Materials and Methods

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2.1 Materials

Anti-GFP monoclonal antibody was purchased from BD Biosciences Clontech, Palo Alto, CA, USA. DNA modifying enzymes were purchased from Roche Molecular Biochemicals, Germany. Protease inhibitors, miconazole, nystatin, cycloheximide, anisomycin, rhodamine 6G, fluorescamine and other molecular grade chemicals were obtained from Sigma Chemical Co, (St. Louis, MO, USA). The radiolabeled $^{125}$I-IAAP (2200 Ci/m mole) from Perkin Elmer Life Sciences (Boston, MA, USA). $[^{32}P] \text{8-azidoATP (15-20 Ci/m mole)}$ from Affinity Labeling Technologies, Inc. (Lexington, KY, USA) and $[^{3}H]\text{-azidopine (60 Ci/m mole)}$ from Amersham Biosciences (Arlington Heights, IL, USA) were procured. The $[^{32}P] \text{8-azidoATP}$ showed no detectable contaminating $[^{32}P] \text{8-azidoADP}$ using thin-layer chromatography with 0.8 M LiCl as the solvent. Pfizer (Sandwich, Kent, UK) kindly provided Fluconazole. $[^{3}H]\text{-fluconazole}$ was custom prepared by Amersham Biosciences, UK.

2.1.1 Bacterial, Yeast Strains and growth media

Plasmids were maintained in Escherichia coli XL-1 blue. E. coli was cultured in Luria Bertani medium (Difco, BD Biosciences, NJ, USA) to which ampicillin was added (100 μg/ml). The S. cerevisiae strains used were AD1234568 (provided by Prof. A Goffeau, Universite Catholique de Louvain, Belgium), AD1-8u' and AD1002 (provided by Prof. Richard D. Cannon, University of Otago, Dunedin, New Zealand). SS1-SS18 were AD 1234568 derivatives expressing mutant Cdr1ps. PSCDR1-GFP, SS5G (F774A), SS6G (ΔF774), and SS15G (T1351F) were AD1-8u' derivatives expressing Cdr1p-GFP and its mutant proteins (mutant Cdr1p-GFPs), respectively (appendix I). The yeast
strains were cultured in YEPD broth (Bio101, Vista CA) or SD-URA (Bio 101, Vista, CA). For agar plates 2% (w/v) bacto agar (Difco, BD Biosciences, NJ, USA) was added to the medium.

2.1.2 Primers

The oligonucleotides used for site-directed mutagenesis are listed in appendix II. The nucleotides, which were changed to incorporate the mutation, are shown as lower case letters. Oligonucleotides were synthesized by Integrated DNA technologies (Iowa, USA)

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2.2.1 Generation of polyclonal antibody to Cdr1p

The peptide CQSNKISKKEKDDYVDY (amino acid 965-979, part of putative NBD2) which represented the predicted most antigenic epitope of Cdr1p was commercially synthesized and KLH conjugated by Princeton Biomolecules, PA, USA. The anti-sera was raised in NZW rabbit after injecting 250 μg of conjugated peptide and giving a booster dose after 2 months. The antiserum was collected after 2 months (done by Covance Research Products Inc, PA, USA) and used in a dilution of 1:500 for western blot analysis.

2.2.2 Molecular cloning

A Green Fluorescent Protein (GFP) tag was attached at the C-terminal end of CDR1. The GFP ORF was amplified from the plasmid pGFP31 (Morschhauser et al. 1998) (kind gift from Prof. Joachim Morschhauser, Zentrum für Infectionsforschung, Universität Würzburg, Würzburg, Germany) using the primers (amplified by forward primer having Sal I (marked bold) 5'-
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ACGCGTCGACATGAGTAAGGGAGAAGAA-3', reverse primer having Sal I (bold) & Spe I (bold italicized) site 5'-ACGCGTCGACGACTAGTTTATTTGTATAGTTCCATCCA-3'). This GFP amplicon was digested with Sal I restriction enzyme. The stop codon of CDR1 was replaced with a Sal I site in p425-GPD-CDR1 (Kind gift from Prof. Martine Raymond, Institut de Recherches Cliniques de Montreal, Montréal, Québec, Canada). It was then digested with Sal I and the digested GFP amplicon was ligated to the C terminal of CDR1. The resulting CDR1-GFP ORF was taken out from the vector p425-GPD-CDR1GFP by Spe I restriction enzyme and cloned in the vector pSK-PDR5 PPUS (Nakamura et al. 2002) (kind gift from R. D. Cannon) at the Spe I site resulting in plasmid pPSCDR1-GFP. After every cloning the orientation was checked by restriction enzyme digestions as well as by sequencing using Big dye terminator™ Cycle sequencing kit (ABI) and ABI 310 DNA sequencer.

