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Candida Drug Resistance Protein 1, a Major Multidrug ATP Binding Cassette Transporter of *Candida albicans*, Translocates Fluorescent Phospholipids in a Reconstituted System†

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Abstract

Candida albicans drug resistance protein 1 (Cdr1p), an ATP-dependent drug efflux pump, contributes to multidrug resistance in *Candida*-infected immunocompromised patients. Previous cell-based assays suggested that Cdr1p also acts as a phospholipid translocator. To investigate this, we reconstituted purified Cdr1p into sealed membrane vesicles. Comparison of the ATPase activities of sealed and permeabilized proteoliposomes indicated that Cdr1p was asymmetrically reconstituted such that ~70% of the molecules had their ATP binding sites accessible to the extravesicular space. Fluorescent glycerophospholipids were incorporated into the outer leaflet of the proteoliposomes, and their transport into the inner leaflet was tracked with a quenching assay using membrane-impermeant dithionite. We observed ATP-dependent transport of the fluorescent lipids into the inner leaflet of the vesicles. With ~6 molecules of Cdr1p per vesicle on average, the half-time to reach the maximal extent of transport was ~15 min. Transport was reduced in vesicles reconstituted with Cdr1p variants with impaired ATPase activity and could be competed out to different levels by a molar excess of drugs such as fluconazole and miconazole that are known to be effluxed by Cdr1p. Transport was not affected by ampicillin, a compound that is not effluxed by Cdr1p. Our results suggest a direct link between the ability of Cdr1p to translocate fluorescent phospholipids and efflux drugs. We note that only a few members of the ABC superfamily of *Candida* have a well-defined role as drug exporters; thus, lipid translocation mediated by Cdr1p could reflect its cellular function.

Overexpression of the multidrug transporter Cdr1p (*Candida* drug resistance protein 1), a member of the ABC[1] (ATP binding cassette) transporter superfamily (1–3), accounts for a clinically significant mechanism of azole resistance in the pathogenic yeast *Candida albicans*. This is especially clear in fluconazole-resistant clinical isolates of *C. albicans*, where enhanced expression of Cdr1p promotes efflux of therapeutic azoles, thus facilitating cell survival (4–6). Cdr1p, like other ABC transporters, uses ATP hydrolysis to power the transport

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of substrates across membranes. Also, like most ABC drug transporters, Cdr1p is promiscuous in its choice of substrates and effluxes a variety of structurally unrelated compounds (2,7,8).

Phospholipids in the plasma membrane of most eukaryotic cells are asymmetrically distributed across the inner and outer leaflets, such that the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are predominantly localized in the inner leaflet (9,10). Maintenance of lipid asymmetry is due to transporters that specifically flip (translocate out-to-in) or flop (translocate in-to-out) phospholipids. P-type ATPases have been identified as phospholipid flippases (11), whereas ABC transporters and members of the family of multidrug resistance proteins such as mouse Mdr1 and human MDR1 and MDR3 have been shown to act as floppases in the plasma membrane of intact cells (12–16). Direct evidence that ABC transporters can translocate phospholipids was derived from experiments in which purified human MDR1/Pgp, human MRP1, and *Lactococcus lactis* LmrA (a human P-glycoprotein (Pgp) homologue) were reconstituted into proteoliposomes and shown to translocate fluorescent phospholipid analogues in an ATP-dependent fashion (17–20).

There are no reports to date of the reconstitution of phospholipid translocation by purified fungal ABC proteins. Previous work by Decottignies et al. demonstrated that the yeast ABC transporters Pdr5p and Yor1p likely translocate the fluorescent PE analogue NBD-PE at the plasma membrane of live cells, since cells lacking these proteins showed increased levels of accumulation of the fluorescent lipid (21). Similar results were obtained with the Pdr5p homologues in *C. albicans*: Cdr1p, Cdr2p, Cdr3p, and Cdr4p. These latter studies suggested that the drug efflux pumps Cdr1p and Cdr2p are able to flop fluorescent phospholipids, while Cdr3p—which does not efflux drugs—acts as a phospholipid flippase (22). In an effort to understand the molecular basis of the lipid translocase activity of Cdr1p, we chose a biochemical reconstitution approach. Here we describe the functional reconstitution of purified Cdr1p and characterization of its ATP-dependent phospholipid translocase activity using fluorescent phospholipid analogues.

EXPERIMENTAL PROCEDURES

Materials

Triton X-100, *n*-dodecyl *β*-D-maltoside, imidazole, ATP, anisomycin, cycloheximide, miconazole, sodium dithionite, egg phosphatidylcholine, creatine kinase, creatine phosphate, and other analytical grade chemicals were obtained from Sigma Chemical Co. Ni^{2+}-NTA Superflow and Q Sepharose fast flow resins, as well as protease inhibitors, were obtained from Amersham Biosciences Ltd. SM2 Bio-Beads were purchased from Bio-Rad. Fluconazole was provided by Ranbaxy Laboratories (New Delhi, India). NBD-PC (1-acyl-2-[1-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]dodecanoyl]-*sn*-glycero-3-phosphocholine, NBD-PE (1-acyl-2-[6-[(7-nitro-2,1,3- benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphoethanolamine, and NBD-PS (1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-*sn*-glycero-3-phosphoserine were obtained from Avanti Polar Lipids, Inc., and *N*-NBD-PE (*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine salt) was obtained from Molecular Probes.

[1]Abbrebiations: ABC, ATP binding cassette; Aniso, anisomycin; CM, crude membrane; DDM, *n*-dodecyl *β*-D-maltoside; ePC, egg phosphatidylcholine; MDR, multidrug resistance; Mic, miconazole; PM, plasma membrane; R6G, rhodamine 6G; TMD, transmembrane domain; TMS, transmembrane segment; NBD-PC, 1-acyl-2-[1-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PE, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphoethanolamine; NBD-PS, 1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoserine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine salt.

Bacterial and Yeast Strains and Growth Media

Plasmids were maintained in *Escherichia coli* XL-1 blue. *E. coli* was cultured in Luria–Bertani medium (Difco) to which ampicillin was added (100 μg/mL). *Saccharomyces cerevisiae* VyCDR1H, SSCH-C193A, SSCH-A1345G, SSCH-T1355A, and SSCH-F1360A (Table 1) were AD1-8u$^-$ derivatives expressing Cdr1p–His_6 and its mutant variants. The yeast strains were cultured in yeast extract–peptone–dextrose (YEPD) broth (Difco). For agar plates, 2.5% (w/v) Bacto agar (Difco) was added to the medium.

Site-Specific Mutagenesis and Development of Transformants

Site-directed mutagenesis was performed by using the QuickChange mutagenesis system from Strategene. Mutations were introduced into plasmid psCdr1H according to the manufacturer's instructions using primers 5′-GAATT-AGCTGATAATGGTGCCAATTTGGCTAC-3′ (forward) and 5′-GTAGCCAAATTGGCACCATTATCAGCTTAAT-TC-3′ (reverse) for mutating Ala1346 to Gly, 5′-GCTACATTGTTATTTGCTATGTGTTTGAATTTC-3′ (forward) and 5′-GAAATTCAAACACATAGCAAATAACAATGTAGC-3′ (reverse) for mutating Thr1355 to Ala, and 5′-CTATGTGTTTGAATGCCTGTGGTGTTTTAGC-3′ (forward) and 5′-GCTAAAACACCACAGGCATTGAAACA-CATAG-3′ (reverse) for mutating Phe1360 to Ala. Nucleotide sequence alterations were confirmed by DNA sequencing of the ORF. The wild-type construct psCdr1H and its mutated versions were linearized with *Xba*I and used to transform AD1-8u$^-$ cells for uracil prototrophy by the lithium acetate transformation protocol (23).

