Ser^{950}) and TM-11 of the yeast transporter also displays the potential to form such an amphipathic helix (*20*). These observations, together with the finding that the Ser^{939} to Phe^{939} substitution in MDR3 affects not only drug transport in mammalian cells (*12*) but also peptide transport in yeast cells, strongly support the proposition that TM-11 may be involved in substrate recognition and transport by the Pgp superfamily of transporters.

The export of a-factor pheromone by the *STE6* gene product has identified a pathway for protein export in yeast cells, different from the classical secretory pathway (*7, 8*). The recent isolation of peptide pumps homologous to Pgp and apparently implicated in transporting fragments of antigenic proteins from the cytosol into the endoplasmic reticulum (*21*) suggests that such transport pathways are also used in mammalian cells. Our finding that a mammalian Pgp can functionally substitute for the yeast *STE6* gene product supports the proposition that the as yet unidentified physiological functions of Pgps in normal cells may include the transmembrane transport of endogenous peptides or proteins. It remains to be determined whether mammalian Pgp protein directs secretion of a-factor or other peptides by a mechanism that requires or is independent of the classical yeast secretory pathway.

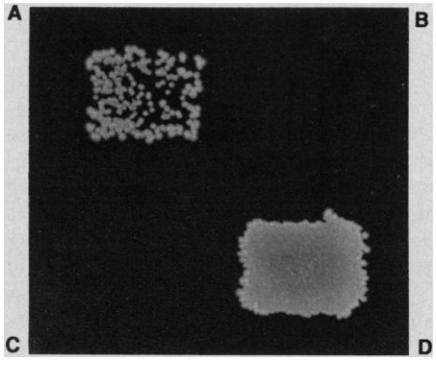

Fig. 2. Determination of a-factor production by mating assay. Patches of JPY201 cells transformed with plasmid pVT-MDR3S (**A**), pVT-MDR3F (**B**), pVT (**C**), or pVT-STE6 (**D**) were grown on SD-URA plates and replicated to rich YPD plates (yeast extract, peptone, and dextrose) on a lawn of yeast strain DC17 (*MATα his1*). Mating was allowed to proceed for 12 hours at 30°C, before transfer onto synthetic dextrose minimal plates to assess diploid formation.

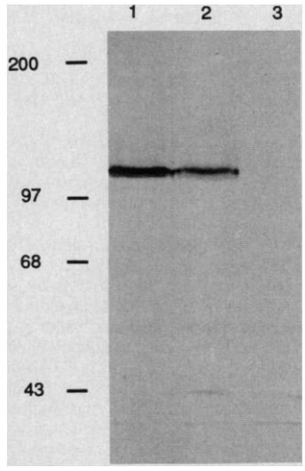

Fig. 3. Expression of the mouse *mdr3* gene products in yeast JPY201 transformants. Membrane proteins (10 μg) (*24*) from JPY201 cells transformed with plasmid pVT-MDR3S (lane 1), pVT-MDR3F (lane 2), or pVT (lane 3) were separated by SDS–polyacrylamide gel electrophoresis and analyzed by Western (RNA) blotting with the monoclonal antibody to Pgp C219 (Centocor Corp., Philadelphia, Pennsylvania). Immune complexes were revealed by incubation with antibodies to mouse immunoglobulin G coupled to alkaline phosphatase (Bio-Rad Laboratories, Ltd., Mississauga, Ontario).

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23. Similar quantitative mating experiments have been performed in two additional yeast *ste6* deletion strains generated in this report. For this purpose, a 5.2-kb Bgl II–Sac I genomic DNA fragment containing the *STE6* gene was cloned at the BamH I–Sac I sites of vector pGEM7Zf to give pSTE6-5.2. We engineered the *ste6* deletion Δ*ste6*::*HIS3* by substituting in pSTE6-5.2 a 3.4-kb Stu I–SnaB I segment of *STE6* with the 1.9-kb Sma I–Pvu II fragment of plasmid pJJ215 [J. S. Jones and L. Prakash, *Yeast* **6**, 363 (1990)] containing the *HIS3* gene. This deletion removes 87% of the STE6 coding sequence (amino acids 39 to 1168). The resulting Δ*ste6*::*HIS3* construction was excised by cleavage with Eco RI and Sac I and used for one-step gene replacement of the wild-type *STE6* gene in strains JPY200 *MAT***a** *his3 leu2 ura3 lys2 trp1* (*7*) and strain T109.6-3C *MAT***a** *his3 leu2 ura3* [M. Whiteway, R. Freedman, S. Van Arsdell, J. W. Szostak, J. Thorner, *Mol. Cell. Biol.* **7**, 3713 (1987)], yielding strains MRY4 and MRY5, respectively. The structure of the resulting deletion alleles in strains MRY4 and MRY5 was confirmed by Southern (DNA) analysis. Strains MRY4 and MRY5 were transformed with the four pVT-based plasmids listed in Table 1 and the mating efficiency of the transformants was quantified by filter assay [G. F. Sprague, Jr., *Methods Enzymol.* **194**, 77 (1991)]. Experiments were performed in duplicates and averaged results of the duplicates are as follows:

