RESULTS & DISCUSSION
MOLECULAR
AND
FUNCTIONAL ANALYSES OF Cdr1p
3. RESULTS AND DISCUSSION

3.1 MOLECULAR AND FUNCTIONAL ANALYSES OF Cdr1p

3.1.1 INTRODUCTION

MDR has been one of the principle causes of failure of cancer chemotherapy where tumour cells exhibit simultaneous resistance to multiple chemically unrelated chemotherapeutic agents (Gottesman, et al., 1996; Gottesman, et al., 1995; Gottesman and Pastan, 1993). The elucidation of the mechanism by which tumour cells develop resistance to toxic effects of potent chemotherapeutic agents has revealed a great deal about the process of drug uptake, metabolism and extrusion. This has also provided basic insights into cellular processes such as regulation of gene expression and gene amplification (Gottesman and Pastan, 1993; Gottesman, et al., 1995; Gottesman, et al., 1996). It has been shown that over-expression of certain ATP Binding Cassette (ABC) proteins in prokaryotes and eukaryotes, is linked to drug resistance phenomenon (Higgins, 1992; Ames, 1986; Ames, 1986). The well characterised mammalian protein Mdr1p (P-glycoprotein) is associated with the development of a drug-induced multidrug resistance phenotype in tumour cells (Endicott and Ling, 1989).

Multidrug resistance phenomenon is not restricted to mammalian or microbial cells (Higgins, 1992). A number of genes homologous to MDR1 have been identified in yeasts during past two decades (Balzi and Goffeau, 1994). Yeast shares similarity in structural and functional organisation with higher eukaryotes and is amenable to genetic manipulations and thus, serves as an excellent model for unraveling eukaryotic pathways of MDR. The studies involving MDR in yeasts have attracted further importance since some yeast species are also pathogenic to humans. Already several genetic determinants associated with multidrug resistance (Pleiotropic Drug Resistance, PDR in yeasts) have been characterised in Saccharomyces cerevisiae and Schizosaccharomyces pombe (Prasad, et al., 1995a; Balzi and Goffeau, 1994a).

The PDR5 gene was cloned from S. cerevisiae as a multicopy plasmid borne DNA fragment capable of conferring PDR (Balzi, et al., 1994). The gene codes for a polypeptide of 1511
amino acid residues with calculated molecular weight of ~170.4 kDa. Pdr5p is predicted to contain twelve “integral” transmembrane spans gathered in two groups of six contiguous membrane spans. Each hydrophobic domain follows a hydrophilic region including a predicted ATP-Binding Cassette (ABC). Thus, Pdr5p seems to have duplicated structure, consisting of two halves each composed of one hydrophilic and a hydrophobic domain (Balzi, et al., 1994; Hyde, et al., 1990). The two similar ABC domains of Pdr5p are conserved within a large super family of ABC transport proteins (Hyde, et al., 1990).

Dimorphic pathogenic yeast, Candida albicans is naturally more resistant to several drugs e.g. cycloheximide, benomyl, methotrexate than S. cerevisiae (Gow, et al., 1994; Ben-Yaakov, et al., 1994; Prasad, 1991; Fling, et al., 1991). In addition, the incidence of C. albicans cells acquiring resistance to azoles and polyenes has increased considerably in recent years, which has posed serious problems towards its successful chemotherapy (Prasad, et al., 1996; Vanden Bossche, 1995; Sternberg, 1994; Wagenvoort, 1993; Medoff, 1993; Hitchcock, 1993; Scholer and Polak, 1984). Therefore, the cloning and sequencing of a PDR5 homologue, CDR1 in C. albicans, was an important step towards an understanding of the mechanism of drug resistance (Prasad, et al., 1995b). CDR1 not only confers multiple drug resistance but also elicits collateral sensitivity to mitochondrial inhibitors. A few copies of CDR1 when expressed in a PDR5 null mutant of S. cerevisiae confer much higher level of resistance to cycloheximide as compared to the multicopy expression of PDR5 in the same background mutant (Prasad, et al., 1995b). Very recently, a few more homologues of CDR1 have been identified in C. albicans (Sanglard, et al., 1997) and some genes specific to fluconazole resistance have also been characterised (Vrana, 1993; Prasad, et al., 1995). The identification of several multidrug resistance loci in this pathogenic yeast has led to several studies where reduced accumulation of fluconazole has been shown to be related to its resistance (Clark, et al., 1996; Sanglard, et al., 1995; Parkinson, et al., 1995).