Southern Analysis and Immunodetection of Cdr1p

Genomic DNA was isolated from *S. cerevisiae* cells, and Southern analysis was performed to check for single-copy integration into the genomic DNA of AD1-8u$^-$ cells as described previously (24). Immunodetection of Cdr1p in plasma membrane fractions was done with anti-penta-His monoclonal antibodies (used at a dilution of 1:5000) according to the manufacturer's protocol (ECL kit, Amersham Biosciences).

FACS Assay for Lipid Translocation in Live Cells

Translocation of NBD-labeled phospholipids at the plasma membrane was assayed by FACS as previously described (21). Briefly, *S. cerevisiae* cells expressing the Cdr1p construct of choice were grown overnight in SDC (except for Ad1-8u$^-$ cells, for which uridine (0.02%) was added to the medium) at 30 °C, diluted, and allowed to grow to an A_{600} of 0.2–0.3. NBD-PC (1 mg/mL in DMSO) was added to the cells at a final concentration of 50 μM and incubated for 60 min at 37 °C. The cells were washed three times with ice-cold $SCNaN_3$ (SDC lacking glucose but containing 2% sorbitol and 20 mM sodium azide) prior to analysis by flow cytometry.

Flow cytometric analysis of the NBD-PC-labeled cells was performed with a FACScan cytometer (Becton-Dickinson Immunocytochemistry Systems) equipped with an argon laser operating at 488 nm. A 10 μL portion of a 50 mg/mL stock solution of propidium iodide was added to approximately 4×10^5 cells in 200 μL of $SCNaN_3$ immediately prior to dilution (3-fold) and flow cytometric analysis. A total of 10 000 cells were analyzed without gating during acquisition. Analysis was performed with Cell Quest Pro (Becton-Dickinson Immunocytochemistry Systems) software. Histograms of the extent of NBD fluorescence accumulation in live cells were generated, and the mean and standard deviation of the fluorescence intensity were calculated.

Purification of Cdr1p

Crude membranes were prepared from Cdr1p-expressing *S. cerevisiae* as described earlier (24). The membranes (generated from ~18 g wet weight of late exponential phase cells) were solubilized in a total volume of 10 mL by stirring with 1% (w/v) Triton X-100 for 5 min at room temperature, followed by stirring at 4 °C for 30 min. The mixture was spun at 18 000 rpm at 4 °C for 45 min, yielding a pellet of insoluble material, a clear yellow-tinted supernatant, and a floating milky layer, likely lipid. The supernatant was collected avoiding the surface lipid layer for further purification of Cdr1p.

Solubilized protein was added to preequilibrated Ni-NTA Superflow resin (Qiagen) in 0.05% (w/v) Triton X-100 and 20 mM Tris (pH 7.5) and mixed for 2 h at 4 °C. The resin was washed extensively with buffer A (20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 30 mM imidazole, and protease inhibitors as described previously (24)) before elution of bound protein with buffer B (50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 10% (w/v) glycerol, 150 mM NaCl, 500 mM imidazole, and 0.05% (w/v) Triton X-100). The eluted fraction was dialyzed for 12 h against 1 L of buffer (50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 10% (w/v) glycerol, 30 mM NaCl, and 0.05% (w/v) Triton X-100). After dialysis, the protein sample was incubated with Q-Sepharose Fast Flow resin (preequilibrated in 50 mM Tris (pH 7.5) and 0.01% (w/v) Triton X-100) (Amersham) for 1 h at 4 °C. The resin was washed with buffer C (50 mM Tris (pH 7.5), 2 mM $MgCl_2$, 50 mM NaCl, 0.05% (w/v) Triton X-100, and 1% (w/v) glycerol) before elution of protein with buffer D (Tris 20 mM (pH 7.5), 2 mM $MgCl_2$, 200 mM NaCl, and 0.1% (w/v) Triton X-100).

Reconstitution of Purified Cdr1p

Purified Cdr1p was combined with a solution of egg phosphatidylcholine (ePC) in a Triton X-100-containing buffer, and proteoliposomes were prepared by treating the mixture with detergent-adsorbing resin. Briefly, 30 μL (~4 μmol) of 100 mg/mL ePC (from a stock solution prepared in chloroform and stored at −20 °C) was transferred to a screw-cap glass tube and dried under a gentle stream of N_2 to remove the organic solvent completely. The dried lipid sample was dissolved in 500 μL of reconstitution buffer (20 mM Tris (pH 7.5), 2 mM $MgCl_2$, and 2% (w/v) Triton X-100) by incubation at room temperature with occasional gentle vortexing (avoiding frothing) until a clear solution was obtained. Purified Cdr1p was then added (500 μL, typically containing 20 μg of protein in buffer D). Detergent was removed by adding 100 mg of freshly washed SM2 Bio-Beads (prepared by being washed with methanol (25 mL/g of beads), three times with water, and finally with buffer containing 20 mM Tris (pH 7.5), 2 mM $MgCl_2$, and 100 mM NaCl; the washes were done with stirring for 15 min at room temperature, and the final buffer was removed to obtain a preparation of damp beads for use in reconstitution) and rotating the sample at room temperature for 3 h, followed by a further addition of 200 mg of Bio-Beads and rotation for an additional 18 h at 4 °C. The turbidity of the sample increased during the incubation as proteoliposomes were formed. After the incubation period, the turbid supernatant was withdrawn and used for further experiments. Residual Triton X-100 was measured by absorbance as described (25). Soluble protein was quantified according to the Bradford method (26), and the result was confirmed on occasion with a BCA (bis-cinchonic acid) protein assay kit (Sigma). The protein content of proteoliposomes was measured according to the procedure of Kaplan and Pedersen (27). Phospholipids were quantitated as described previously (28). Protein and phospholipid recovery was ~60% reproducible.

Determination of the Leakiness of the Proteoliposomes

To assess the intactness of the proteoliposomes, vesicles were prepared exactly as described above except that 0.3 mol % NBD-PC was dried down along with 4 μmol of ePC to prepare vesicles containing fluorescent lipid symmetrically distributed in both leaflets. Protein-free

liposomes were prepared in parallel. A 100 μL portion of the vesicle preparation (~0.24 μmol of phospholipid) was added to 1.9 mL of transport buffer (10 mM Tris–Cl (pH 7.5), 200 mM sucrose, and 5 mM $MgCl_2$). After a brief incubation (5 min) in the dark at room temperature, the fluorescence intensity of the sample was measured (excitation, 470 nm; emission, 540 nm). This value was defined as 100%. Sodium dithionite (1 M stock solution prepared in 1 M Tris–Cl (pH 10)) was then added to a final concentration of 5 mM, and the fluorescence of the sample was recorded at time intervals for a total time of 10 min. Triton X-100 was then added (1% (w/v) final concentration) to permeabilize the membranes, and fluorescence was measured for another 10 min.