2.2.3 Site-specific mutagenesis and development of transformants.

Site-directed mutagenesis was performed by using the Quick Change Mutagenesis system from Stratagene (La Jolla, USA). The mutations were introduced into plasmid pS12-35 and pPSCDR1-GFP according to manufacturer's instructions and the desired nucleotide sequence alterations were confirmed by DNA sequencing of the ORF. The primers used for sequencing the ORF are listed in appendix III. A representative set of wild type and mutant sequences showing few mutants are shown in appendix IV. The mutated plasmid pS12-35 or the mutated pPSCDR1-GFP after linearizing with Xba I was used to transform AD1234568 or AD 1-8u- cells respectively for uracil prototrophy.
2.2.4 Bacterial miniprep DNA isolation

1.5 ml culture (from 3 ml culture) was taken in 1.5 ml eppendorf tubes and centrifuged for 5 minutes at 10,000 rpm. Plasmid DNA was isolated using alkaline lysis method. Contaminated proteins and E. coli DNA was removed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) twice. The DNA was precipitated with 2.5 volumes chilled absolute ethanol. Precipitated DNA pellet was subjected to 70% ethanol wash and then dried under vacuum and resuspended in 50 µl TE buffer (10 mM Tris.HCl pH 8.0, 1 mM EDTA).

2.2.5 Yeast transformation

Cells were grown in 10 ml YEPD medium overnight at 30°C with shaking at 200 rpm. 2.5 ml cells were inoculated into 50 ml YEPD media and allowed to grow at 30°C. When the OD reaches between 0.4–0.6 harvest the cells in to four falcons (10 ml each) at 2500 rpm at 4°C. Cells of each pellet was washed with 10 ml TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0) and finally resuspended in 0.4 ml of TE and 0.4 ml of 0.2 M LiCl and mixed vigorously. This mixture was incubated at 30°C for one hour shaking at 150 rpm. After incubation 0.1 ml of the mixture was added into the eppendorf tube containing 10 µl DNA (~1-5µg). Appropriate controls with no DNA and only vector (pYEURA3) were also kept. The mixture was incubated at 30°C with no shaking for 30 min. To this mixture 0.1 ml of 70 % PEG M.W. 3500 (take 0.7g PEG in 0.4 ml Milli Q water and heat at 70–80°C to dissolve the PEG) was added with blunt tips and mixed properly with same tips. It was incubated for one hour at 30°C with no shaking. Then the mixture was given heat shock at 42°C for 5 minutes and then centrifuged at room temperature at 4000 rpm for 1 min. The cells were washed twice in sterile Milli Q water (to remove residual PEG).
and resuspended in 100 μl sterile Milli Q water and plated on the selective media-agar plate.

2.2.6 Yeast miniprep DNA isolation

Yeast transformants were grown overnight in 10 ml of SD-URA at 30°C. The cells were harvested and resuspended in 1 ml of water. The cell suspension was then transferred to microfuge tube and centrifuged at 14,000 rpm for 30 s. The pellet was then resuspended in 0.2 ml digestion mixture (0.1 M Tris-Cl, pH 7.5, 10 mM EDTA pH 8.0, 0.2 mg ml⁻¹ Zymolyase and 1% β-mercapto ethanol) and incubated at 37°C for 2 h. To the above suspension 0.2 ml of lysis buffer (0.2 N NaOH, 10% SDS) was added and mixed gently by inverting the tube and incubated at 65°C for 20 min. The heated mixture is chilled for 2 minutes in ice and 0.2 ml of 3 M potassium acetate was added and incubated for 15 minutes in ice. The above mixture is centrifuged at 14,000 rpm for 15 minutes and the supernatant was recovered in fresh microfuge tube. DNA was precipitated by adding 0.6 volumes of isopropanol to the supernatant and centrifuging at 14,000 rpm for 15 min. The pellet was washed with 70% ethanol, dried and resuspended in 30 μl TE. 5 μl of this DNA was used for E. coli transformation and 15-20 μl was used for Southern analysis.