The broad specificity of ABC pumps (ability to efflux a number of hydrophobic compounds), role of ATP binding to nucleotide binding domain (NBD) and its subsequent hydrolysis, dissection of drug binding domains and search for physiological substrate(s) and function(s) are some of the interesting aspects which have been receiving considerable attention. The work embodied in this section deals specifically with the putative drug binding domains of Cdr1p.
is shown that the truncation of TM 12 of a yeast P-glycoprotein (Cdr1p) results in selective loss of drug resistance, since the strain expressing truncated CDR1 (ΔCDR1) was still able to confer resistance to large number of drugs and also that the ability of Cdr1p and its truncated form (ΔCdr1p) to efflux [³H]-β-estradiol and Rhodamine 123 remained unaltered. In order to understand the functionality of Cdr1p domains, ΔCDR1 was expressed both in S. cerevisiae and the baculovirus expression system. The results demonstrate that when expressed in baculovirus expression system, the deletion of TM 12 (ΔCdr1p) neither affects its membrane localization nor affects drug stimulatable ATPase activity.

3.1.2 Results

3.1.2.1 Drug resistance studies of both truncated and intact Cdr1p in yeast

S. cerevisiae transformed with plasmid carrying the complete CDR1 gene and truncated CDR1 were analyzed for drug resistance. The truncated CDR1 clone was constructed (Prasad, et al., 1995b) by a deletion of ~237 bp BamHI fragment from clone pS12-4, carrying the complete CDR1 cloned into pYEURA3 (Clontech, USA), resulting in a plasmid pB3.3 (ΔCDR1) (Fig 12a). Both clones were transformed into JG436, which had disrupted PDR5 and was hypersensitive to cycloheximide. The region removed from the complete gene is shown as yellow/bold colour (Fig 12b).

The deletion of TM12 resulted in a very interesting drug resistance profile. While the growth of pB3.3 was much slower compared to JG436 and S-12 as a result of truncation (Fig 13) it continued to confer resistance to number of drugs on filter disk assay (Table XVI, Fig. 14 a, b & c). The truncation resulted in a selective loss of drug resistance, which was most noteworthy in case of cycloheximide, anisomycin, sulfomethuron methyl and nystatin. These results suggest that the drug binding domains, are not exclusively localised in TM 12 but rather scattered to other parts of the molecule. Interestingly, TM 11 and 12 and TM 5 and 6 of human Mdr1p are also the possible sites for drug binding (Zhang, et al., 1995; Gottesman, et al., 1995; Gottesman, et al., 1996) (discussed below).
FIG. 12 Construct of CDR1 and ΔCDR1 in pYEURA3 vector. (a) Linearised map of pS-12-4 and pB3.3 carrying CDR1 and ΔCDR1, respectively, as described earlier in "Methods". Xm, XmnI; H, HindIII; B, BamHI. Yellow box depicts the truncated part. The arrow indicates the ORF length. (b) The C-terminal amino acid sequence of Cdr1p is depicted which shows TM11 and 12. The truncated amino acids are depicted in bold and yellow box. The amino acids in TM 11 and 12 domains are underlined. TM= transmembrane.
FIG. 13 Comparison of growth curve of both the transformants S-12-4 and B3.3 with their hosts JG436. Both the transformants and the host were grown in YNB medium with their supplements as described in the "Methods". Aliquot of cells was taken every two hours (after 10 hrs as shown in the fig.) till 26 hrs and OD was measured at $A_{595}$. 
### Results and Discussion

