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Functional expression of human *mdr1* in the yeast *Saccharomyces cerevisiae*

(P glycoprotein/plasma membrane/drug resistance/valinomycin)

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ABSTRACT **Development of multiple drug resistance in tumor cells involves amplification of the *mdr1* gene product, a 170-kDa plasma membrane glycoprotein that is an ATP-driven pump that extrudes the drugs. Human *mdr1* (also designated as *PGY1*) cDNA was expressed in yeast cells by using the promoter and translational initiation signal of a related yeast gene, *STE6*. Immunoblotting of subcellular fractions showed that all of the Mdr1 (also known as P glycoprotein) was associated with the particulate material. Immunofluorescence microscopy revealed that the majority of the Mdr1 was localized to the plasma membrane (although a significant amount was also found in the endoplasmic reticulum). In contrast to mammalian cells, Mdr1 was not glycosylated in yeast. Nevertheless, some, if not all, of the Mdr1 made in yeast was properly folded and functional because it could be photoaffinity labeled specifically with 8-azido-ATP and because cells overexpressing Mdr1 displayed increased resistance towards valinomycin, an ionophore known to interact with Mdr1 in animal cells. Hence, a human polytopic membrane protein was correctly inserted into the yeast plasma membrane, and glycosylation was not required for its function.**

In response to treatment of mammalian tumor cells with any one of several anticancer drugs, neoplastic lines are selected that display pleiotropic cross-resistance against a wide variety of different chemotherapeutic agents (1–3). This multiple drug resistance is due to amplification of the *mdr1* gene and the consequent overexpression of its product, Mdr1 (also known as P glycoprotein), an integral membrane protein that is a member of a superfamily of both prokaryotic (4) and eukaryotic (5) ATP-dependent transport proteins. More than 30 members of this family have been described to date in eukaryotes.

Mdr1 appears to catalyze the ATP-dependent efflux of a broad spectrum of apparently unrelated drugs (1–3); however, the true physiological function of Mdr1 and the determinants of its substrate specificity are still ill-defined (6). Furthermore, other members of the Mdr-like family are expressed in normal cells and in many different tissues, yet are unable to confer multiple drug resistance when overexpressed, raising additional questions about the substrates and *in vivo* role of this class of membrane proteins.

The *STE6* gene of *Saccharomyces cerevisiae* encodes a transporter that is homologous in amino acid sequence and predicted topology to Mdr1 (7, 8). Moreover, we demonstrated that Ste6 is required, and rate limiting, for secretion of the mating pheromone a factor (7), a 12-residue lipopeptide (9) that is secreted independently of the classical secretory pathway (10–12). Therefore, to our knowledge, Ste6 is the first eukaryotic Mdr-like protein whose physiologically relevant substrate has been identified. By analogy, we proposed that other members of this large family of ATP-driven transporters might catalyze the translocation of peptides or proteins across membranes (7), expanding on an original suggestion by Gerlach *et al.* (13) that Mdr1 might be involved in some aspect of secretion. We and others have suggested further that some Mdr-related proteins could be the machinery responsible for export of another class of secretory proteins, those lacking a typical N-terminal hydrophobic signal sequence (14, 15), and that other Mdr-like proteins could be responsible for the import of peptides or proteins into organelles (7, 15). Increasing evidence supports these hypotheses (16).

The member of this transporter superfamily that is most closely related to human Mdr1, in amino acid sequence, length, and structural organization, is yeast Ste6 (7, 8). This striking similarity suggested that Ste6 and Mdr1 might also share conservation of function. Therefore, we sought to express human *mdr1* (also designated as *PGY1*) cDNA in *S. cerevisiae* to determine whether Mdr1 could be produced in functional form in yeast cells and, if so, whether Mdr1 could substitute for Ste6 in the transport of a factor. This report demonstrates that yeast is a useful system for expressing, and studying the structure and function of, human Mdr1 and perhaps other members of this extended family of transport proteins.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions. Yeast strains used throughout this study were W303-1A (*MATa ura3-52 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) and an isogenic *ste6Δ* derivative, WKK7 (*ste6Δ::HIS3*), constructed as described (7). Mating tester strains were RC757 (*MATα sst2-1 rme1 his6 met1 can1 cyh2*) (17) and L1543 (*MATα lys9*) (18). Synthetic medium [minimal salts containing dextrose (SD)], supplemented with appropriate nutrients for maintenance of plasmids, and rich medium (yeast extract/peptone/glucose), were prepared as described (19). Cells were grown routinely at 30°C.