Plasmids	Mating frequency	
	MRY4 transformants	MRY5 transformants
pVTSTE6	1.1×10^{-1}	4.6×10^{-1}
pVT-MDR3S	2.5×10^{-4}	1.2×10^{-3}
pVT-MDR3F	$<5.2 \times 10^{-6}$	$<1.0 \times 10^{-7}$
pVT	$<5.0 \times 10^{-6}$	$<1.5 \times 10^{-7}$

24. Exponentially growing cells in selective SD-URA- medium 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 pounds per square inch. Unlysed cells were removed by centrifugation (1000*g*, 10 min) and crude membranes concentrated by centrifugation (100,000*g*, 30 min). Membranes were then resuspended in TNE containing 45% sucrose (w/v), loaded into a discontinuous sucrose gradient (60, 45, 35, and 30%), and centrifuged (100,000*g*, 3 hours). Membrane fractions at the 30 and 35% interface were pooled, washed in TNE, concentrated by centrifugation (100,000*g*, 30 min), and stored frozen in TNE containing 30% glycerol. All steps of the extraction were performed at 4°C in the presence of a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride at 1 mM; leupeptin, pepstatin A, and trasylol, each at 5 μg/ml).
25. We thank J. McGrath and A. Varshavsky for providing the pUCSTE6-Sal plasmid and JPY201 strain and T. Vernet for providing the pVT101-U vector. Supported by a grant to D.Y.T. from the National Research Council and the Medical Research Council (MRC) of Canada and to P.G. from the National Cancer Institute. M.R. is supported by a postdoctoral fellowship from MRC.

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Specific Binding of Chromosomal Protein HMG1 to DNA Damaged by the Anticancer Drug Cisplatin

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The mechanism of action of the anticancer compound *cis*-diamminedichloroplatinum(II) (cisplatin) involves covalent binding to DNA. In an effort to understand the tumor-specific cytotoxicity of such DNA damage, the interactions of these lesions with cellular proteins have been studied. One such protein has been identified as the high-mobility group protein HMG1. Recombinant rat HMG1 binds specifically (dissociation constant $3.7 \pm 2.0 \times 10^{-7}$ molar) to DNA containing cisplatin d(GpG) or d(ApG) intrastrand cross-links, which unwind and bend DNA in a specific manner, but not to DNA modified by therapeutically inactive platinum analogs. These results suggest how HMG1 might bind to altered DNA structures and may be helpful in designing new antitumor drugs.

Recent cancer statistics reveal the annual ratio of deaths to incidence of new solid tumors of the testes to be nearly zero (*1*). This result is thought to be largely the consequence of chemotherapy afforded by the anticancer drug cisplatin. The antitumor activity of cisplatin is generally accepted to involve binding to DNA, a process about which there is substantial chemical and structural information (*2*). The biological activity of the drug cannot be explained solely on the basis of its ability to damage DNA, however, because the geometric isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP) binds DNA and can block replication (*3–5*) but is ineffective as a chemotherapeutic agent (*6*). Stereochemical differences in the adducts formed by the two isomers imply that the antitumor activity of cisplatin arises from the formation of a specific structural motif on DNA which, in turn, triggers a cellular response leading to cell death. To investigate this possibility, attention has turned to identifying cellular proteins that recognize cisplatin-DNA lesions with the ultimate aim of understanding how such interactions might lead to the selective toxicity of the drug to tumor cells.

The existence of proteins in mammalian cellular extracts capable of binding to cisplatin adducts with high affinity and specificity has been established by gel mobility shift and Southwestern blot assays (*7–11*). Until recently, however, none of these proteins has been identified. The Southwestern blot analyses revealed the existence of two classes of proteins that recognize cisplatin-damaged DNA having molecular masses of ~28 kD and 80 to 100 kD (*8, 11, 12*). A gene that encodes an 81-kD cisplatin-DNA structure-specific recognition protein (SSRP1) has been cloned (*8*) and

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