#### Table XVI. Drug resistant profile of yeast transformants

<table>
<thead>
<tr>
<th>Drugs (μg)a</th>
<th>JG436b</th>
<th>S-12-4c</th>
<th>B3.3d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide (0.5)</td>
<td>3.8</td>
<td>1.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Anisomycin (50)</td>
<td>2.0</td>
<td>N#</td>
<td>2.1</td>
</tr>
<tr>
<td>o-Phenanthroline (20)</td>
<td>1.6</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>4-Nitroquinolinoxide(10)</td>
<td>2.6</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Cerulenin (20)</td>
<td>2.8</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Fluconazole (100)</td>
<td>3.2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ketoconazole (10)</td>
<td>1.5</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Itraconazole (5)</td>
<td>1.7</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Miconazole (100)</td>
<td>3.4</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Oligomycin (0.2)</td>
<td>1.8</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Erythromycin (50)</td>
<td>2.0</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Chloramphenicol (500)</td>
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<td>1.1</td>
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<tr>
<td>Benomylo (50)</td>
<td>1.6</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Sulfomethuron methyl (20)</td>
<td>1.8</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Nystatin (1.0)</td>
<td>1.6</td>
<td>1.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

a The results are determined by using filter disk assay (Leppert et al, 1990) as described in "Methods". The values are mean of three independent experiments and represent the diameter of inhibition zone.

# N, no zone (no inhibition of growth).

b JG436, disrupted pdr5 (Δpdr5)

c S-12-4, Δpdr5+CDR1

d B3.3, Δpdr5+ΔCDR1

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#### 3.1.2.2 NTPase activity of Cdr1p and ΔCdr1p

Pdr5p of *S. cerevisiae*, a homologue of Cdr1p was earlier shown to elicit ATPase and UTPase activities (Decottignies, *et al.*, 1994). UTPase activity is native to Cdr1p, as it has not been shown by any other MDR transporters till now, so this can be used to show the intactness of functionality of truncated Cdr1p. Purified plasma membrane (PM) preparations were used to demonstrate the NTPase activity of Cdr1p. NTPase assay was performed as mentioned in Materials and methods. **Fig. 15a** shows that S-12-4 (Cdr1p) has ATPase (180 nmoles/mg protein /min) and UTPase (250 nmoles/mg protein/min) activities which were sensitive to vanadate (**Fig. 15b**). The truncated Cdr1p also had both ATPase (140 nmol/mg protein/min)
FIG. 14. Panels showing the representative drug resistance profile of yeast transformants and their host, obtained as a result of filter disc assay. Amount of drugs used is as mentioned in "Methods". The results of this assay are shown in Table XVI. Cyh, cycloheximide; Nys, nystatin; Smm, sulfometuron methyl; Ben, benomyl; Phe, phenanthroline; Ani, anisomycin; Cer, cerulenin; NQO, 4-nitroquinoline-N-oxide; Oli, oligomycin; Ery, erythromycin; Chl, chloramphenicol.
FIG. 15. Properties of NTPase activity of Cdr1p in yeast transformants. (a) ATPase and UTPase activity of purified plasma membrane from transformants was assayed as described in “Methods”. NTPase assay was done in presence of 5 mM Mg-NTP and 60 mM Tris-Cl (pH 7.5) and 10 mM NaN₃ (mitochondrial ATPase inhibitor). (b) Effect of vanadate was seen by performing NTPase assay in the presence of 50 μM vanadate as described in “Methods”. The bar shows mean of values in three independent experiments.
and UTPase (165 nmol/mg protein/min) activities, which were higher than the host JG436 (Fig. 15a). Cdr1p ATPase activity peaked over a broad pH range (4.5 to 7.5) which was similar to Pdr5p and mammalian P-glycoprotein (Fig. 16). UTPase activity on the other hand had a narrow pH range and peaked around pH 6.5 as shown in Fig. 16. The NTPase activities, which were detected in PM of host strain (JG436) had optimal pH between 5-6 and were sensitive to orthovanadate at those pH values as shown in Fig 15a. However, the ATPase activity of host was insensitive to orthovanadate at pH 7.5 (Fig. 15b). Interestingly, the NTPase activities of ΔCdr1p was insensitive to vanadate, however, when expressed in baculovirus expression system, the activity was sensitive to vanadate (discussed below) (Fig. 15b).