Fluorescence Quenching Assay for Phospholipid Translocation in Reconstituted Vesicles

Stock solutions (1 mg/mL) of NBD-PC and NBD-PS were made by dissolving the lipids in transport buffer (10 mM Tris–Cl, 200 mM sucrose, and 5 mM $MgCl_2$ (pH 7.5)), whereas NBD-PE (1 mg/mL) was dissolved in 10% dimethyl sulfoxide (DMSO). *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (headgroup labeled, with dihexadecanoyl acyl chains) was dissolved in DMSO by incubation at 37 °C with intermittent vortexing. Proteoliposomes (100 μL, ~0.24 μmol of phospholipid) were diluted with 1.9 mL of transport buffer, and the NBD-lipid of choice was added (final 1.5 μM). The sample was incubated for 10 min at room temperature to ensure complete incorporation of the NBD-lipid probe into the outer leaflet of the vesicles. To initiate lipid transport, 50 μL of transport buffer or 50 μL of transport buffer containing ATP and an ATP-regenerating system (17) was added (the final concentrations were 3 mM ATP, 30 μg/mL creatine kinase, and 3 mM creatine phosphate). The samples were kept at 30 °C in the dark for the desired length of time (0–90 min) before addition of dithionite to a final concentration of 5 mM and incubation for 10 min at room temperature. The samples were then taken for fluorescence measurement. Incubation with dithionite for 10 min was enough to completely quench the fluorescence of labeled lipids present in the outer leaflet (prolonged incubation did not make any significant difference).

The percentage maximal quenching (ΔF_{max} (%)) in each case was calculated as follows:

$$\Delta F_{max}(\%) = [F_T - F_D/F_T] \times 100$$

where F_T is the total fluorescence of the sample before addition of dithionite and F_D is the fluorescence of the sample after quenching with dithionite. The percentage maximal quenching reflects the movement of labeled lipids from the outer leaflet to the inner leaflet. If Cdr1p in proteoliposomes mediates ATP-dependent translocation of NBD-phospholipids from the outer monolayer to the inner monolayer of the vesicle, there will be less quenching by dithionite compared to that of the controls, where no transport occurs.

To monitor lipid translocation activity in the presence of drugs, drug substrates were added to the desired concentrations after labeling of the vesicles with NBD-lipids. The samples were incubated for 10 min before addition of transport buffer and assaying of lipid translocation as described above.

RESULTS

Cdr1p-Mediated Translocation of NBD-PC at the Plasma Membrane of Intact Cells

In preliminary experiments, we tested the phospholipid floppase activity of a His-tagged variant of Cdr1p that was overexpressed in *S. cerevisiae* (strain VyCdr1H). To do this, we used flow cytometry to determine the extent to which an exogenously supplied fluorescent analogue of phosphatidylcholine (NBD-PC) accumulated in the cells (21,29,30). When NBD-PC is added to intact cells, it is translocated across the plasma membrane and subsequently, on account of its slight water solubility, partitions spontaneously into various organellar

membranes, where it accumulates (31). In comparison with that in wild-type cells, less NBD-PC is expected to accumulate in cells that overexpress a plasma-membrane-localized in-to-out phospholipid translocator. VyCdr1H cells were incubated with NBD-PC for 1 h at 37 °C, and the extent of fluorescence accumulation was analyzed by flow cytometry. The host strain (Ad1-8u^{-}, lacking Cdr1p) was analyzed in parallel. The fluorescence overlay histogram plot in Figure 1 shows that VyCdr1H cells (trace c) accumulate ~28% less NBD-PC than the host strain (trace b), consistent with the expression of Cdr1p floppase activity at the plasma membrane in these cells. To investigate this further, we tested a functionally impaired variant of Cdr1p (Cdr1p-C193A). We previously showed that the Cdr1p-C193A mutant is severely impaired in its ATPase and drug efflux activities and that its expression in place of wild-type Cdr1p renders cells hypersensitive to drugs (24,32). Figure 1 shows that the extent of NBD-PC accumulation in SSCH-C193A cells overexpressing His-tagged Cdr1p-C193A (trace d) is comparable to that of the host strain Ad1-8u^{-}. These results suggest that Cdr1p acts as a plasma-membrane-localized phospholipid floppase when expressed in *S. cerevisiae*.

Asymmetric (Inside-Out) Reconstitution of Purified Cdr1p with Unimpaired ATPase Activity

To explicitly test and characterize the proposed ATP-dependent phospholipid translocase activity of Cdr1p, we purified the protein and reconstituted it into membrane vesicles. Our previous work indicated that reconstitution of dodecyl maltoside (DDM)-solubilized protein results in proteoliposomes where Cdr1p is predominantly (75–80%) in a right-side-out (RO) orientation; i.e., its ATP binding site is oriented toward the vesicle lumen, making it difficult to measure ATP-dependent transport. In testing various detergents, we discovered that solubilization of VyCdr1H cells in Triton X-100 followed by a two-step purification protocol yielded pure Cdr1p (Figure 2A) with essentially unimpaired ATPase activity (~0.70 (μmol/mg)/min, similar to ~0.81 (μmol/mg)/min measured for DDM-solubilized Cdr1p). Reconstitution of this material into ePC vesicles yielded proteoliposomes with Cdr1p in a predominantly inside-out (IO) orientation (ATP binding sites facing out) as described below.

For a standard reconstitution, purified Cdr1p (20 μg) was combined with ~4 μmol of Triton X-100-solubilized ePC to prepare mixed micelles. The sample was incubated with SM2 Bio-Beads, initially for 3 h at room temperature (24 °C) and then for another 18 h at 4 °C, to remove detergent quantitatively (25). The detergent removal procedure led to the formation of sealed unilamellar vesicles (data not shown), ~150 nm in diameter, with ~20% loss of Cdr1p ATPase activity (measured after solubilization in Triton X-100) and ~40% loss of ePC and protein (data not shown). On the basis of the protein:phospholipid ratio of the recovered vesicles (5 μg/μmol, corresponding to a 1:160 (w/w) protein:phospholipid ratio), the molecular mass of Cdr1p (~170 kDa), and the average vesicle diameter, we estimate about six copies of Cdr1p per vesicle.

We assessed the orientation of Cdr1p in the proteoliposomes by determining the ratio of its ATPase activity in intact and detergent-permeabilized vesicles. ATPase activity was ~0.55 (μmol/mg)/min in intact vesicles and increased to ~0.81 (μmol/mg)/min after permeabilization with 0.05% (w/v) Triton X-100 (Figure 2B). Since some detergents are known to increase ATPase activity, we excluded the possibility of any stimulatory effect of Triton X-100 on Cdr1p activity by using DDM (0.1%, w/v) or Brij 58 (0.05%, w/v) instead (33). Permeabilization of Cdr1p vesicles with these detergents yielded values of ATPase activity comparable to that seen with Triton X-100-permeabilized vesicles (Figure 2B). Thus, if one compares the ATPase activities of Cdr1p between nonpermeabilized and detergent-permeabilized proteoliposomes, it is apparent that ~70% of the Cdr1p molecules (or about four molecules per vesicle) are in an IO orientation in the reconstituted proteoliposomes.

