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Two-hybrid-based analysis of protein–protein interactions of the yeast multidrug resistance protein, Pdr5p

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Abstract The ATP-binding cassette (ABC) transporters are a large family of proteins responsible for the translocation of a variety of compounds across the membranes of both prokaryotes and eukaryotes. The inter-protein and intra-protein interactions in these traffic ATPases are still only poorly understood. In the present study we describe, for the first time, an extensive yeast two-hybrid (Y2H)-based analysis of the interactions of the cytoplasmic loops of the yeast pleiotropic drug resistance (Pdr) protein, Pdr5p, an ABC transporter of *Saccharomyces cerevisiae*. Four of the major cytosolic loops that have been predicted for this protein [including the two nucleotide-binding domain (NBD)-containing loops and the cytosolic C-terminal region] were subjected to an extensive inter-domain interaction study in addition to being used as baits to identify potential interacting proteins within the cell using the Y2H system. Results of these studies have revealed that the first cytosolic loop (CL1) – containing the first NBD domain – and also the C-terminal region of Pdr5p interact with several candidate proteins. The possibility of an interaction between the CL1 loops of two neighboring Pdr5p molecules was also indicated, which could possibly have implications for dimerization of this protein.

Keywords Yeast two-hybrid system · ABC transporter · Pdr5p · NBD1

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Introduction

The ATP binding cassette transporters (ABC transporters) transport a large variety of compounds, from ions to large lipophilic compounds and even peptides (Taglicht and Michaelis 1998). These ATPases form one of the largest superfamily of proteins and are present in virtually all known organisms (Holland and Blight 1999). In humans, several diseases have been correlated with defects in different ABC transporters (Karttunen et al. 1999; Akabas 2000). In addition, multidrug resistance (MDR) to various pathogens is conferred by the ABC transporters of the MDR and multidrug resistance protein (MRP) families. These transporters have a modular structure and are characterized by the presence of two nucleotide-binding domains (NBDs; ATPase) and two membrane-spanning domains. In prokaryotes these domains are made up of many different subunit proteins, while in eukaryotes all are found in a single polypeptide.

The crystal structure of the ATPase subunit of the bacterial histidine transporter (HisP) was solved a few years ago (Hung et al. 1998). Very recently, the complete crystal structure (at 4.5°Å resolution) of the prokaryotic ABC transporter MsbA was reported (Chang and Roth 2001) and shown to exist as a dimer. The complete crystal structure of a eukaryotic transporter has, however, yet to be solved. In the absence of complete crystallographic information, electron image analysis studies have been carried out on the mammalian P-glycoprotein (Pgp), an ABC transporter involved in MDR, suggesting a monomeric protein with a channel-like structure (Loo and Clarke 1996; Rosenberg et al. 1997). A monomeric structure was also proposed, from a similar analysis, for the cystic fibrosis transmembrane regulator (CFTR), an ABC protein that functions as a chloride channel regulator. However, other biochemical studies with CFTR have revealed that the protein may possibly exist as a dimer (Marshall et al. 1994; Eskandari et al. 1998; Zerhusen et al. 1999). More recently, structural studies at 22°Å resolution on 2-D crystals of mammalian MRP1, reconstituted

with lipids, have revealed the dimeric structure of this protein (Rosenberg et al. 2001).

Numerous studies have revealed that both the NBDs of the ABC transporters, although asymmetric in their function, are required for the functional activity of these proteins. Mutation in either one of the two domains leads to a non-functional transporter. When the two identical halves are expressed separately, a non-functional protein results, but the combined expression of both halves together leads to a functional protein, indicating that the two halves can interact within the cell to yield a functional transporter (Ostedgaard et al. 1997; Sheppard and Welsh 1999).

Despite the importance of the ABC transporters, very little is known about their protein interactions. In CFTR, however, the C-terminal tail of the protein which contains the second NBD as well as the 'R' (regulatory) domain has been demonstrated to bind to the PDZ motif in Ezrin-binding protein, EBP-50, and is postulated to play a role in the apical localization of the protein (Short et al. 1998). Also, the N-terminus of this protein is predicted to interact with the membrane protein Syntaxin-1 (Naren et al. 1997). However, CFTR protein is slightly distinct from the other ABC transporters in that it functions as a chloride channel regulator rather than a transporter per se.

In several bacterial ABC transporters, particularly those which import substrates, an interaction has been demonstrated both genetically and biochemically between the NBD domains and the membrane-spanning protein subunits (Schneider et al. 1995; Mourez et al. 1998; Liu et al. 1999). Very little else is known, or has been reported, about the possible interactions of the different domains of the other ABC transporters with cellular proteins as well as with other domains of the same protein. Several methods exist for examining protein–protein interactions. Of these, the yeast two-hybrid (Y2H) method is one of the most powerful for quickly identifying candidate interacting proteins. In the present report, we have systematically analyzed the protein–protein interactions of the different cytoplasmic loops of a yeast ABC transporter, the pleiotropic drug resistance protein Pdr5p (Balzi et al. 1994; Bissinger and Kuchler 1994) using the Y2H system. Results of these studies indicate that some of the loops in the cytoplasmic portion of this protein possibly interact with several cellular proteins. The results also suggest the possibility of NBD1–NBD1 interactions between two adjacent Pdr5p molecules.

Materials and methods

Chemicals and reagents

All the chemicals and reagents used were of analytical grade. Media components, such as yeast nitrogen base, peptone, yeast extract, and tryptone were obtained either from Difco, USA, or Hi Media, India. Galactose (Gal), raffinose (Raff), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), ampicillin, and dimethyl sulfoxide were procured from Sigma (St. Louis, Mo.). Oligonucleotides were obtained from Ransom Hill Biosciences (Ramona, Calif.) or Integrated DNA Technologies (Coralville, Ind.). The Y2H system of Roger Brent, including plasmids, strains, and the corresponding library, was obtained from Roger Brent (Department of Molecular Biology, Massachusetts General Hospital, Boston, Mass.). The Y2H system DupLEX-A, which contained additional strains and plasmids, was purchased from OriGene Technologies (Rockville, Md.).