Production of Polyclonal Antibodies. Rabbit antiserum (no. 4007) directed against the C-terminal 359 amino acids of Mdr1 was supplied by M. M. Gottesman (National Institutes of Health). Anti-yeast carboxypeptidase Y antibodies were the gift of R. Schekman (University of California, Berkeley).

Recombinant DNA Manipulations. Multicopy (2-μm DNA-based) plasmids and low-copy [yeast centromeric DNA (CEN)-based] plasmids for expression of *mdr1* cDNA in yeast were constructed. A 370-base-pair *Sac* I/*Bsp*HI fragment from pKAK1 (K.K. and J.T., unpublished results)

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Abbreviations: YMdr1, a chimeric protein consisting of authentic human Mdr1 (1280 residues) lacking its 17 N-terminal amino acids fused in-frame to the methionine and 6 amino acids encoded by the vector used for expression; SD, a synthetic minimal salts medium containing dextrose; CEN, yeast centromeric DNA.

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containing the entire *STE6* promoter (including the initiator ATG) was converted to blunt ends by treatment with Klenow fragment of *Escherichia coli* DNA polymerase I and inserted into the *Sac* I/*Sma* I sites of the CEN vector pRS316 (20) to give pRK7. A 3991-base-pair *Aha* III fragment, obtained from pMDR2000XS (21), containing all but 17 N-terminal amino acids of the *mdr1* coding sequence, was inserted into the *Eco*RV site of pRK7, to generate pRKM7. In this construct, 7 amino acids were added in-frame to the N terminus of the truncated *mdr1* cDNA, and expression of Mdr1 was governed by the yeast *STE6* promoter and initiator methionine. The 2-μm plasmid pYKM77 was constructed by cloning the 4.3-kilobase *Sac* I/*Hin*dIII fragment of pRKM7 into the corresponding sites of YEp352 (22). To permit simultaneous overexpression of both *mdr1* and yeast *MFa1* (the structural gene encoding the **a** factor precursor), pYMA177 was constructed by inserting a 4.3-kilobase *Sac* I/*Hin*dIII fragment from pRKM7, which was converted to flush ends by treatment with bacteriophage T4 DNA polymerase, into the *Sma* I site of pKK1 (7). Transformation of yeast cells was performed by standard procedures (23). Standard methods and conventional *E. coli* strains were used for the propagation and isolation of plasmid DNA (24).

Preparation of Membranes and Membrane Extracts. Total cell extracts and partially purified membrane fractions for immunoblot analysis were prepared from cells grown to mid-exponential phase (A_{600} = 0.5–1) in SD medium by using minor modifications (K.K. and J.T., unpublished results) of procedures recommended by Franzusoff *et al.* (25). If membranes were to be used for photoaffinity labeling, 2-mercaptoethanol was omitted from all buffers. Protein concentration was measured by the Lowry method (26) in the presence of SDS to solubilize membrane proteins, using bovine serum albumin as the standard. For immunoblots, about 50–100 μg of protein was suspended in SDS/PAGE sample buffer (27) and heated at 55°C for 10 min prior to loading. Transfer of proteins from SDS/PAGE gels (27) to nitrocellulose (Schleicher & Schüll BA85, pore size = 0.45 μm) was carried out by standard procedures (28). Immune complexes were detected with ^{125}I-labeled protein A (specific activity 70–100 μCi/μg; 1 Ci = 37 GBq; ICN).