3.1.2.3 Rhodamine 123 transport in transformants

Rhodamine 123 is a fluorescent dye, which is taken up by a wide variety of cells expressing MDR1 and is effluxed out (Homolya, et al., 1993; Canitrot and Lautier, 1995). Rhodamine 123 (Rh 123) and rhodamine 6G are taken up by yeast cells also and recently quenching of its fluorescence has been linked to Pdr5p activity (Kolaczkowski, et al., 1996). In the present study, the accumulation of Rh 123 was followed to ascertain if the deletion of TM 12 would in any way affect the putative drug effluxing ability of Cdr1p. Our results demonstrate that the dye was accumulated much more abundantly (increase in relative fluorescence) in JG436 which had disrupted PDR5 (Fig. 17a). Strains expressing Cdr1p and ΔCdr1p showed almost equal amount of accumulation, which were about 50% less as compared to JG436. The ability of pS-12-4 and pB3.3 to efflux out Rh 123 was comparable (Fig 17a). This suggests that the removal of ~79 aa residues (including TM 12) did not affect their ability to efflux Rh 123 indicating the intactness of transport property of ΔCdr1p. Since JG436 does not have CDR1 or for that matter PDR5, it was unable to efflux Rh123 and exhibited maximum accumulation of the dye.

3.1.2.4 β-estradiol transport in transformants

Recently, human steroid hormone β-estradiol was reported as a possible physiological substrate effluxed by Pdr5p/Snq2p of S. cerevisiae (Mahe, et al., 1996). In order to assess the functionality of Cdr1p and ΔCdr1p, transport of [3H]-β-estradiol was checked in transformants as mentioned in Materials and Methods. As can be seen in Fig 17b, JG436 accumulated ~7.5
FIG. 16. pH profile of ATPase activity of Cdr1p in PM of S-12-4. ATPase and UTPase activity of purified plasma membrane from transformants was assayed as described in "Methods". NTPase assay was done in presence of 5 mM Mg-NTP and 60 mM Tris-Cl (pH 7.5) and 10 mM NaN₃ (mitochondrial ATPase inhibitor). NTPase assay was done in presence of buffer containing 5 mM Mg-NTP and 60 mM MES-KOH (pH values 4.5 -6.5) Tris-Cl (pH 7.5). Values shown are mean of three independent experiments.
FIG. 17 Rhodamine 123 and [3H]-β-estradiol uptake in JG436 and transformants, S-12-4 and B3.3. (a) Rhodamine 123 uptake was done as described in "Methods". Mid log phase cells were harvested and washed twice in PBS and 2% cell suspension was used for each experiment. The results mentioned are mean of three independent experiments. ± SD are shown. (b) Uptake of [3H]-β-estradiol was done as described in "Methods". Mid log phase cells were harvested and resuspended as 10% cells in fresh medium and the reaction performed for 1 h. Aliquots (0.5 ml) were taken and filtered rapidly following with three washes of buffer (PBS pH 7.4 containing 2% glucose). Radioactivity retained on the filter was measured as described in the "Methods". The results mentioned are mean of three independent experiments.
folds more $[^{3}\text{H}]-\beta$-estradiol as compared to transformants expressing either complete Cdr1p or Cdr1p. Here again, less accumulation of $[^{3}\text{H}]-\beta$-estradiol by the transformants as compared to host was related to Cdr1p dependent rapid efflux, which remained unaltered in Cdr1p as well (Fig 17b). Thus, truncation of Cdr1p did not affect the transport of $[^{3}\text{H}]-\beta$-estradiol also, significantly and therefore correlated well with the efflux pattern of rhodamine 123 (Fig 17a).