Bacterial and yeast strains and growth conditions

Escherichia coli DH5α [*SupE 44 ΔlacU169(ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was the transformation recipient for all plasmid constructions. *E. coli* KC8 (*pyrF leuB600 trpC hisB463*) was used to isolate the library-insert-containing plasmid (pJG4-5) from the yeast DNA. Both *E. coli* strains were grown at 37°C in LB medium. For selection of the pJG4-5 library plasmid, transformants of *E. coli* KC8 were selected on minimal medium plates lacking tryptophan (KC8 plates; Golemis and Brent 1997). Yeast strain EGY48 (*MATα trp1 his3 ura3 leu2::6LexAop-LEU2*) was used for all transformations and was grown at 30°C in either YPD or SD medium. RAX2 wild-type yeast strain (*MATα leu2–3 ura3–52 his3 lys2–801 trp1 suc2Δ9*) and *rax2* (*rax2::HIS3*) in the wild-type background were grown at 30°C in YPD.

Plasmids and their construction

Plasmid pYEplac 195-STS1, which contains the *PDR5* gene (Kaur and Bachhawat 1999), was a derivative of a plasmid obtained from Karl Kuchler (Bissinger and Kuchler 1994). This plasmid was used as a template DNA for PCR amplifications. Plasmid pEG202-CL1, containing the CL1 region of Pdr5p from amino acids 1–520 (nucleotides 1–1560) in the bait plasmid pEG202, was constructed by amplifying CL1 (cytosolic loop1) and digesting the purified PCR product with *Eco*RI, followed by ligation into the *Eco*RI site of pEG202. Plasmid pEG202-CL2, containing the CL2 region of Pdr5p from amino acids 575–612 (nucleotides 1726–1836) in the bait plasmid pEG202, was constructed in the same way as pEG202-CL1 but with an amplified CL2 fragment. Plasmid pEG202-CL4, containing CL4 of Pdr5p from amino acids 793–1236 (nucleotides 2380–3708) in the bait plasmid pEG202, was constructed by amplifying CL4, digesting the purified PCR product with *Xho*I, followed by ligation into the *Xho*I site of pEG202. Plasmid pEG202-C-tail, containing the C-tail (cytoplasmic tail) of Pdr5p from amino acids 1494–1511 (nucleotides 4483–4536) in the bait plasmid pEG202, was constructed by amplifying the C-tail, digesting the purified PCR product with *Eco*RI and *Bam*HI, followed by ligation into the *Eco*RI-*Bam*HI sites of pEG202. All the bait plasmids were sequenced to ensure the absence of PCR-induced mutations and that the fusions to LexA were in-frame.

pNLexA-CL1 (containing CL1 fused to the N-terminal region of LexA), PGEX-1-CL1 (containing CL1 fused to the C-terminal region of a GST tag) and pJG4-5-CL1 (containing CL1 fused to the C-terminal region of acid blob B42) were constructed by excising the CL1 fragment from pEG202-CL1 with *Eco*RI, followed by cloning into the *Eco*RI site of the appropriate vectors. pJG4-5-CL2, containing CL2 fused to the C-terminal region of acid blob B42 was constructed by excising CL2 from pEG202-CL2 with *Eco*RI, followed by cloning into the *Eco*RI site of the pJG4-5 vector.

pEG202-CL1D(*Sal*I-*Xho*I), a *Sal*I-*Xho*I deletion derivative of pEG202-CL1 containing amino acids 1–464 of the CL1 region (nucleotides 1–1392) was constructed by digesting pEG202-CL1 with *Sal*I and *Xho*I, followed by religation of the plasmid. pEG202-CL1(*Eco*RI-*Bgl*II), containing the *Eco*RI-*Bgl*II fragment of CL1 containing amino acids 1–388 of the CL1 region (nucleotides 1–1164), was constructed by digesting pJG4-5-CL1 with *Eco*RI and *Bgl*II, followed by cloning of the CL1 deletion fragment in the *Eco*RI-*Bam*HI sites of pEG202. pEG202-

CL1(*Bam*HID), a *Bam*HI deletion construct of pEG202-CL1 containing amino acids 1–326 of the CL1 region (nucleotides 1–978) was constructed by digesting pEG202-CL1 with *Bam*HI, followed by religation of the plasmid. pEG202-CL1(*Bgl*II-*Xho*I), containing the *Bgl*II-*Eco*RI fragment of CL1, was constructed by digesting pJG4-5-CL1 with *Bgl*II and *Xho*I, followed by cloning of the CL1 deletion fragment in the *Bam*HI-*Xho*I sites of pEG202.

ABE 980 contains RAX2 expressed downstream of the GAL promoter (Chen et al. 2000).

Yeast two-hybrid screen

The Y2H procedures were conducted as described earlier (Golemis and Brent 1997). All bait plasmids were subjected to the recommended control tests before proceeding further. These include: (1) the auto-activation test, which was done by using EGY48 containing pBait and pSH18-34; and (2) the repression assay, which was carried out by using EGY48 containing pBait and pJK101. Baits which passed these tests were used for library transformations.

The library screening was performed by transforming the yeast strain EGY48 containing pBait and pSH18-34 with a *Saccharomyces cerevisiae* genomic two-hybrid library (pJG4-5). Transformants were selected on glucose (Glc)/complete medium (CM) –His–Ura–Trp plates. The colonies thus obtained were pooled and frozen. Transformants were induced in galactose medium and plated on Gal/Raff –His–Ura–Trp–Leu selection plates for putative positive colonies, which were subsequently patched onto a Glc/CM –His–Ura–Trp master plate. These positive transformants were subjected to reporter gene activation for both *LEU2* and *lacZ*. Plasmid DNA was isolated from yeast interactors that showed a positive phenotype for both the reporter genes. Interactive library plasmids were isolated by transforming the isolated yeast DNA into *E. coli* strain KC8, and the transformants thus obtained were selected on minimal medium plates lacking tryptophan. To confirm the Y2H interaction, the isolated library plasmids were re-transformed into EGY48 containing pBait and pSH18-34 and transformants were again selected for the activation of both reporters. DNA sequencing of the interactive library plasmids was performed to identify the protein interacting with the bait.

β-Galactosidase plate assay

X-Gal-containing plates were prepared as described earlier (Golemis and Brent 1997). Yeast transformants containing pSH18-34 along with the other plasmids were patched on both Glc/CM –Ura +X-gal and Gal/Raff –Ura +X-gal plates, and were incubated at 37°C for 24–36 h. Interaction was detected by observing the blue color of the transformants on Gal plates and white on Glc plates.