Photoaffinity Labeling, Radiolabeling, and Immunoprecipitation. Carrier-free 8-azido-[α-^{32}P]ATP (10 Ci/mmol, ICN) was added to a final concentration of 2–5 μM to 1 mg of yeast membranes prepared from cells overexpressing Mdr1 from pYKM77. The membranes were suspended at 10 mg/ml in labeling buffer (10 mM Tris·HCl, pH 7.6/5 mM $MgCl_2$/0.1 mM NaH_2PO_4/0.1 mM NaF/0.1 mM $Na_4P_2O_7$) in a total volume of 100 μl and incubated on ice for 20 min. Photolysis and cross-linking were achieved by irradiating the samples with a hand-held shortwave UV lamp (254 nm, UVG-54; Ultraviolet-Products, San Gabriel, CA) at 0°C from a distance of 2.5 cm (lamp output, 100 μA; equivalent to 77 erg·mm^{-2}·sec^{-1}) for two 1-min intervals with a 1-min cooling period in between. Reactions were quenched by addition of dithiothreitol to a final concentration of 2 mM. When added as competitors, ATP and GTP were present at a 1 mM final concentration. After quenching, samples were diluted to a volume of 1 ml in immunoprecipitation buffer (25) and heated to 55°C for 10 min; affinity-labeled products were immunoprecipitated with the polyclonal anti-Mdr1 serum.

Radiolabeling of cells in low sulfate medium, tunicamycin treatment, lysis of radiolabeled cells, immunoprecipitation with polyclonal antibodies, and fluorography were conducted by methods described in detail elsewhere (7, 25, 29).

Drug-Resistance Assays. An agar plate assay was used to monitor drug resistance in yeast cells. A stock solution (25 mg/ml) of valinomycin (Calbiochem) was prepared by dissolving the drug in ethanol. This stock was added to molten (48°C) SD medium containing 1.5% agarose (GIBCO/BRL) to give a range of final concentrations from 100 to 200 μM valinomycin. Cells to be tested for valinomycin resistance were streaked out on these plates, or alternatively, an equal number of cells was spotted on the plates. Growth was monitored after incubation at 30°C for 36–48 hr.

Indirect Immunofluorescence. Fixation and permeabilization of cells, treatment of the primary rabbit anti-Mdr1 antiserum (no. 4007) to reduce nonspecific staining, reaction with secondary fluorescein-conjugated goat anti-rabbit immunoglobulin (Cappel Laboratories), and fluorescence photomicroscopy were carried out by procedures recommended by Pringle *et al.* (30).

RESULTS AND DISCUSSION

Expression of Human Mdr1 in Yeast. Human *mdr1* cDNA was expressed in *MAT***a** cells of *S. cerevisiae* by using the expression plasmid pRK7, which contains the *STE6* promoter and its natural ATG codon inserted into the polylinker of the vector pRS316 (20). The ATG is followed by several unique restriction sites. In derivatives of pRK7 (pRKM7 and related plasmids pYKM77 and pYMA177), an *mdr1* cDNA lacking the coding sequence for its first 17 amino acids was joined in-frame to the ATG of *STE6*. The resulting chimeric Mdr1 protein (YMdr1) contains 6 additional amino acids (Gly-2 to Asp-7) derived from the polylinker of pRK7 (Fig. 1). Expression from the *STE6* promoter is *MAT***a** cell specific (31). The calculated size of YMdr1 is 140,424 Da. Yeast *MAT***a** cells expressing the *mdr1* cDNA produced a protein of about 125 kDa that was recognized by polyclonal antibodies raised against the C terminus of human Mdr1 and was found exclusively in the membrane fraction (Fig. 2). pYKM77, a multicopy plasmid, supported a 10- to 15-fold higher level of expression than a low-copy plasmid, pRKM7. Despite the overall sequence similarity between Mdr1 and Ste6 [up to 42% identity in two domains (7)], antiserum no. 4007 did not cross-react with membranes from cells overproducing Ste6 (data not shown). Although the estimated size of YMdr1 was somewhat smaller than that predicted from its primary sequence, it has been amply documented that Mdr1 extracted from animal cells shows variable mobility (apparent size ranges from 120 to 200 kDa), depending on the gel system used (32).

To determine whether expression of YMdr1 could substitute for the function of yeast Ste6, two tests for the ability of YMdr1 to complement a *ste6*Δ mutant were applied. The first was a halo bioassay, which is a direct measure of the ability of cells to secrete **a** factor pheromone (7, 29). The second was a quantitative mating assay (33) that measures the ability of *MAT***a** cells to mate with an appropriate *MAT*α partner, a process that requires production of extracellular **a** factor. Expression of YMdr1 at either a high or a low level in a *MAT***a** *ste6*Δ mutant was unable to restore either the ability to secrete detectable amounts of **a** factor or the ability to mate. Despite its broad specificity for drugs, Mdr1 was apparently unable to accept **a** factor as a substrate for transport. Alternatively, however, the Mdr1 expressed in yeast could perhaps be misfolded, mislocalized, proteolytically degraded, or