3.1.2.5 Construction of recombinant Baculovirus containing $\Delta$CDRI cDNA

In order to further check the functionality of $\Delta$Cdr1p, dissect the drug resistance domains and to obtain protein in large quantities for raising antibodies, it was expressed in the baculovirus expression system. Baculovirus expression system is one such system, which has been successfully used to overexpress membrane proteins, thus enabling the investigator to purify even less abundant membrane proteins. Baculoviruses are increasingly being used as helper-independent viral vectors for the high-level expression of foreign genes in insect cell lines (Spodoptera frugiperda) which provide eukaryotic environment. These viruses have a very late but highly abundantly expressed polyhedrin gene during the occlusion stage. AcNPV (Ac nuclear polyhedrosis virus) is the most commonly used virus and has been isolated from Autographa californica (alfalfa looper). Polyhedrin is not essential for the infection process, so it is possible to replace the polyhedrin gene with a heterologous gene while retaining the polyhedrin regulatory signals. The gene coding for the protein of interest is expressed under control of the polyhedrin promoter and the recombinants are selected on the basis of their occlusion-negative (occ) phenotype, which can be visually detected as plaques. The protein is reclaimed after lysing the cells and can be further purified using routine methods. Both membrane localised as well as cytosolic proteins have been purified using the baculovirus expression system. This study represents first attempt at expressing multidrug transporter (P-glycoprotein) of yeast in the insect cell system.

Truncated CDRI was cloned into pBacPAK8™ (Clontech, USA) as a BamHI fragment as outlined in Fig. 18 resulting in a plasmid pSKCDR1Δ which included a truncated coding region preceded by ~100 bp of 5'-untranslated region under the transcriptional control of the very strong polyhedrin promoter. Homologous recombination in vivo between the AcNPV sequences in transfer vector and AcNPV DNA generated a recombinant baculovirus
FIG. 18 Construct of \(\Delta CDRI\) in BacPAK8 vector. Schematic diagram depicting construction of pSKCDRI\(\Delta\). \(\Delta CDRI\) was subcloned into baculovirus shuttle vector BacPAK8 \(^\text{TM}\) as described in "Methods".
vSKCDR1Δ, which was identified by plaque assays and southern hybridisation screening (Hasnain and Nakhai, 1990) (Fig 19a & b). The integration of ΔCDR1 at the polh locus of vSKCDR1Δ was confirmed by Southern hybridization and dotblot of recombinant viral DNA with radiolabeled CDRI probe (Fig 19a). The recombinant virus which gave best results was then amplified to ~10^8 pfu/ml as mentioned in “Methods” section.

3.1.2.6 Expression of ΔCDR1 in Insect cells

For checking the expression of ΔCdr1p, vSKCDR1Δ infected Sf9 cells as well as uninfected and wild type AcNPV-infected control cell extracts were analysed on SDS-PAGE. Coomassie blue stained gel showed a protein band exclusively in the vSKCDR1Δ infected cells which was absent in both uninfected or wild type AcNPV infected Sf9 cells (Fig. 20a). Using [³⁵S]-methionine, the time course analysis of ΔCdr1p synthesis in vSKCDR1Δ infected Sf9 cells revealed a ~130 kDa band as early as 36 hpi and by 60-84 hpi this protein was one of the main protein synthesised (Fig. 20b). In comparison, the wild type virus AcNPV infected Sf9 cells expressed polyhedrin protein as a prominent band. The kinetics of ΔCdr1p synthesis matched with polyhedrin synthesis of wild type AcNPV (O'Reilly, et al., 1992). The recombinant truncated multidrug transporter ΔCdr1p synthesised in insect cells however, displayed a lower apparent molecular weight than its calculated molecular weight (~160.9 kDa).