We considered the possibility that our conclusions concerning the IO orientation of reconstituted Cdr1p could be due to leakiness of the vesicles. To investigate this, we tested to

see whether the vesicles were sealed to small molecules such as the dianionic reductant dithionite. Cdr1p-containing proteoliposomes were prepared from a mixture of ePC and ~0.3 mol % NBD-PC, resulting in the NBD-PC tracer being located evenly between both leaflets of the vesicles (34). When dithionite was added to the vesicles, a ~50% decrease in fluorescence was observed corresponding to the reduction of NBD fluorophores located in the outer leaflet of the vesicles (Figure 2C). No further decrease was observed on addition of additional dithionite, indicating that the reductant was not limiting. These results indicate that ~50% of the NBD-PC population is located in the inner leaflet of the vesicles and protected from dithionite reduction. Permeabilization of the proteoliposomes with 1% (w/v) Triton X-100 allowed dithionite access to the inner leaflet pool of NBD-PC, resulting in rapid loss of all the fluorescence (Figure 2C). These results indicate that the Cdr1p-containing proteoliposomes are sealed and reinforce our conclusion that ~70% of the reconstituted Cdr1p molecules are in an IO orientation.

Reconstituted Cdr1p Displays Phospholipid Translocase Activity

For phospholipid translocase activity measurements, Cdr1p-containing vesicles were incubated with a trace amount of NBD-PC in transport buffer for 10 min at room temperature. Insertion of NBD-PC into the outer leaflet of the vesicles was determined by monitoring the increase in NBD fluorescence (the quantum yield of NBD is low in water and increases considerably when the fluorophore associates with the membrane bilayer (34**)**. As shown in Figure 3A, labeling was completed within ~3 min for both Cdr1p-containing proteoliposomes and protein-free liposomes. To ensure that all the exogenously supplied NBD-PC was inserted into the vesicles, we routinely performed the labeling reaction for 10 min at room temperature. Addition of dithionite to vesicles labeled in this way resulted in >98% loss of fluorescence, confirming that the NBD-PC was localized to the outer leaflet (see below).

To test the ATP-dependent phospholipid floppase activity of Cdr1p, we added ATP and an ATP-regenerating system to the NBD-PC-labeled proteoliposomes. At various time points over the course of 60 min, samples were withdrawn and treated with dithionite to determine the amount of NBD-PC remaining in the outer leaflet. As shown in Figure 3B, the pool of NBD-PC in the outer leaflet decreased monoexponentially ($t_{1/2} \approx 30$ min) to reach a predicted plateau value of ~85% when translocase activity was monitored with proteoliposomes reconstituted with 20 μg of protein (filled squares). This indicates translocation of up to ~15% of the NBD-PC from the outer to the inner leaflet. Experimentally, however, only ~8% translocation of NBD-PC was observed even with extended incubations and ATP supplementation; this could be a consequence of loss of activity of Cdr1p or because of lipid buildup in the inner leaflet of the vesicles. Translocase activity also depended on the protein concentration used for the reconstitution: translocation was slower when vesicles were prepared with less Cdr1p (Figure 3B, filled and open triangles corresponding to reconstitutions performed with 10 and 15 μg of Cdr1p, respectively). No transport was observed when ATP was omitted or when protein-free liposomes were analyzed (Figure 3B). By employing symmetrically labeled proteoliposomes, as well as proteoliposomes containing fluorescent lipid solely in the inner leaflet (generated after dithionite treatment of symmetrically labeled vesicles), we ruled out the possibility that Cdr1p can act as a scramblase, i.e., translocate lipids in both directions (data not shown). These results indicate that Cdr1p mediates ATP-dependent translocation of NBD-PC from the outer to the inner monolayer of vesicles.

We further investigated the ATP dependence of transport by examining the floppase activity of Cdr1p preparations with impaired ATPase activity. We tested the floppase activity of the Cdr1p-C193A ATPase mutant as well as that of *N*-ethylmaleimide (NEM)-treated wild-type Cdr1p (35). Reconstituted Cdr1p-C193A exhibited severely impaired—albeit nonzero ATPase activity (Figure 3C) and a corresponding loss in floppase activity (Figure 3D; the floppase

activity was ~55% lower for the C193A mutant compared with the wild type (as assessed at the 60 min time point). NEM treatment inhibited the ATPase activity of Cdr1p by ~65% (Figure 3C) and reduced its phospholipid translocase activity by ~45% (Figure 3D). Taken together, these data confirm that purified, reconstituted Cdr1p exhibits ATP-dependent phospholipid translocase activity.

Cdr1p Translocates a Variety of Fluorescently Tagged Glycerophospholipids

To assess the specificity of the Cdr1p phospholipid translocase activity, proteoliposomes were labeled with NBD-PE, NBD-PS, or NBD-PC, and ATP-dependent transport of these lipids from the outer to the inner leaflet of the vesicles was monitored as described above. Figure 4A,C,D shows that, like NBD-PC, NBD-PE and NBD-PS are both transported by Cdr1p in an ATP-dependent fashion. Transport of all NBD-lipids ceased after ~60 min; no further translocation was observed even if the incubation time was prolonged to 90 min or a fresh aliquot of ATP was added. Of note, translocase activity was also observed with *N*-NBD-PE (Figure 4B), a headgroup-modified NBD-phospholipid possessing hexadecanoyl acyl chains, indicating that the slight water solubility of the acyl-NBD-phospholipid analogues (assayed in Figure 4A,C,D) did not promote their translocation by Cdr1p. A comparison of the transport assays for the four different lipids tested indicated that the maximum protection of fluorescence, i.e., maximum extent of transport, was observed with NBD-PE (~9%), followed by NBD-PC (~7.6%), NBD-PS (~5.3%), and *N*-NBD-PE (~5%) (Figure 4).

Drug Efflux and Phospholipid Translocase Activity of Cdr1p

We next tested whether the phospholipid translocase activity of Cdr1p is linked to its ability to efflux hydrophobic drugs from cells. To do this, we assayed phospholipid translocation in the presence of drugs that are known to be effluxed by Cdr1p; we also tested ampicillin, a compound that is not a substrate for Cdr1p's drug efflux activity. We observed that a variety of structurally unrelated drug substrates (anisomycin, cycloheximide, fluconazole, and miconazole) blocked NBD-PE translocation in a concentration-dependent manner (Figure 5A), with cycloheximide being the most efficacious blocker and fluconazole the least effective (Figure 5B). Phospholipid translocase activity was not blocked by ampicillin (Figure 5B), confirming that only drugs that are effluxed by Cdr1p are able to inhibit phospholipid translocation by the protein. These data suggest that the translocations of NBD-phospholipids and drugs by Cdr1p are linked.