Measurement of β-galactosidase activity

β-Galactosidase activity was measured in permeabilized yeast cells as described earlier (Guarente 1983). The yeast cultures were grown overnight in Glc/CM –His–Ura–Trp medium and reinoculated in fresh medium to an initial OD_{600} of 0.1. After 5–6 h incubation at 30°C, one third of the culture was harvested and the pellet was washed once with Gal/Raff –His–Ura–Trp medium. Finally, the cells were suspended in the same medium to an OD_{600} of 0.5–0.6. The rest of the Glc-grown culture was diluted to an OD_{600} of 0.1 with fresh Glc/CM –His–Ura–Trp medium. After 8 h of incubation at 30°C, approximately 1×10^8 cells were harvested, washed once with water followed by a wash with ice cold *lacZ* buffer (Ausubel et al. 1995) and the cell pellet was resuspended in 50 µl of *lacZ* buffer (ice cold). To permeabilize the yeast cells, 50 µl of chloroform and 20 µl of 0.1% SDS were added to the cell suspension on ice and the suspension was vortexed vigorously for 10 s. The cells were equilibrated at 30°C for 5 min. β-Gal substrate, ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (0.7 ml of 2 mg/ml stock prepared in *lacZ* buffer) was added and the cell suspension further incubated at 30°C for 10–20 min. The reaction was terminated by adding 0.5 ml of 1 M sodium carbonate solution. The cells were separated by a quick spin and the absorbance of the yellow-colored supernatant was measured at 420 nm. β-Galactosidase activity was calculated per cell OD_{600} for both Gal and Glc cultures and the relative β-gal activity (Gal/Glc) was determined.

Purification and solublisation of GST-CL1

E. coli BL21 (DE3) harboring plasmid pGEX-1-CL1 was grown at 37°C in LB medium containing 100 mg ampicillin/ml, until the absorbance at 600 nm reached 0.5–0.8. Expression of fusion proteins was induced with 0.2 mM IPTG for 3 h and the cells were harvested by centrifugation at 8,000 *g* for 10 min at 4°C. The pellet was suspended either in phosphate-buffered saline (PBS) (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3, 150 mM NaCl) or in phosphate buffer (PB) containing different detergents [CHAPS (0.5%), Triton X-100 (1%), deoxycholate (0.5%) or Sarkosyl (0.5%)]. The protease inhibitors DTT (1 mM), leupeptin (2 µg/ml) and pepstatin (1 mg/ml) were added to the cell suspensions. The cells were lysed by sonication and centrifuged at 15,000 *g* for 30 min at 4°C. The supernatants and pellets were analyzed by SDS-PAGE.

Binding of sarkosyl-solubilized GST-CL1 to glutathione agarose beads

The (0.4–0.5%) sarkosyl-solubilized GST-CL1 cell lysate was mixed with Triton X-100 (0, 1, or 2%) and the suspension was added to glutathione agarose beads at 4°C which were equilibrated with PBS. After incubation for 30 min under mild stirring, the beads were washed with the same buffer, and then without Triton X-100. Gel loading dye was added to the beads and the samples were analyzed by western blot using a GST antibody.

Drug sensitivity assay

The transformants containing pTEF426, pTEF426-CL1, pGPD426 and pGPD426-CL1 were pregrown in SD broth lacking uracil to late log phase and then reinoculated in fresh medium and grown to an OD of 0.1. After an incubation of 6–7 h at 30°C, the absorbance of cells was measured at 600 nm and the number of cells per ml of the culture was calculated. Serial dilutions of 10^7, 10^6, 10^5 and 10^4 cells/ml were made in sterile water and 10 µl of each serially diluted culture was spotted onto YPD plates containing drugs (cyclohemixide and emetine) at different concentrations. The plates were incubated at 30°C and examined for growth after 2 days.

Results and discussion

Identification of cytosolic proteins that may interact with the individual cytosolic loops of Pdr5p

The predicted two-dimensional topology of Pdr5p indicating the various cytoplasmic loops is shown in Fig. 1. The location of the cytoplasmic loops and membrane spanning domains is based on an earlier report (Balzi et al. 1994). CL1 and CL4 are the two major cytoplasmic loops that also contain NBDs 1 and 2, respectively. Loops CL3 and CL6 consist of only a few amino acids; we therefore considered these loops to be too small to investigate using the Y2H system. CL2 and CL5 are approximately 35 amino acids long, but we used only

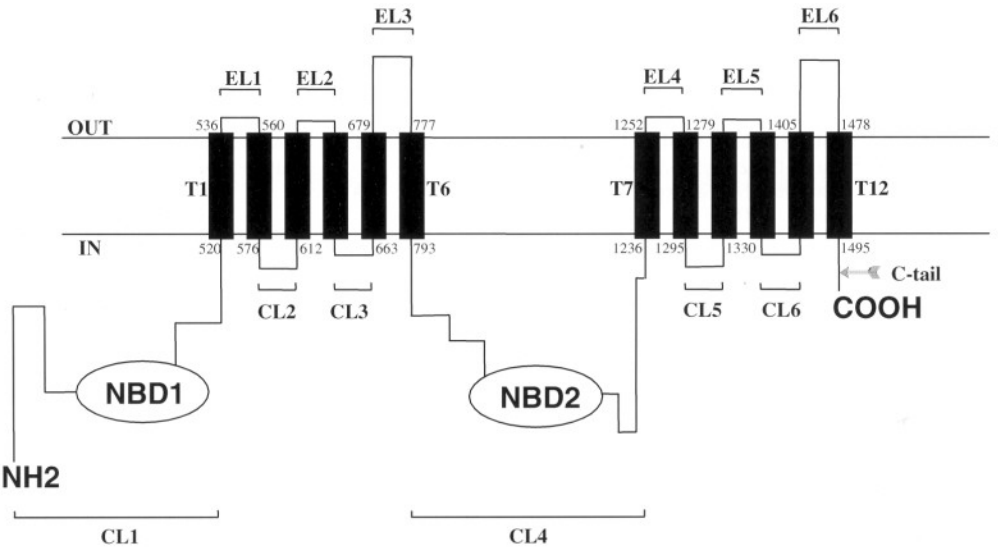


Fig. 1 A schematic representation of Pdr5p displaying the predicted topologies. *CL* Cytosolic loop, *EL* extracellular loop, *T* transmembrane domain, *NBD* nucleotide binding domain, *C-tail* cytoplasmic tail. *Numbers* refer to amino acid residue numbers

CL2 in our analysis. The C-tail of the protein is also predicted to be cytoplasmic and contains 16 amino acids. This region was also included in our analysis despite being relatively small.