2.2.7 Yeast Genomic DNA isolation

Yeast genomic DNA was prepared from overnight cultures grown on 100 ml YEPD. The cells were harvested and washed with water in SS34 tubes and were finally resuspended in 3 ml SOE (0.9 M Sorbitol, 0.1 M EDTA, pH 8.0). 0.2 ml of DTT (0.5 M) and 0.5 ml of Zymolyase (2 mg/ml) was added to the SOE suspension and incubated at 37°C for 30 minutes with gentle shaking (the turbidity
should decrease 10 fold from the start value). The mixture was centrifuged at 2000 rpm for 5 minutes and the protoplasts were resuspended with 2 ml of SOE gently with. 10 ml of TCES (0.2 M Tris-HCl pH 8.0, 0.2 M NaCl, 50 mM EDTA and 2% SDS), 50 µl of β-mercaptoethanol and 100 µl of 20% proteinase K was added to the protoplast suspension and incubated at 65°C with gentle shaking for 30 min. To remove proteins the lysate was mixed with phenol:chloroform (1:1 v/v) mixture and centrifuged at 12000 rpm for 5 min. The upper aqueous phase was taken and the step was repeated twice. Finally, the DNA was precipitated by adding 0.6 volumes of isopropanol to the upper aqueous phase and centrifuging at 12000 rpm for 5 min. The DNA pellet was dried and suspended in TE (10 mM Tris and 1 mM EDTA pH 8.0) after RNAse treatment with subsequent phenol chloroform step. The DNA was stored in aliquots at -20°C until further use.

2.2.8 Southern analysis of CDR1 gene in S. cerevisiae

Plasmid or genomic DNA was isolated from S. cerevisiae cells as described above. Genomic DNA was digested with restriction endonuclease (Eco RV, Bam HI and Pst I, Roche Biochemicals). Plasmid DNA (5 µg) or Digested genomic DNA (10 µg) was separated on 1% agarose gel. The gel was denatured in 0.2 N HCl for 15 minutes and then in 1.5 M NaCl, 0.5 M NaOH solution for 45 minutes followed by rinsing in water and leaving it in 20X SSC for 1 h. The gel was then transferred to a Hybond- nylon membrane (Amersham) by capillary transfer overnight. The membrane was cross-linked in UV cross-linker and then put for prehybridization at 65°C in 3 ml 1 M sodium phosphate pH 7.0, 7 ml 10% SDS and 20 µl 0.5 M EDTA. Membranes were hybridized with [α-32P] dATP labeled CDR1 specific probe (ORF nucleotides 1 to 280) in hybridization solution overnight and washed
under high stringency conditions (Sanglard et al. 1997) at 65°C. The membrane was exposed to X-ray film in a cassette containing two intensifying screens, at -80°C.

2.2.9 Immuno detection of Cdr1p in plasma membrane

Crude membrane (CM) were prepared from S. cerevisiae cells grown in YEPD to late exponential phase. The cells were broken with glass beads by vortexing the cells 4 times for 30 sec followed by 30 sec interval on ice. The homogenization medium contained 50 mM Tris pH 7.5 and 2.5 mm EDTA and the protease inhibitors cocktail (1mM PMSF, 1µg/ml leupeptin, pepstatin A and aprotinin). The CM were recovered by centrifuging at 3500 rpm to remove unbroken cells and pelleting the CM by ultracentrifugation at 25,000 rpm for 1 hr. Plasma membrane (PM) fractions were obtained from CM fractions by sucrose gradient centrifugation as described by Monk et al. (Monk et al. 1991). The protein samples (20 µg) were separated on 8% SDS-PAGE gel and either stained with colloidal Coomassie G250 or electroblotted (40V, 1h, 4°C) on to nitrocellulose membranes (Invitrogen Life Technologies, CA, USA). The membranes were incubated with 1:500 dilution of anti-Cdr1p polyclonal antibody or 1:1000 dilution anti-GFP monoclonal antibody. Immunoreactivity was detected with HRP labeled goat anti rabbit antibody at 1:20,000 in 20% fat-free milk for Cdr1p antibody and 1:10,000 antimouse antibody in 5% fat-free milk for GFP antibody. Proteins on immunoblots were visualized using the enhanced chemiluminescence assay system (ECL kit, Amersham Biosciences, Arlington Heights, IL, USA).