We previously generated a battery of mutant variants of Cdr1p that display different degrees of drug transport (36–38). In the present study, we employed some of these mutants (Cdr1p-A1346G, Cdr1p-T1355A, and Cdr1p-F1360A (36)) in an attempt to define potential common drug and phospholipid binding sites of Cdr1p. The cells expressing these mutants displayed different substrate specificities as monitored by rhodamine 6G efflux (Figure 6B). For example, efficient competitors of rhodamine 6G efflux such as ketoconazole, itraconazole, and miconazole became totally ineffective in cells expressing Cdr1p-F1360A. The inability of ketoconazole to compete with rhodamine 6G in cells expressing Cdr1p-T1355A and of miconazole to compete with rhodamine 6G in cells expressing the A1346G variant further suggests differential interactions of Cdr1p residues with various substrates. For example, it appears that while F1360 may represent part of the substrate binding pocket for all three azoles, A1346 and T1355 may be specific to miconazole and ketoconazole binding, respectively (Figure 6B). We purified His-tagged variants of the Cdr1p mutants (Figure 6A) and reconstituted them into ePC vesicles. Interestingly, all three purified mutant proteins were impaired in their ability to translocate NBD-PE (Figure 6C).

DISCUSSION

Several indirect lines of evidence suggest that fungal ABC transporters such as the *S. cerevisiae* proteins Pdr5p and Yor1p and the *C. albicans* proteins Cdr1p–Cdr4p can translocate fluorescent glycerophospholipid analogues across membranes (reviewed in ref 12). In this study we directly demonstrate the lipid translocation activity of a member of this class of fungal proteins by showing that, on reconstitution into membrane vesicles, purified Cdr1p acts as an ATP-driven transporter capable of translocating different fluorescently modified glycerophospholipids between leaflets of the vesicle membrane.

We developed a procedure to reconstitute Cdr1p into unilamellar ePC vesicles asymmetrically such that ~70% of the reconstituted protein molecules had an inside-out orientation, i.e., ~70% of the reconstituted molecules had their ATP binding sites accessible in intact vesicles. Our reconstitutions generated vesicles with a typical phospholipid:protein ratio of 160:1 (w/w), with about six Cdr1p molecules per vesicle. We labeled the outer leaflet of the vesicles with NBD-labeled phospholipids (either glycerophospholipids bearing an NBD fluorophore covalently linked to a short 2-acyl chain or *N*-NBD-PE, a long-chain phospholipid with its ethanolamine headgroup covalently modified by NBD) and incubated the samples at 30 °C with or without ATP and an ATP-regenerating system. We assessed translocation of the fluorescent lipids to the inner leaflet of the vesicles by measuring the fraction of NBD fluorescence that could be irreversibly reduced by dithionite, a membrane-impermeant dianion. In the absence of ATP, essentially all (>98%) of the NBD-phospholipid in Cdr1p proteoliposomes was accessible to dithionite over the time course of the experiment, indicating that the lipids remained localized to the outer leaflet of the vesicles. However, on addition of ATP and an ATP-regenerating system, the pool of dithionite-accessible NBD-phospholipid decreased over time, consistent with translocation to the inner leaflet. Translocation stopped after ~ 60 min when 5–10% of the lipid reporter had been translocated (Figure 4), even if the samples were supplemented with additional ATP. This situation can be anticipated since vectorial translocation of lipid from the outer to the inner monolayer of a vesicle would cause an imbalance in monolayer surface area and limit the capacity of proteoliposomes to accumulate lipids in the inner leaflet while remaining intact. We note that the ability of Cdr1p to translocate phospholipids in the reconstituted system is not fully reflected in the flow-cytometry-based assay (Figure 1), where Cdr1p-expressing cells accumulated only 28% less NBD-PC than their wild-type counterparts: this could simply be explained by low expression levels of the Cdr1p in intact cells or the simultaneous up-regulation of compensatory transport activities.

Previous studies of the phospholipid translocation activity of purified and reconstituted ABC transporters used fluorescent NBD-labeled phospholipids as transport reporters (16–20). These studies did not explicitly assay transport of natural glycerophospholipids. Our experiments, likewise, were done with NBD-labeled phospholipids and do not address the issue of whether Cdr1p can also translocate natural phospholipids. This remains an open question. Since the acyl-chain-labeled NBD-phospholipids are slightly water-soluble (39), it is possible that they are not flopped by Cdr1p, but rather follow an aqueous translocation route where they desorb from the membrane and are extruded into the opposing aqueous phase by Cdr1p before partitioning into the exoplasmic leaflet (40). However, the ability of Cdr1p to translocate *N*-NBD-PE, which more closely resembles natural lipids because of its two long fatty acyl chains, argues against the “desorption and aqueous translocation” route being a general mechanism for translocation of NBD-phospholipids. Instead, translocation of *N*-NBD-PE is consistent with a floppase translocation mechanism since this lipid cannot readily desorb from the membrane.

The NBD fluorophore is relatively hydrophilic compared with typical drug substrates of Cdr1p. Nevertheless, it is possible that NBD-phospholipids, by virtue of their NBD tag, sufficiently

resemble the various drug substrates of Cdr1p so that they are recognized as xenobiotics and thus transported. Our result that specific mutations in Cdr1p that affect its ability to recognize azoles also affect its ability to transport NBD-PE (Figure 6) would appear to support this proposal. However, the three Cdr1p mutations tested (A1346G, T1355A, and F1360A) affected drug recognition quite differently (Figure 6B) while having a comparable inhibitory effect on NBD-PE transport (Figure 6C). This suggests that Cdr1p transports NBD-phospholipids by a path that overlaps but does not precisely follow the path used for drug extrusion. Also, the ability of Cdr1p to transport a variety of acyl-chain-labeled NBD-phospholipids as well as *N*-NBD-PE at comparable rates suggests that if the NBD group constitutes the recognition motif for Cdr1p-mediated translocation of NBD-phospholipids, then it would not need to be precisely located with respect to the membrane to be "seen" by Cdr1p. Resolution of these issues will require the development of sensitive assays to measure the translocation of natural phospholipids. An attractive possibility is the recently reported use of shape changes of giant unilamellar vesicles to detect differences in monolayer surface area that result from lipid translocation (41,42). Studies along these lines are under way.

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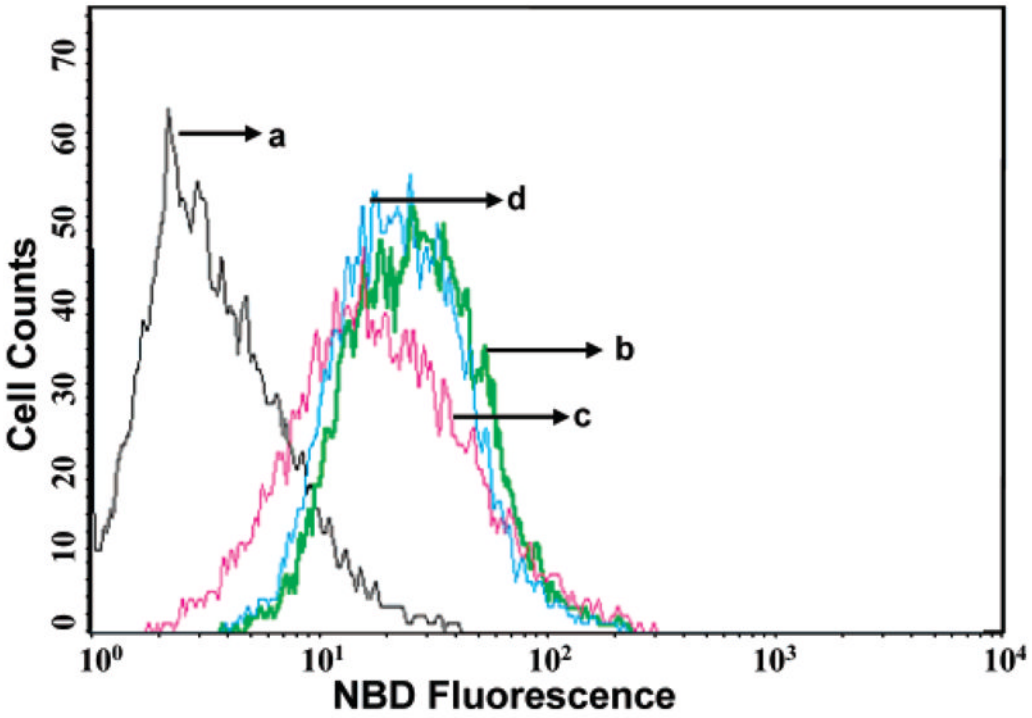

Figure 1.