Identification of proteins interacting with the loop containing NBD1 (CL1)

The first cytoplasmic loop, CL1, has a free N-terminus. We therefore initially attempted to use the vector pNLexA, which allows the bait to be fused to the N-terminus of LexA giving rise to a bait with a free N-terminus. However, the plasmid thus constructed did not pass the preliminary repression assay test, and therefore could not be pursued further. CL1 was subsequently cloned into the classical bait plasmid pEG202 fused to the C-terminal region of LexA. The bait displayed the correct characteristics in all the preliminary tests. It was subsequently used in a screen for candidate interacting proteins. Yeast strain EGY48, containing plasmids pEG202-CL1 and pSH18-34, was transformed with the two-hybrid library and approximately 10^5 transformants were obtained on selection medium lacking histidine, uracil and tryptophan. After pooling, and subsequent induction as indicated in Materials and methods, the Gal-induced transformants were plated on Gal medium lacking leucine to allow for selection of the reporter gene (*LEU2*). A total of 34 transformants were obtained which were then patched on Gal and Glc medium containing X-gal. Transformants that displayed a blue color after overnight growth were picked for further analysis. Plasmids were isolated from these interactors, and *TRP1* plasmids were selected by passage through *E. coli* KC8. These were rechecked for their phenotypes, and only those that showed a positive phenotype using both the reporters (*LEU2* and *lacZ*) were subjected to sequencing (Fig. 2). A total of seven clones were obtained and are shown in Table 1 together with the region of the protein responsi-

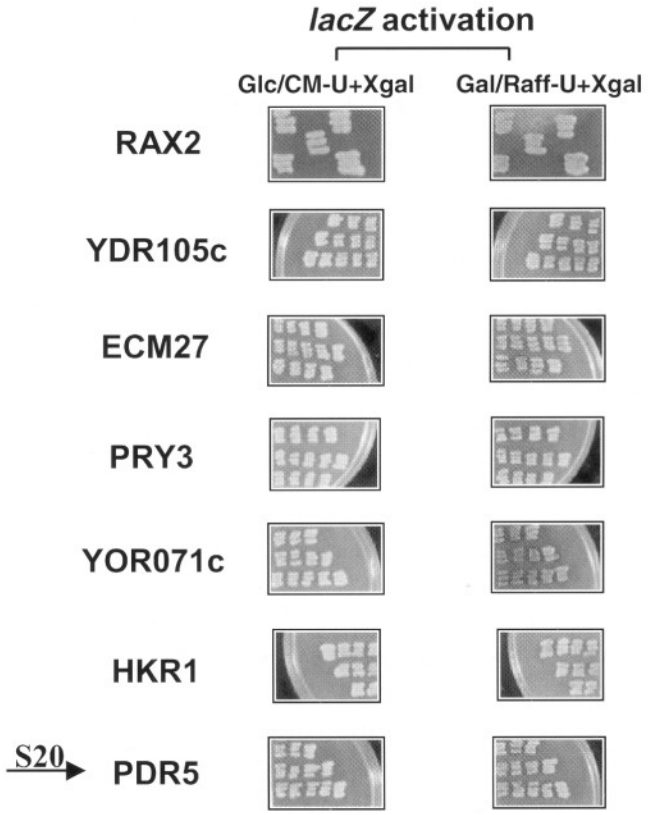


Fig. 2 Yeast two-hybrid (Y2H) interaction of CL1 with different interacting proteins as seen by the β-galacatosidase reporter assay. Transformants of EGY48 containing pEG202-CL1, pSH18-34 and interacting library plasmid were selected on Glc/CM –His–Ura–Trp plates followed by incubation at 37°C for 4 days. The transformants were repatched onto *LEU2* activation (Glc/CM–His–Ura–Trp–Leu and Gal/Raff–His–Ura–Trp–Leu) (not shown) and *lacZ activation* (Glc/CM–Ura+Xgal and Gal/Raff–Ura+Xgal) plates followed by incubation at 37°C. X-Gal plates and Leu$^-$ plates were observed after 36–48 h and after 3 days, respectively

Table 1 Proteins interacting with cytoplasmic loop 1 CL1 of Pdr5p

S. no.	Gene name[a]	Known function	Protein size [amino acids (aa)]	Interacting region[b]	Interaction with:	
					CL1 bait (relative β-gal activity)	CL4 bait
1	RAX2 (YLR084c)	Protein involved in bipolar budding	1221	aa 1117–1221 = 104 aa C-teminal end	204.2	–[c]
2	YDR105C	Unknown protein	474	aa 328–474 = 116 aa C-teminal end	196.3	–
3	ECM27 (YJR106w)	Involved in cell wall structure or biosynthesis	726	aa 200–478 = 278 aa towards N-terminal end	221.5	–
4	PRY3 (YJL078c)	Similarity to plant pathogenesis related protein	882	aa 684–882 = 198 aa C-terminal end	70.8	–
5	YOR071c	Possible low affinity thiamine transporter	599	aa 360–599 = 239 aa C-terminal end	258.9	–
6	HKR1 (YDR153w)	Hansenula mrakii K9 killer toxin-resistance protein	1803	aa 1262–1803 = 541 aa C-terminal end	162.0	–
7	PDR5 (YOR153w) (S20)	Drug efflux pump, ABC family member	1512	aa 4–520 = 516 aa N-terminal end	312.3	–

[a] Gene name, size of the protein and its predicted function were obtained from the Yeast Protein Database (http://www.proteome.com/YPD)

[b] N-terminal amino acids were determined by DNA sequencing and C-terminal amino acids were predicted according to library construction

[c] No activation

ble for the interaction and their putative assigned function. The possible 'auto-activation' of the 'prey' proteins was also examined; in no case did we observe any auto-activation (data not shown).