2.2.10 Drug susceptibility of S. cerevisiae

The susceptibilities of S. cerevisiae cells to different drugs were determined by two different methods. The following stock solutions
were prepared: fluconazole 1 mg/ml (water), cycloheximide 0.1 mg/ml (water), nystatin 1 mg/ml (DMSO), ketocoanzole 1 mg/ml (methanol), miconazole 1 mg/ml (methanol), anisomycin 1 mg/ml (DMSO). The solvents used to solubilize different drugs were also tested and there was no inhibition of growth due to the solvents used.

(a) Microtitre assay: Cells were grown on SD-URA plates for 48 h at 30°C to obtain single colonies, which were resuspended in saline solution to give an A600 of 0.1. The cells were then diluted 100-fold in SD-URA medium. The diluted cell suspensions were added to the wells of round bottom 96-well microtitre plates (100 μl/well) containing equal volumes of medium (100 μl/well) and different concentrations of drugs (Kohli et al. 2002; Talibi and Raymond 1999). A drug free control was also included. The plates were incubated at 30°C for 48 h. The MIC test end point was evaluated by reading A600 in a microplate reader and is defined as a lowest drug concentration that gave >80% inhibition of growth compared with the growth of the drug free control.

(b) Spot assay: The yeast cells were grown overnight on SD-URA plates. The cells were then resuspended in normal saline to an A600 of 0.1. Five microlitres of five-fold serial dilutions of each strain was spotted on to SD-URA plates in the absence (control) and presence of the following drugs, fluconazole (2 μg/ml), cycloheximide (20 ng/ml), miconazole (250 ng/ml) and nystatin (500 ng/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs.
2.2.11 ATPase assay

The Cdr1p associated ATPase activity of the purified PM was measured as oligomycin sensitive release of inorganic phosphate. Membrane suspension (10 μg of PM protein as determined by Amido Black B protein estimation) (Schaffner and Weissmann 1973) was incubated at 30°C in 0.1 ml of a medium containing 59 mM Tris pH 7.5 and 7 mM MgCl₂ (ATPase assay buffer) and 20 μM oligomycin where indicated. To eliminate possible contributions from non-specific phosphatases and vacuolar or mitochondrial ATPases, 0.2 mM ammonium molybdate, 50 mM KNO₃, and 10 mM NaN₃, respectively were included in the reaction mixture. The reaction was started by addition of 5 mM ATP and was stopped by the addition of 0.1 ml of 5% SDS solution. The amount of inorganic phosphate released was determined immediately as described previously (Sarkadi et al. 1992).

2.2.12 [³H]-fluconazole accumulation in S. cerevisiae cells

The accumulation of [³H]-fluconazole was determined by modification of method described elsewhere (Sanglard et al. 1996). Cells from midlog phase were centrifuged at 500 x g for 3 minutes and resuspended in fresh YNB medium as a 5% cell suspension. 1.5 ml of cell suspension was incubated in shaking water bath for 150 rpm at 30°C and [³H]-fluconazole was added at a final concentration of 100 nM (0.7 TBq/m mole). A 100 μl aliquot of cells was taken after 45 min, filtered rapidly and washed twice with 10 mM PBS, pH 7.4 on Millipore manifold filtration assembly using 0.45 μm cellulose nitrate filter (Millipore, USA). The filter discs were dried and put in scintillation cocktail for radioactivity measurement by using a liquid scintillation counter (Packard, USA). The [³H]-fluconazole accumulation was expressed as pmoles/mg dry weight.
2.2.13 **Rhodamine 6G efflux**

Efflux of rhodamine 6G was determined essentially by a protocol as described previously (Maesaki et al. 1999). Approximately $10^7$ cells from an overnight culture were inoculated in 250 ml of YEPD and grown for 5 h at 30°C. The cells were pelleted and washed three times with phosphate buffered saline without glucose. The cells were subsequently resuspended as 2% cell suspension in PBS, to which rhodamine 6G was added to a final concentration of 10 $\mu$M and incubated for 2 h at 30°C. The cells were then washed and resuspended in PBS with 2% glucose. An aliquot of 1 ml was taken after 45 minutes and centrifuged at 9000 X g for 2 min. The absorbance of the supernatant was measured at A527.