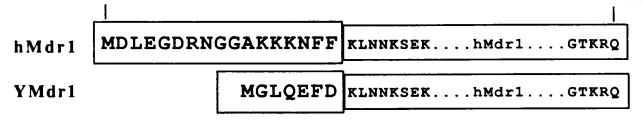

FIG. 1. N-terminal sequence of YMdr1 construction for yeast expression. The N terminus of authentic human Mdr1 (hMdr1) is compared, using the one-letter amino acid code, to the construct (YMdr1) used for expression in yeast. The residues from Gly-2 to Asp-7 are derived from the nucleotide sequence of the multiple cloning site of pRK7.

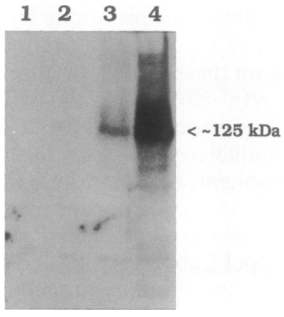

FIG. 2. Expression of YMdr1 in yeast. Immunoblot analysis of membrane fractions from *MATa ste6Δ* cells carrying the YEp352 parent control vector alone (lane 2), or expressing YMdr1 from pRKM7 (lane 3) or from pYKM77 (lane 4) was carried out as described in *Materials and Methods*. The soluble fraction derived from cells overexpressing YMdr1 from pYKM77 is also shown (lane 1). An SDS/7% PAGE gel was loaded with 50 μg of protein per lane (except lane 1, where 100 μg was loaded). Polyclonal antibodies raised against the C terminus of human Mdr1 (antiserum no. 4007) were used. Exposure of Kodak XAR-5 x-ray film was for 2 hr.

have aberrant posttranslational modification. We examined several independent criteria to assess whether human Mdr1 expressed in yeast was functional.

YMdr1 Is Localized to the Yeast Plasma Membrane. To determine if YMdr1 reached its correct destination, its subcellular localization was examined by indirect immunofluorescence. Intense staining of the cell surface was found, indicating that the bulk of the YMdr1 was delivered to the plasma membrane (Fig. 3). No staining was observed in cells not expressing YMdr1. In addition, a readily detectable portion of the YMdr1 was found in the endoplasmic reticulum as indicated by perinuclear staining; a pattern identical to that observed with antibodies against known components of the yeast endoplasmic reticulum like Kar2 (34) or Sec63 (D. Feldheim and R. Schekman, personal communication) was seen. Endoplasmic reticulum localization of YMdr1 did not appear to be simply a consequence of massive overproduction because similar staining, although less intense, was seen in cells expressing YMdr1 from the low-copy CEN plasmid pRKM7 (data not shown).

YMdr1 Is Not Glycosylated in Yeast. To examine glycosylation, cells expressing YMdr1 were radiolabeled in the presence or absence of tunicamycin, an inhibitor of asparagine-linked glycosylation (35). The apparent mobility of ^{35}S-labeled YMdr1 was identical whether the cells were treated with tunicamycin or not (Fig. 4), suggesting that YMdr1 is not N-glycosylated. The mobility of a known yeast glycoprotein, carboxypeptidase Y, was clearly affected by tunicamycin treatment. Mammalian P glycoprotein, as its name implies, carries a single asparagine-linked oligosaccharide (1–3); however, even in animal cells, this glycosylation does not appear to be required for Mdr1 function (36, 37).

YMdr1 Made in Yeast Binds ATP. Extrusion of drugs by Mdr1 is an ATP-dependent process (1–3); it seems that ATP