Although it was not obvious if any of these interactions were physiologically significant, the following two aspects were interesting: (1) the majority of proteins identified displayed membrane-spanning regions and some of these domains were present also in the interacting region (data not shown), and (2) amongst the candidate proteins identified was the N-terminal domain of Pdr5p itself.

Identification of proteins interacting with the second cytoplasmic loop, CL2

CL2, when cloned in-frame with LexA protein in the bait plasmid pEG202, showed auto-activation on its own. As a result, it was not possible to use this plasmid further as bait in the two-hybrid screen. However, when cloned in the prey plasmid pJG4-5, CL2 did not auto-activate and therefore we used this construct in the analysis of the potential domain-domain interactions described in subsequent sections.

Identification of proteins interacting with CL4 (containing NBD2)

CL4 was used as bait in vector pEG202 in a manner similar to that described for CL1. Although approximately 1.5×10^4 transformants were initially obtained with the library transformation, none of the transformants displayed a positive interaction phenotype. After prolonged growth on Leu$^-$ plates, a few colonies were obtained but they failed to develop blue color in the X-gal test. This result was a little surprising in light of the fact that the numerous colonies obtained with CL1 contained NBD regions that were fairly homologous. To further investigate this point, we evaluated all the proteins that were obtained as potential CL1-interacting proteins (Table 1) for their interaction with CL4. However, none of the proteins showed any interaction with CL4.

Identification of proteins interacting with the cytoplasmic tail

The C-tail consisted of only 16 amino acids but we nevertheless decided to use it as a bait since the C-terminus of CFTR (though much larger in size) had revealed binding to the Ezrin Binding Protein, EBP-50 (Short et al. 1998). The bait was used to screen a library. A total of 5×10^5 transformants were screened. The initial positives were again checked with both the reporters and also after purification of the plasmid from the yeast colonies. Several interacting proteins were isolated and these are shown in Table 2. The C-tail of Pdr5p appeared to interact with at

Table 2 Proteins interacting with the C-terminal tail (C-tail) of Pdr5p

S. no.	Gene name[a]	Known function	Protein size (aa)	Interacting region[b]	Interaction with:		
					CL1 bait (relative β-gal activity)	CL1 bait	CL4 bait
1	YJL109c	Protein with weak similarity to DRS2	1769	aa 752–1073 = 321 aa towards N-terminal end	153.7	–[c]	–
2	YPL136w	Protein of unknown function	122	aa 92–122 = 30 aa C-terminal end	59.8	–	–
3	YAP3 (YHL009c)	Transcription factor of the basic leucine zipper (bZip) family	330	aa 1–330 = 330 aa + 32 aa of upstream sequence; full protein + upstream sequence	94.2	–	–
4	YFL044c	Protein of unknown function, has a single C2H2 type zinc finger	301	aa 191–301 = 110 aa C-terminal end	33.8	–	–
5	SCP160 (YJL080c)	Involved in control of mitotic chromosome transmission	1222	aa 160–499 = 339 aa towards N-terminal side	22.9	–	–

[a] Gene name, size of the protein and its predicted function were obtained from the Yeast Protein Database (http://www.proteome.com/YPD)

[b] N-terminal amino acids were determined by DNA sequencing and C-terminal amino acids were predicted according to library construction

[c] No activation

least five proteins in the two-hybrid system. Since the C-tail of Pdr5p consists of only 16 amino acids, we initially believed that most of the observed interactions could well be false positives since the physiological relevance of the proteins identified were not apparent. All the interactors were therefore subjected to analysis for possible auto-activation. However, none of the proteins displayed auto-activation. We also checked if these proteins might also interact with either CL1 or CL4. However, the proteins that interacted with the C-tail did not interact with CL1 or CL4. This indicated that the interactions were both specific, and real, at least as seen by Y2H analysis.

Investigations into possible inter-domain interactions within Pdr5p

Interaction between CL1 and CL2, or CL1 and CL4, was studied by transforming the yeast with pEG202-CL1 as bait and pJG4-5-CL2 or pJG4-5-CL4 as prey. The transformants thus obtained were checked for activation of both reporter genes. The results of this study showed that there was no direct interaction between CL1 and CL2, or CL1 and CL4 (NBD1 and NBD4), loops (data not shown). Earlier studies have suggested that the two NBD domains in multidrug transporters function cooperatively, perhaps through their close interaction in the native membrane (Ames and Lecar 1992; Carson et al. 1995; Gunderson and Kopito 1995; Wilkinson et al. 1996; Gadsby and Nairn 1999; Zeltwanger et al. 1999). This has been further supported by a recent study (Qu and Sharom 2001), which showed close interactions between the NBD1 and NBD2 domains of Pgp by fluorescence resonance energy transfer analysis. However, our two-hybrid analysis failed to detect any interaction between CL1 and CL4 of Pdr5p. The possible reasons for the discrepancy between these two studies are, however, not clear to us at this moment.

Investigations into possible CL1-CL1 interactions

The isolation of a Pdr5p-encoding clone (clone S20) among the proteins interacting with CL1 was very intriguing. It suggested a possible domain-domain interaction that might play a role in the homo-dimerization of Pdr5p. Initial restriction analysis of the clone suggested that the S20 clone included the CL1 region of Pdr5p and the first two trans-membrane domains adjacent to CL1. It was possible that, in addition to homo-dimerization, the transmembrane domains might also play a role in the transport process, in a manner similar to the physical association followed by physical disengagement seen with the HisP permease and the membrane spanning domain subunits (Liu et al. 1999). We therefore decided to examine this interaction in greater detail.