2.2.14 **Phospholipid translocation measurements**

Fluorescamine, which specifically labels exposed amino phospholipids (Diaz and Schroit 1996), was used for labeling of *S. cerevisiae* cells expressing Cdr1p or its mutant variants. The protocol of labeling and quantitative estimation of asymmetry was followed as described previously (Smriti et al. 1999). Briefly, cells were harvested in midlog phase by centrifuging at 3000 rpm (Beckman model TJ-60) for 5 minutes at 4°C and washed twice with buffer A (100 mM potassium phosphate-5 mM EDTA, pH 7.5). The harvested cells (0.6 g wet weight) were resuspended in 5 ml of buffer B (100 mM potassium phosphate and 600 mM KCl, pH 8.2) and kept at 4°C with gentle swirling. Fluorescamine (15.6 mM) in dehydrated dimethyl sulfoxide was added to the cell suspension with gentle and constant swirling. After 30 s the reaction was stopped by adding an equal volume of 1 M ammonia in 600 mM KCl. The cells were centrifuged and washed at 4°C three to four times till the color of the dye disappeared from the supernatant. Resolved phospholipids and derivatized
phosphatidylethanol amine (PE) were scraped off from the thin-layer chromatography (TLC) plates, and their phosphate content was estimated.

**2.2.15 Confocal microscopy**

The cells were grown in SD-URA media to late log phase. The drugs were added 4 h after the inoculation wherever indicated. The cells were washed and resuspended in appropriate volume of 50 mM HEPES pH 7.0. The cells were placed on the glass slides and a drop of antifade reagent (Fluoroguard™ high performance antifade reagent, Biorad, Hercules, CA) was added to prevent photobleaching. The cells were directly viewed with 100X oil immersion objective on a Biorad confocal microscope (MRC 1024).

**2.2.16 Flow Cytometry of the cells**

Flow cytometric analysis of the Cdr1p-GFP carrying *S. cerevisiae* cells was performed with a FACSort flow cytometer (Becton-Dickinson Immunocytometry systems, San Jose, CA, USA). Cells were grown to midlog phase and $10^6$ cells were harvested and washed with 50 mM HEPES pH 7.0. Cells were resuspended in 500 μl of 50 mM HEPES pH 7.0. Ten thousand cells were analyzed in acquisition. Analysis was performed with CellQuest software (Becton-Dickinson Immunocytometry systems). The mean fluorescence intensity was calculated using the histogram stat program.

**2.2.17 Photoaffinity labeling with IAAP**

The PM (15 μg) protein was incubated with the indicated competing drug for 10 minutes at 37°C in 50 mM Tris-HCl, pH 7.5. The samples were brought to room temperature and 3-6 nM [$^{125}$I]-IAAP (2200 Ci/mmole) was added and incubated for an additional 5
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minutes under subdued light. The samples were then illuminated with a UV lamp assembly (PGC Scientifics, Gaithersburg, MD, USA) fitted with two Black light (self-filtering) UV-A long wave-F15T8BLB tubes (365 nm) for 10 minutes at room-temperature (21-23°C). Following SDS PAGE on a 8% Tris-glycine gel at constant voltage, gels were dried and exposed to Bio-Max MR film (Eastman Kodak, Rochester, NY, USA) at -80°C for 12-24 h. The radioactivity incorporated into the Cdr1p band was quantified using the STORM 860 phosphorimager system (Molecular Dynamics, Sunnyvale, CA, USA) and the software ImageQuaNT as described previously (Sauna and Ambudkar 2000).

2.2.18 Photoaffinity labeling with [3H]-azidopine

The PM (30 μg) protein was incubated with the indicated competing drug for 10 minutes at 37°C in 50 mM Tris-HCl, pH 7.5. The samples were brought to room-temperature and treated with 0.5 μM [3H]-azidopine (60 Ci/mmole) for 5 minutes and then photo-crosslinked at 365 nm at room-temperature (21-23°C) for 10 minutes and 5X SDS sample buffer was added. Following electrophoresis, the gel was incubated with Fluro-Hance (Research Products Inc., Illinois, USA) for 30-45 min, dried under vacuum and the dried gel was exposed to an X-ray film for 3-6 days at -80°C.

2.2.19 Photoaffinity labeling with [α-32P] 8-azidoATP

PM (15 μg) protein was incubated in the ATPase assay buffer containing 10 μM [α-32P] 8-azidoATP (10 μCi/nmole) in the dark at 4°C for 5 minutes in the presence or absence of 10 mM ATP. The samples were then illuminated with an UV lamp assembly (365 nm) for 10 minutes on ice (4°C) as described (Sauna and Ambudkar 2001). Following SDS PAGE on a 8% Tris-glycine gel at constant voltage, gels
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were dried and exposed to Bio-Max MR film at -80°C for 12-24 h. The radioactivity incorporated into the Cdr1p band was quantified as described above.