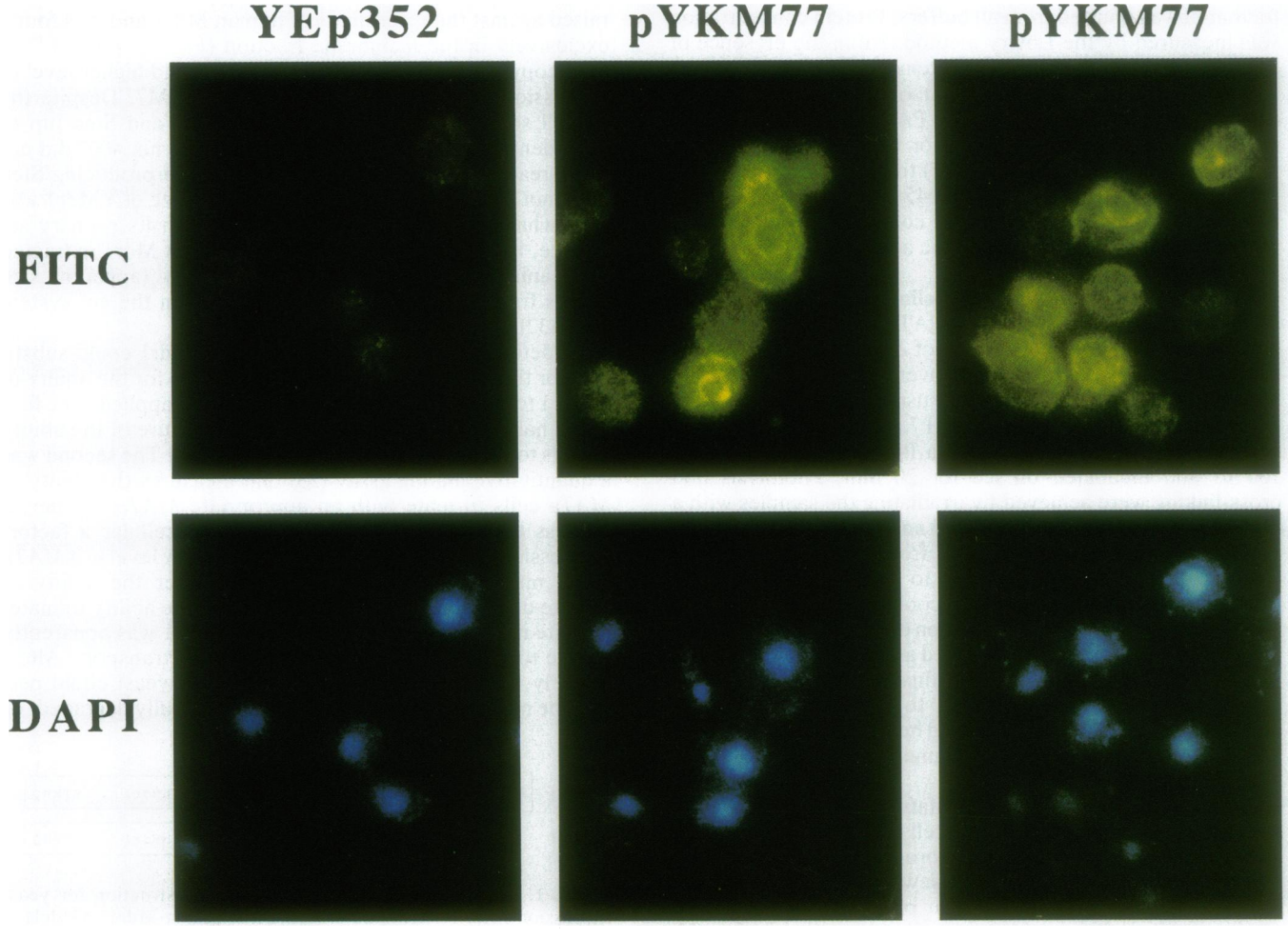

FIG. 3. Localization of YMdr1. *MATa ste6Δ* cells were transformed with YEp352 (*Left*) or pYKM77 (*Middle* and *Right*). (*Upper*) Formaldehyde-fixed cells were probed with polyclonal anti-Mdr1 antibody followed by secondary decoration using fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin. (*Lower*) DNA was stained with 4′,6-diamidino-2-phenylindole. Exposure time was ≈5 sec. (×750.)