Several deletion constructs of CL1 were constructed in the bait vector (Fig. 3A), and their Y2H interaction

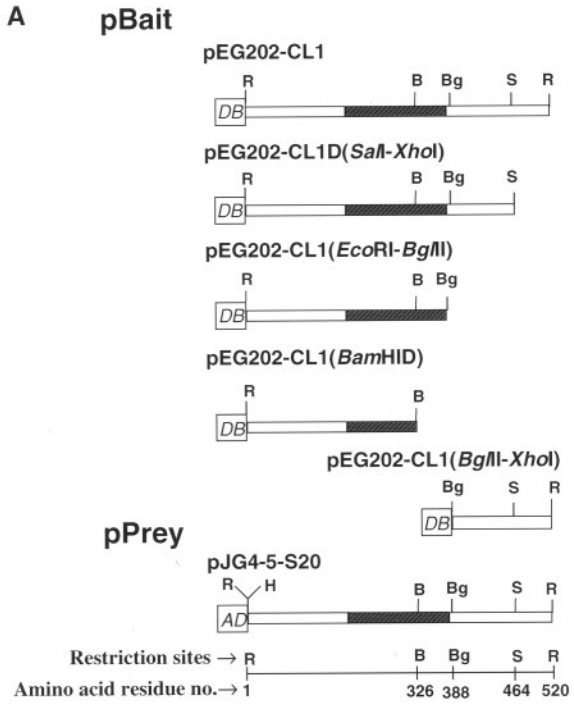
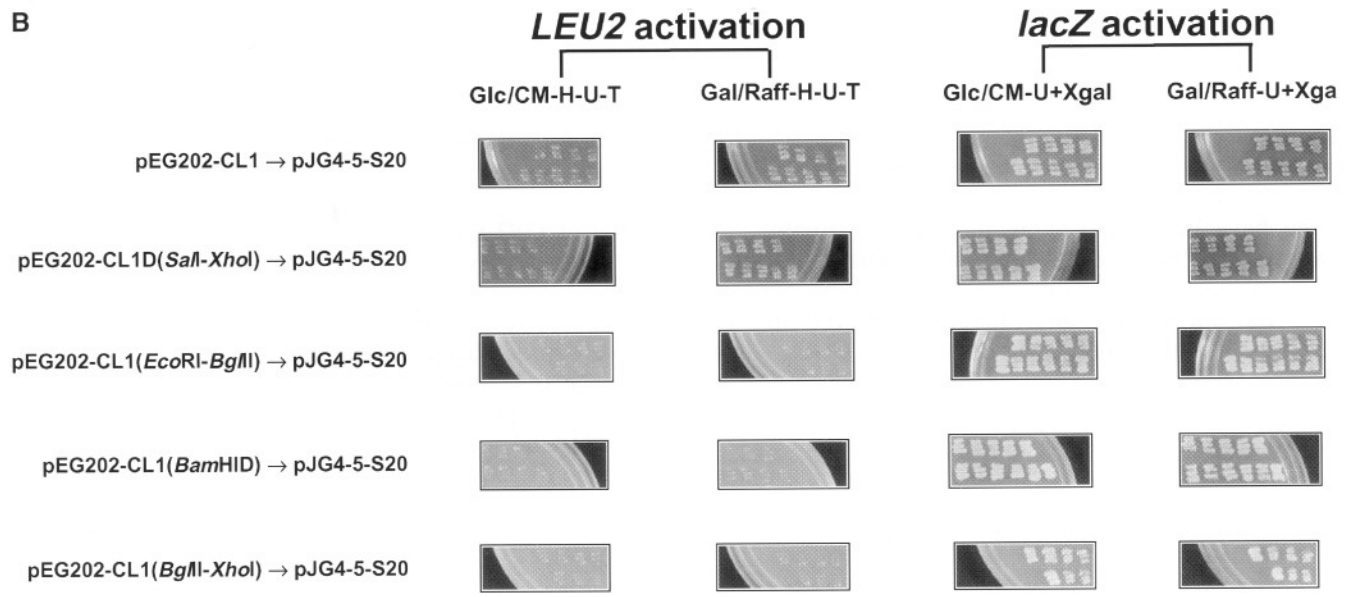


Fig. 3 **A** Deletion constructs of CL1 in *bait* vector and S20 in *prey* vector. Deletion constructs of *pEG202-CL1*, namely *pEG202-CL1D* (*Sal*I-*Xho*I), *pEG202-CL1*(*Eco*RI-*Bgl*II), *pEG202-CL1* (*Bam*HID) and *pEG202-CL1*(*Bgl*II-*Xho*I) were constructed as described in Materials and Methods. *PJG4-5-S20* was a library isolate and contains CL1 similar to pEG202-CL1. *DB* DNA binding domain, *AD* activation domain, *shaded portion* nucleotide binding domain (NBD). Restriction sites: *R Eco*RI, *B Bam*HI, *Bg Bgl*II, *S Sal*I, *H Hin*dIII. **B** Y2H interaction between different deletion constructs of CL1 bait and S20 as seen by *LEU2* and β-galactosidase reporter assays. Transformants of EGY48 containing pEG202-CL1 or deleted derivatives, pSH18-34 reporter and pJG4-5-S20 were selected on Glc/CM–His–Ura–Trp plates followed by incubation at 37°C for 3 days. The transformants were repatched onto *LEU2 activation* (Glc/CM–His–Ura–Trp–Leu and Gal/Raff–His–Ura–Trp–Leu) and *lacZ activation* (Glc/CM–Ura+Xgal and Gal/Raff–Ura+Xgal) plates followed by incubation at 37°C. X-Gal plates and Leu⁻ plates were observed after 36–48 h and after 3 days, respectively



with S20 was analyzed. Results shown in Fig. 3B indicated that deletion of 56 amino acids from the C-terminus of CL1 did not affect the interaction. However, none of the other constructs displayed any interaction phenotype.

To investigate the S20 clone in more detail, we sequenced the complete clone to determine both the end and start points of the clone. Sequencing indicated that the N-terminus started from amino acid number 4 at an *Alu*I site in the DNA. This was in accordance with the fact that the library was constructed by partial *Alu*I digestion, in addition to *Hae*III digestion, followed by *Eco*RI linker addition and cloning into the *Eco*RI sites of the pJG4-5 vector. Restriction map analysis of the *PDR5* gene indicated that the next *Hae*III site was at nucleotide position 1742, which indicated that the S20 clone probably contained CL1, transmembrane (T) domain 1, extracellular loop (EL) 1, T2 and part of CL2. However, when the C-terminal region of the S20 was sequenced, it was found to be similar to the CL1 clone in pEG202 (including the *Eco*RI site that we engineered) and the remaining part of S20 was identical to the pJG4-5 vector. These