Cdr1p mediates translocation of NBD-PC in vivo. The figure shows histograms obtained by flow cytometry analysis of cells after internalization of NBD-PC: unstained cells (black spectrum, a), host cells AD1-8u$^-$ (green, b), Cdr1p-overexpressing cells VyCdr1H (pink, c), and cells expressing the catalytically impaired mutant variant SSCH-C193A (blue, d).

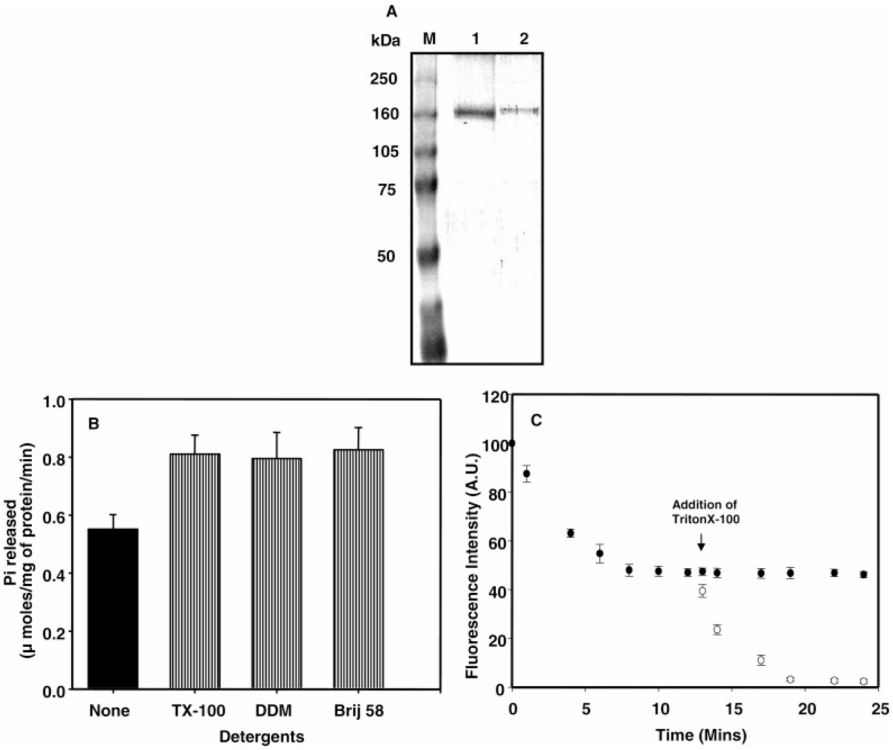

Figure 2.

Purification, ATPase activity, and determination of the leakiness of purified reconstituted Cdr1p. (A) A Coomassie-stained 8% SDS–PAGE gel is shown. Lanes: M, protein molecular mass markers; 1, Triton X-100-solubilized purified Cdr1p–His_6 (0.8 μg) after passing over Q-Sepharose resin; 2, 0.2 μg of Cdr1p reconstituted in proteoliposomes. (B) Analysis of the orientation of reconstituted Cr1p: ATPase activity of reconstituted Cdr1p as well as of Cdr1p-containing liposomes preincubated with Triton X-100 (0.05%), DDM (0.1%), and Brij 58 (0.05%). The values are given as the mean ± standard deviation for three independent experiments. (C) Measurement of the leakiness of proteoliposomes: Cdr1p proteoliposomes containing 0.3 mol % NBD-PC in both leaflets (●) were incubated for 5 min in transport buffer at 30 °C before fluorescence was recorded (excitation, 470 nm; emission, 540 nm). After addition of 5 mM dithionite at time zero, fluorescence was recorded at regular intervals. Once a stable signal was established (after ~10 min), the sample was separated into two different cuvettes. At the indicated time, Triton X-100 was added to one sample (○), whereas the other sample (●) received only buffer. The values plotted represent the average (± standard deviation) of three independent experiments.

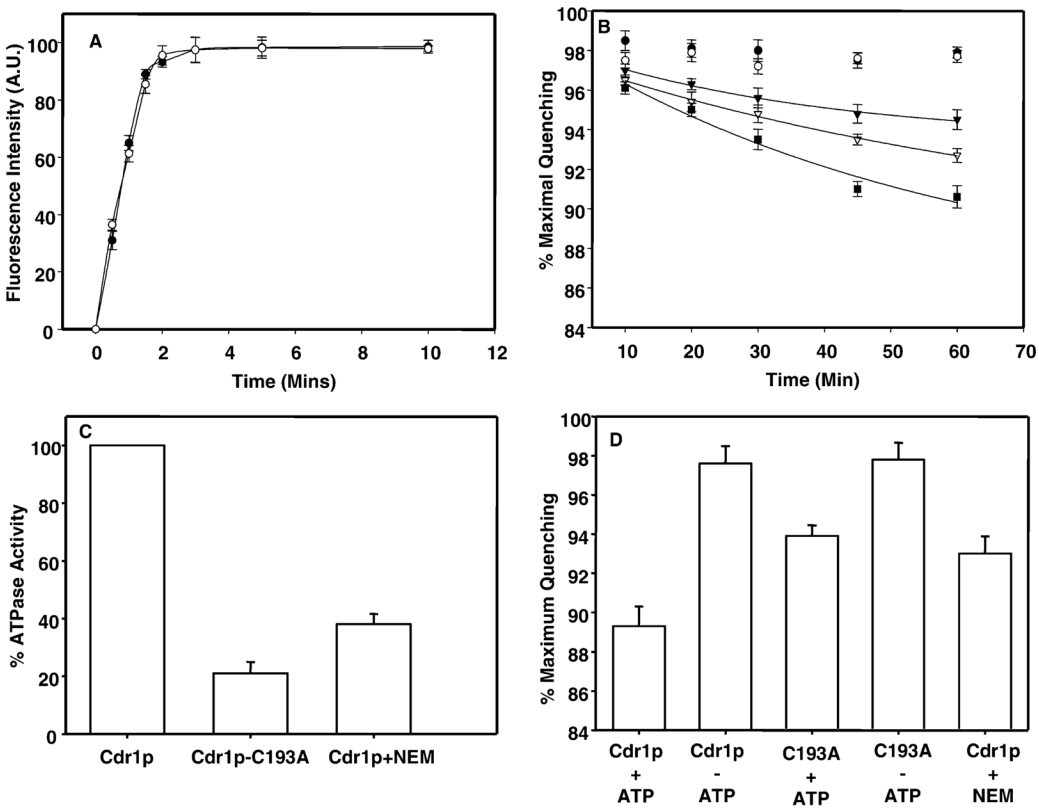

Figure 3.