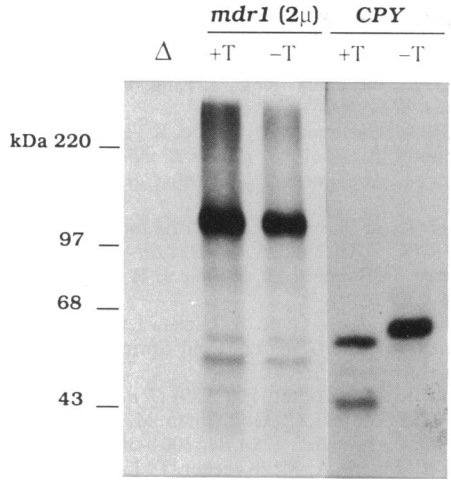

FIG. 4. YMdr1 is not a glycoprotein in yeast. *MATa ste6Δ* cells carrying pYKM77 [*mdr1* (2μ)] or the YEP352 vector alone (Δ) were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine in the presence (+T) or absence (−T) of the glycosylation inhibitor tunicamycin. Labeled proteins were immunoprecipitated using anti-Mdr1 antibody and a control serum recognizing yeast carboxypeptidase Y (CPY). Samples were analyzed by electrophoresis and fluorography. Exposure of Kodak XAR-5 film was for 3 hr.

binding is needed for both drug binding and subsequent drug efflux (38–40). Mdr1 can be tagged by the ATP analog, 8-azido-ATP (39), which can also cross-link to prokaryotic members of the superfamily of ATP-driven transporters (4). When total membranes from *MATa ste6Δ* cells overexpressing YMdr1 were labeled with 8-azido-[α-^{32}P]ATP, only one radioactive protein species was immunoprecipitable with the anti-Mdr1 antibody, and this band had the apparent molecular mass expected for YMdr1 (Fig. 5). This cross-linking was specific because no labeled species was detectable in cells not expressing YMdr1, because labeling was effectively blocked

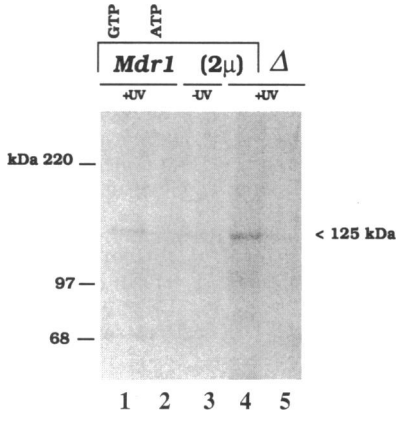

FIG. 5. YMdr1 is an ATP-binding protein. Yeast membranes (1 mg of total protein) from *MATa ste6Δ* cells expressing YMdr1 from pYKM77 [Mdr1 (2μ)] (lanes 1–4) or from the same cells carrying the YEp352 vector alone (Δ; lane 5) were incubated with 8-azido-[α-^{32}P]ATP and then irradiated with ultraviolet light (+UV) or not irradiated (−UV). YMdr1 protein was immunoprecipitated from each sample using specific antibodies and subjected to electrophoresis in a 7% polyacrylamide gel containing SDS; the content of radioactivity in the gel was visualized by using a Phosphoimager (Molecular Dynamics, San Diego). Lane 1, YMdr1 photolabeled in the presence of excess (1 mM) GTP; lane 2, YMdr1 photolabeled in the presence of excess (1 mM) ATP; lane 3, YMdr1 incubated with 8-azido-[α-^{32}P]ATP without irradiation; lane 4, YMdr1 photolabeled in the absence of added competing nucleoside triphosphate; lane 5, control membranes lacking YMdr1 photolabeled in the absence of added competing nucleoside triphosphate.

by competition by either ATP or GTP [as also observed in animal cell membranes (39)] and because appearance of the photolabeled product required UV irradiation (Fig. 5).

Phenotypic Consequence of YMdr1 Expression. If YMdr1 was correctly folded, localized to the plasma membrane, and capable of binding ATP, it should confer on yeast cells the drug-resistance phenotype that is the hallmark of Mdr1 overproduction in animal cells. Nearly all compounds used for selection of Mdr1 overexpression are not toxic to yeast cells, presumably because they cannot pass through the yeast cell wall (41). One agent, valinomycin, a peptide antibiotic shown to be toxic to yeast cells (7), affects Mdr1 function in animal cells (42). Therefore, a simple agar plate assay was used to determine whether yeast cells expressing YMdr1 exhibited a drug-resistance phenotype. Cells carrying the parent control vector (YEp352) were unable to form single colonies on plates containing 125 μM valinomycin. Expression of YMdr1 from the low-copy CEN plasmid (pRKM7) permitted the cells to grow as isolated single colonies at the same drug concentration. More abundant overexpression of YMdr1 from the 2-μm multicopy plasmid (pYKM77) permitted the cells to grow almost normally in the presence of the drug (Fig. 6 *Upper*). Growth of the cells carrying pYKM77 could be observed at concentrations of valinomycin as high as 200 μM. When 10^5 cells were spotted on the surface of a plate containing 150 μM valinomycin and incubated at 30°C, cells overexpressing YMdr1 from pYKM77 were again able to grow (Fig. 6 *Lower*), even though on medium containing valinomycin the growth rate of cells carrying pYKM77 was