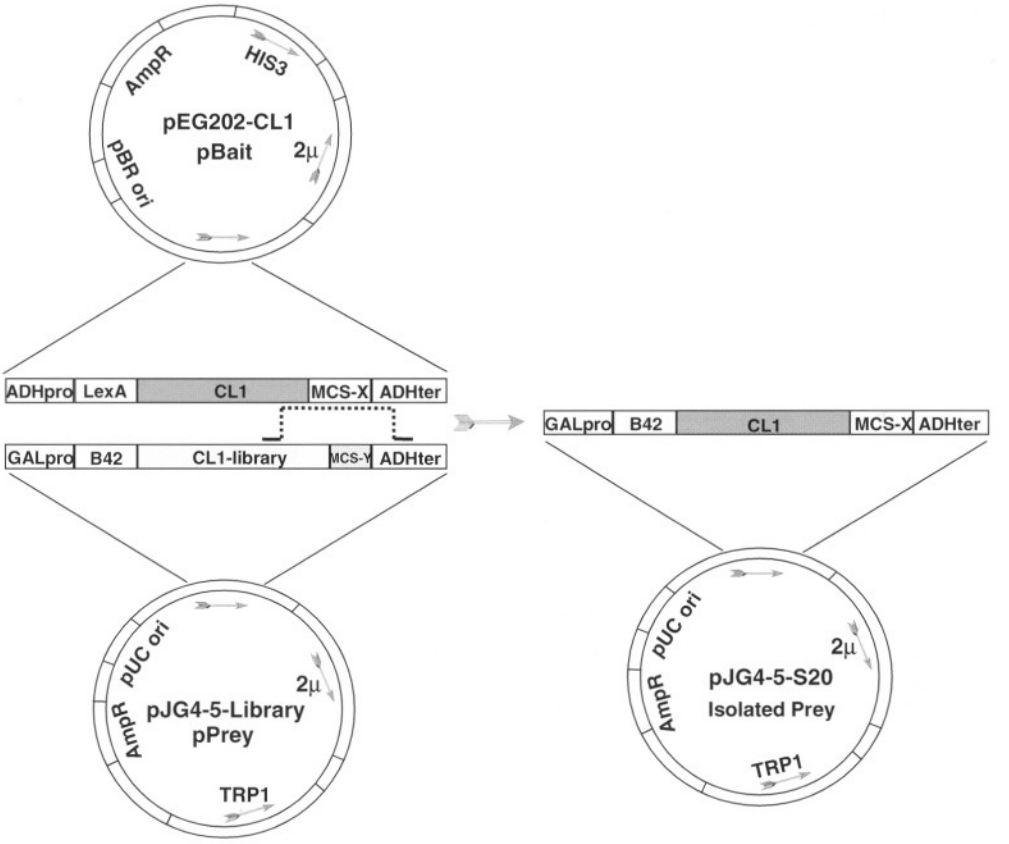


Fig. 4 Schematic representation of the possible recombination event resulting in the formation of S20. Recombination between *pEG202-CL1* (*bait*) and *pJG4-5-library* (*library prey*) resulted in isolation of *pJG4-5-S20*. The portion involved in the recombination of pEG202-CL1 contains the *ADH1 promoter*, *LexA* DNA binding domain, *CL1* of Pdr5p, the multiple cloning site (*MCS-X*) (*Eco*RI.*Xma*I.*Bam*HI.*Sal*I.*Nco*I.*Not*I.*Xho*I.*Sal*I) and the *ADH1 terminator*. The portion involved in the recombination of pJG4-5-library contains the *GAL1 promoter*, SV40 nuclear localization sequence, acid blob *B42*, *CL1-library* (CL1+T1+EL1+T2+part of CL2), *MCS-Y* (*Eco*RI.*Xho*I) and *ADH1 terminator*. All plasmids have *HIS3* and *TRP1* to act as selection markers, 2μ origin (*2μ*) to allow propagation in yeast, the ampicillin resistance gene (*Amp*^{R}), pUC origin (*pUC ori*) and pBR origin (*pBR ori*) to allow propagation in *Escherichia coli*

results suggested that recombination of the bait and the library-prey plasmid had occurred (shown schematically in Fig. 4). It may therefore be inferred that the S20 clone could have been derived by the above recombination. Surprisingly, however, when we examined CL1-CL1 interactions in the conventional bait and prey plasmids, we could not observe any interaction between the two domains. It is therefore possible that the CL1-CL1 interaction is context-dependent and the genuineness of the interaction will be authenticated only by biochemical experiments.

Purification of the GST-CL1 fusion protein

To investigate protein interactions mediated by CL1 in vitro, it was necessary to purify the CL1 protein. This was attempted by using a GST-CL1 fusion protein. The GST-CL1 protein could be induced to significant levels in *E. coli* but all of this protein entered into an insoluble inclusion body fraction. Several attempts were made to obtain at least a significant portion in the soluble fraction by using a variety of induction conditions. These included variations in IPTG concentrations and induction times as well carrying out induction at different temperatures (e.g. 25°C, 30°C or 37°C), conditions that have previously been shown to enhance the amount of protein going into the soluble fraction that would otherwise go into inclusion bodies. However, in the present case, none of the conditions tested increased the solubility of the protein. We therefore resorted to resolubilization of the GST-CL1 protein from inclusion bodies. The resolubilization of GST-CL1 in different detergents showed that the protein was soluble in PB containing 0.4–0.5% sarkosyl (Fig. 5A). However, the presence of sarkosyl in the cell lysate affected the binding of GST-CL1 to glutathione agarose beads. Frangioni et al. reported that the presence of Triton X-100 improved protein binding to the affinity column (Frangioni and Neel 1993). However, the GST-CL1 fusion did not bind to the beads even in the presence of 2% Triton X-100 (Fig. 5B). The inability to obtain functionally active, soluble, CL1 prevented further experiments at the in vitro level with the purified protein.