Phospholipid translocase activity shown by purified reconstituted native and mutant variant proteins. Experiments were performed in triplicate, and the values plotted represent the average ± standard deviation. (A) Labeling of proteoliposomes and liposomes with NBD-PC. Cdr1p proteoliposomes (●) and liposomes (○) were incubated with 1.5 μM NBD-PC in transport buffer at room temperature, and fluorescence was recorded (excitation, 470 nm; emission, 540 nm) at the indicated times. (B) The phospholipid translocase activity was assayed with proteoliposomes containing different amounts of Cdr1p, using NBD-PC as the transport reporter. Reconstitutions were done with 10 μg (▼), 15 μg (▽), and 20 μg (■) of protein. ATP and an ATP-regenerating system were added after labeling of the vesicles with NBD-PC. The samples were kept at 30 °C; translocation was assayed by adding 5 mM dithionite to sample aliquots at the indicated time points and measuring fluorescence. Cdr1p proteoliposomes containing 20 μg of Cdr1p without an energy source (●) and liposomes (○) were taken as the negative control. (C) Comparison of ATPase activities. The bars represent the ATPase activity of the reconstituted mutant protein Cdr1p-C193A and reconstituted Cdr1p in the presence of 0.5 mM NEM, normalized to the ATPase activity of reconstituted Cdr1p. (D) Comparison of phospholipid translocase activities. Phospholipid translocase activities of reconstituted Cdr1p proteins were determined as the amount of NBD-PC translocated after 60 min at 30 °C. The bars show the percentage maximal quenching of reconstituted Cdr1p in the presence and absence of the ATP-regenerating system and also in the presence of 0.5 mM NEM. The bars also represent the translocase activity of the mutant purified reconstituted protein Cdr1p-C193A in the presence and absence of an energy source.

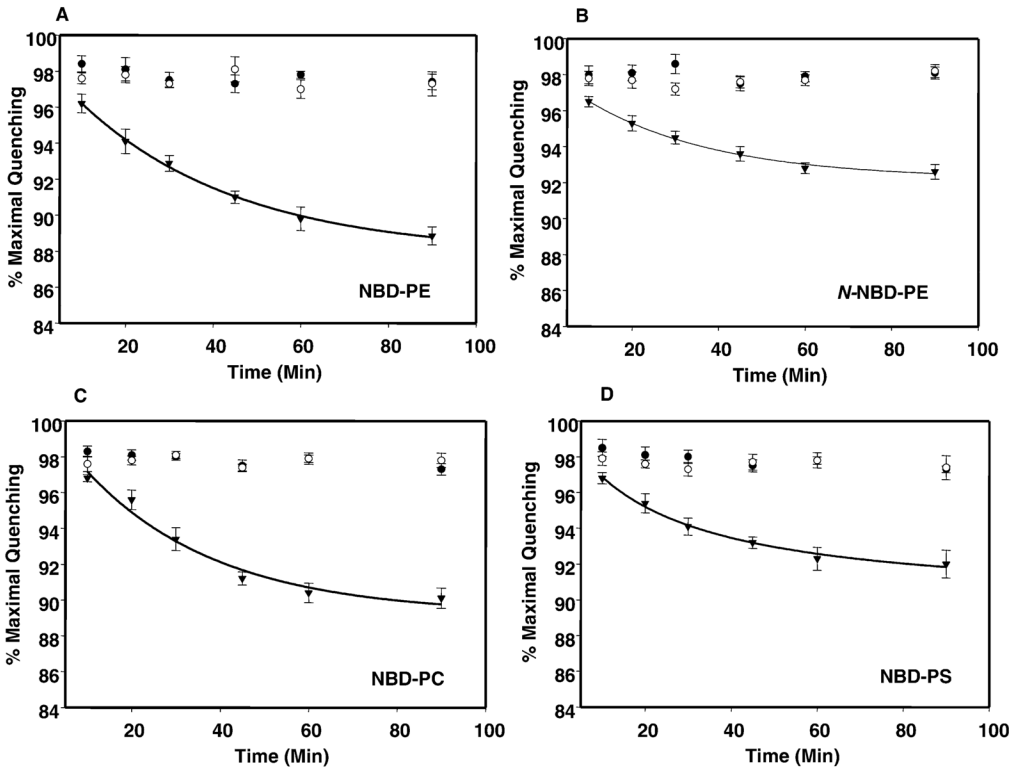

Figure 4.

Cdr1p-mediated translocation of different labeled phospholipids. Proteoliposomes either with (▼) or without (○) ATP and an ATP-regenerating system, as well as liposomes (●), were incubated for different time periods, and dithionite reduction was used to determine the extent of translocation of the NBD-labeled phospholipids indicated: (A) NBD-PE, (B) *N*-NBD-PE (1,2-dihexadecanoyl-PE, NBD covalently linked to ethanolamine), (C) NBD-PC, and (D) NBD-PS. The values plotted represent the average (±SD) of three independent experiments.