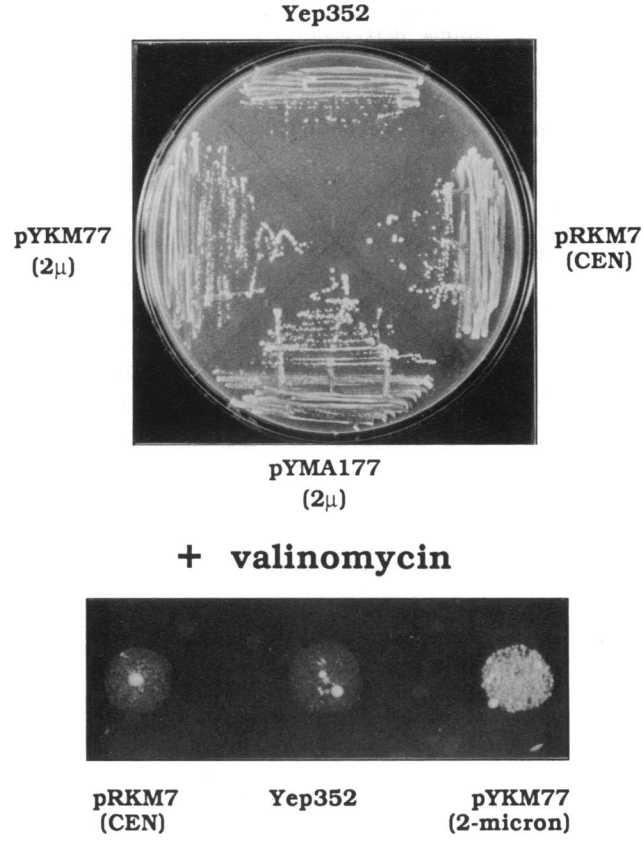

FIG. 6. YMdr1 expression confers valinomycin resistance. (*Upper*) *MATa ste6Δ* cells carrying different plasmids were streaked on an SD plate containing 125 μM valinomycin and incubated at 30°C for 48 hr. (*Lower*) Equal numbers of *MATa ste6Δ* cells (10^5) carrying plasmids pYKM77, pRKM7, and YEp352 were spotted on an SD plate containing 150 μM valinomycin, and growth was monitored at 30°C for 48 hr.

estimated to be 2- or 3-fold slower than that of wild-type cells on the same medium lacking the drug (data not shown).

These results suggest that at least some fraction of the YMdr1 expressed in yeast is functional. The inability of YMdr1 to complement a *ste6Δ* mutation indicated that it was unable to catalyze membrane translocation of **a** factor. Despite the lack of transport, however, it was possible that **a** factor could bind to YMdr1. If so, overexpression of intracellular **a** factor might compete for drug binding to YMdr1 and thereby decrease or eliminate valinomycin resistance. However, cooverexpression of both *MFa1* and YMdr1 from plasmid pYMA177 did not detectably reduce valinomycin resistance (Fig. 6 *Upper*), suggesting that YMdr1 may not be capable of even interacting with **a** factor.

The ability of YMdr1 to be targeted to the plasma membrane, to bind ATP, and to confer a drug-resistant phenotype indicates that the structure and function of YMdr1 have been preserved in *S. cerevisiae*. Although YMdr1 did not compensate for the loss of Ste6 function, it should be noted that the *mdr1* cDNA used in our experiments was isolated from a drug-resistant cell line and carries a G185V mutation that enhances its selectivity for colchicine over vinblastine (43). Perhaps the same mutation interferes with the ability of YMdr1 to recognize **a** factor.

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