The inability to obtain active, soluble, CL1 prompted us to evaluate the authenticity of the interaction by other strategies. In the first approach, we examined the effects of deletion and overexpression of one of the CL1-prey proteins, Rax2p. Rax2p has been identified as a protein involved in bipolar budding (Chen et al. 2000). From the Y2H screen, only 103 amino acids of the C-terminal end of Rax2p were found to interact with CL1 (Table 1) and this was also confirmed at the protein level (Figure6). We decided to overexpress the complete Rax2p protein and examine its effects on Pdr5p function in a functional assay. However, neither Rax2p overexpression nor *rax2* deletion strains conferred any significant drug sensitive/resistance phenotype as seen using the drugs cycloheximide

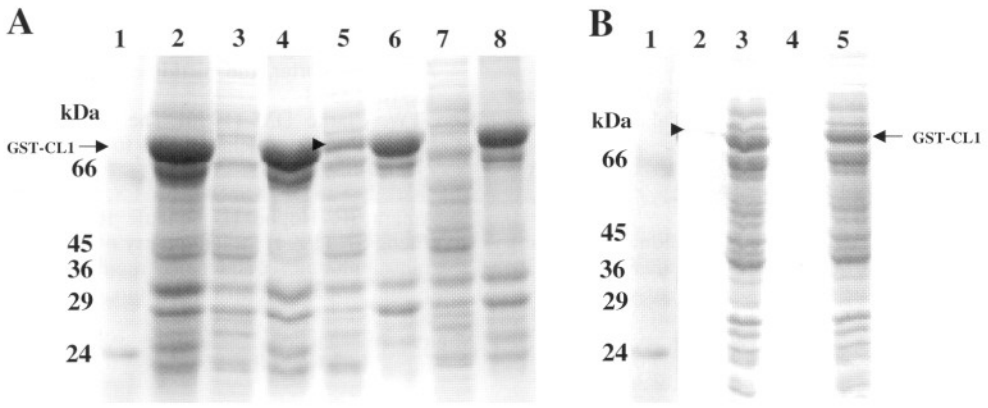


Fig. 5A, B Purification of the GST-CL1 fusion protein (SDS-PAGE). **A** Lanes: *1* molecular weight marker, *2* crude cell extract of GST-CL1 in phosphate-buffered saline (PBS), *3* supernatant of GST-CL1 in PBS, *4* pellet of GST-CL1 in PBS, *5* supernatant of GST-CL1 in sarkosyl-containing phosphate buffer (PB) (*arrowhead*), *6* pellet of GST-CL1 in sarkosyl-containing PB, *7* supernatant of GST-CL1 in Triton X-100-containing PB, *8* pellet of GST-CL1 in Triton X-100-containing PB. **B** Lanes: *1* molecular weight marker, *2* binding of GST-CL1 to GST beads without Triton X-100 (*arrowhead*), *3* protein sample after binding, *4* binding of GST-CL1 to GST beads in the presence of Triton X-100 (2%), *5* protein sample after binding

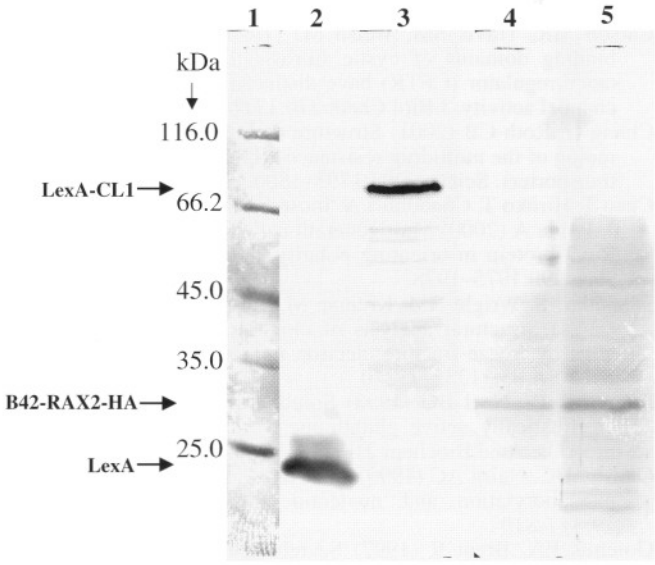


Fig. 6 Expression of two-hybrid fusion proteins (Western blot). Plasmid-containing yeast EGY48 was grown overnight and reinoculated in the same medium to an OD of 0.1. The cultures were incubated at 30°C for 5–6 h. Plasmids containing the *GAL1* promoter were subjected to Gal induction as described in the β-galactosidase assay. A cell suspension containing ~10^7–10^8 cells was harvested and the cell pellet was suspended in 50 µl of 1× SDS-PAGE gel loading dye. Lanes: *1* molecular weight marker, *2*, *3* EGY48 cells containing pEG202 and CL1/pEG202. The expression of these proteins was probed with anti-LexA antibody; *4*, *5* EGY48 cells containing RAX2/pJG4-5 with cells grown in Glc and Gal medium, respectively. Protein expression was probed with anti-HA antibody

and Emetine over a range of concentrations (data not shown). These drugs are substrates of Pdr5p (Kaur and Bachhawat 1999). We also examined a second, more general strategy, wherein we overexpressed the CL1 domain in yeast using both the TEF and GPD promoters to see if it might function to titrate out any interacting proteins and thereby interfere with Pdr5p function. However, no malfunctioning of Pdr5p was seen as examined by drug sensitivities to emetine and cycloheximide over a range of concentrations (data not shown). However, as interaction with Pdr5p does not presuppose interference with function, additional strategies will be required to verify the authenticity of the interactions.

In conclusion, this study is perhaps the first of its kind wherein the different cytoplasmic loops of a multiple membrane spanning protein have been systematically subjected to an analysis searching for interacting partners within the cell, as well as in searching for interaction within the different domains of the same protein. Furthermore, it provides preliminary evidence that Pdr5p, via CL1, might interact with another Pdr5p molecule. In addition, it suggests that both CL1 and the C-tail of Pdr5p could be involved in mediating the interaction of this protein with other proteins. These results may therefore provide a useful road map to other investigators for further analysis. Although the Y2H system is an excellent system for rapidly obtaining potential interacting proteins, it needs to be rigorously followed up with biochemical studies. Our attempts to carry out biochemical studies using the purified CL1 protein have so far been unsuccessful owing to the tendency of CL1 to form inclusion bodies. Despite using a variety of procedures (growth temperatures, different solubilization conditions and unfolding-refolding strategies) that have been successfully applied for the NBD regions of higher eukaryotes, we have so far failed to obtain a functional, solubilized (or resolubilized), CL1 protein suitable for use in in vitro studies. Additional strategies may therefore be needed to authenticate the results that we have obtained with the Y2H analysis.

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