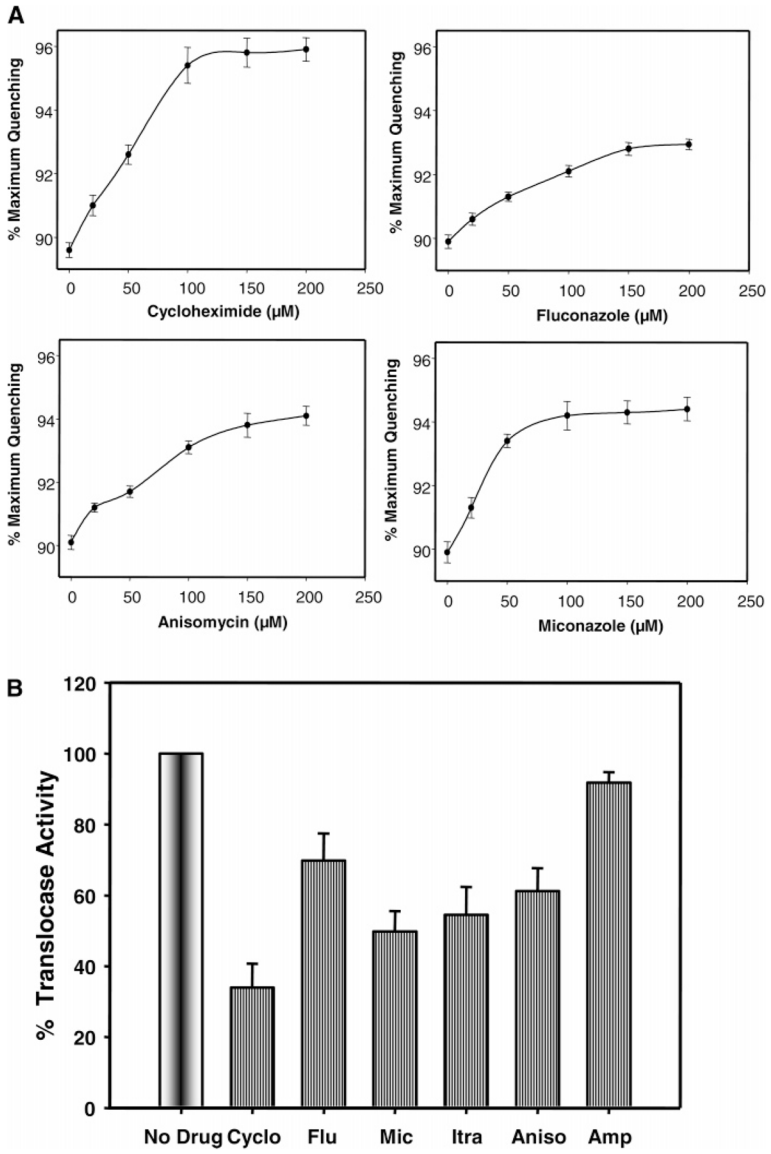

Figure 5.

Inhibition of ATP-dependent translocation of NBD-PE by Cdr1p drug substrates. Panel A shows the extent of translocation of NBD-PE in 60 min in the presence of cycloheximide, fluconazole, anisomycin, and miconazole at the indicated concentrations. Data points represent the mean (±SD) of three independent experiments. Panel B shows the extent of NBD-PE translocation in the presence of a 200 μM concentration of the drugs indicated (data are normalized to the extent of translocation in the absence of drugs). The values plotted (±SD) represent the average of three independent experiments.

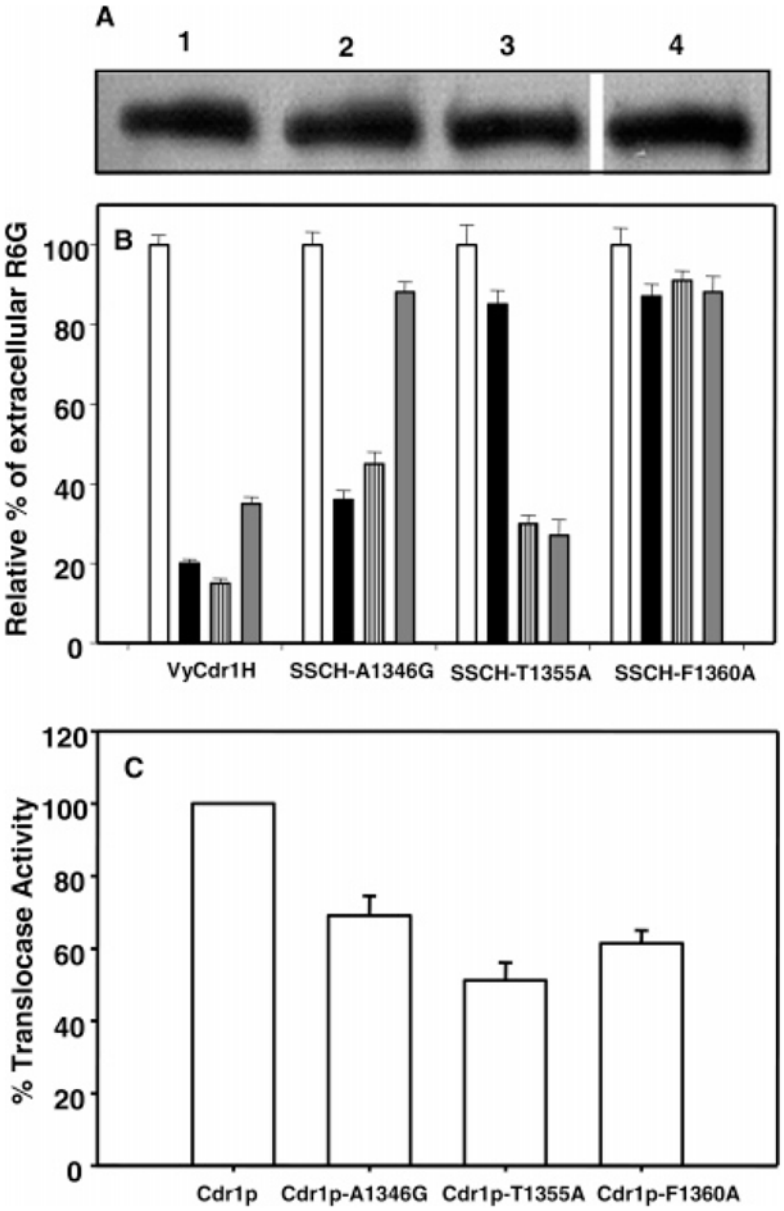

Figure 6.

NBD-PE translocation and drug efflux activities of Cdr1p point mutants. (A) Western blot of membrane fractions of cells expressing wild-type Cdr1p–His_6 (1) and mutant variants SSCH-A1346G (2), SSCH-T1355A (3), and SSCH-F1360A (4) probed with anti-penta-His antibody. The vertical white bar indicates that lane 4 is taken from a portion of the gel not contiguous with lane 3. (B) R6G efflux assay from wild-type VyCdr1H cells and mutant variants. Glucose-mediated efflux of R6G was performed as described elsewhere (35). The histogram shows the relative percentage of R6G efflux from cells overexpressing either wild-type Cdr1p–His_6 or the Cdr1p mutants SSCH-A1346G, SSCH-T1355A, and SSCH-F1360A in the presence of a 5-fold molar excess of substrates (ketoconazole, black bar; itraconazole, striped bar; miconazole, gray bar; without any drug, white bar). The values plotted (±SD) represent the average of three independent experiments, normalized to R6G efflux in the absence of drug (R6G efflux in cells expressing SSCH-A1346G, SSCH-T1355A, and SSCH-F1360A was 40%,

100%, and 45%, respectively, compared to that measured in cells expressing wild-type Cdr1p). (C) Comparison of NBD-PE translocation (determined after 60 min at 30 °C) of proteoliposomes reconstituted with SSCH-A1346G, SSCH-T1355A, or SSCH-F1360A. The extent of translocation is normalized to that seen with wild-type Cdr1p. The values plotted (±SD) represent the average of three independent experiments.

Table 1

List of Strains Used

strain	Description	ref
AD1-8u$^-$	*MATa pdr1-3 his1 ura3Δyor1::hisG Δsnq2::hisG Δpdr5::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisGΔpdr3::hisG Δpdr15::hisG*	24
VyCDR1H	AD1-8u$^-$ cells harboring *CDR1*-His$_6$ ORF integrated at the *PDR5* locus	24
SSCH-C193A	*CDR1*-His$_6$ cells carrying a C193A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	24
SSCH-A1346G	*CDR1*-His$_6$ cells carrying an A1346G mutation in the *CDR1* ORF and integrated at the *PDR5* locus	this study
SSCH-T1355A	*CDR1*-His$_6$ cells carrying a T1355A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	this study
SSCH-F1360A	*CDR1*-His$_6$ cells carrying an F1360A mutation in the *CDR1* ORF and integrated at the *PDR5* locus	this study