3.1.2.7 Localisation of ΔCdr1p in insect cells

Cdr1p is a putative membrane bound multidrug efflux pump, however, its localization has not been established. The expression of ΔCdr1p in baculovirus expression system enabled us to study its intracellular localisation. Membranes were isolated from vSKCDR1Δ infected Sf9 cells and were analysed on SDS-PAGE. Fig. 20c shows a band of ~130 kDa appearing only in recombinant virus infected samples. As can be seen from the Fig. 20c both low speed (LP) and the high speed (HP) pellets showed the band, however, it was more prominent in LP. The excised protein band corresponding to ΔCdr1p was excised from SDS-PAGE, pooled and used to raise polyclonal antibodies. Using the polyclonal antisera, a single band of ~130 kDa
FIG. 19. **Confirmation of recombinant Baculovirus carrying truncated CDR1 gene.**

(a) Southern blot showing the integration of 4.3 kb truncated CDR1 gene in *AcNPV* genome. Southern blot was performed according to standard procedure as mentioned in Sambrook, *et al* (1992). λ, indicates the DNA marker (Sigma, USA); E, *EcoRI*; B, *BamHI*; H, *HindIII*; Mock, DNA from uninfected *Sf9* cells; vSKCDR1Δ(#6), DNA from *Sf9* cells infected with recombinant virus clone no.6; vSKCDR1Δ(#8), DNA from *Sf9* cells infected with recombinant virus clone no.8; *AcNPV*, DNA from cells infected with wild type virus.

(b) Dot blots showing the 1st and 2nd screening for isolation of recombinant virus. +, positive control; -, BacPAK8 DNA negative control; Ac, DNA from wild type virus infected cells; M, DNA from uninfected cells.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Mock (Sf9)</th>
<th>vSKCDR1Δ(#6)</th>
<th>vSKCDR1Δ(#8)</th>
<th>λ #6</th>
<th>AcNPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>E</td>
<td>B</td>
<td>H</td>
<td>E</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
FIG. 20. Analysis of expression and the targeting of ΔCdr1p in Sf9 cells. (a) Coomassie blue stained SDS-PAGE analysis of vSKCDR1Δ infected sample using uninfected and wild type (AcNPV) as control. Sf9 cells infected with recombinant virus were harvested at different h pi (as mentioned in the panel) and lysed in SDS-PAGE sample buffer and were electrophoresed on 10% SDS-PAGE, stained with Coomassie blue, as described in “Methods”. (b) Time course [35S]-methionine labeling of Sf9 cells expressing ΔCdr1p. [35S]-methionine labeling of cells infected with recombinant virus was done as described in “Methods”. Different hpi is as mentioned in the panel. (c) ΔCdr1p is targeted to the membrane of Sf9 infected with recombinant virus. Membranes were purified from Sf9 cells after infection with recombinant virus for 72 h and gel was run as described in “Methods”. 25 μg of membrane protein was loaded on to each lane. Abbreviations: LP, low speed pellet; HP, high speed pellet; Ac, AcNPV; Rec, recombinant virus (vSKCDR1Δ). (d) Western blot analysis of membranes isolated from Sf9 infected with recombinant virus for 72 h. 25 μg of membrane protein were loaded on to each lane and blotted on to the Nylon membrane (Amersham, UK) as described in “Methods”. LP and HP is same as mentioned legend (c).
was detected from recombinant virus in both LP and HP membrane fractions (Fig. 20d). The polyclonal antisera did not elicit any significant cross reactivity with other membrane proteins. In order to confirm that expressed ΔCdr1p is indeed targeted and assembled into plasma membrane, immunofluorescence microscopy was done. Sf9 cells infected with either AcNPV or vSKCDR1Δ were harvested 72 hpi and stained with polyclonal antibody and anti rabbit IgG conjugated to FITC. Cell surface fluorescence was detected only on cells infected with recombinant virus (panel 3, Fig. 21) but not on AcNPV or uninfected cells (panels 1 & 2, Fig 21). Preimmune control antibody also gave no fluorescence on staining of recombinant virus infected cells. The ΔCdr1p was thus correctly inserted into plasma membrane of insect cells and deletion of C-terminal end from the complete Cdr1p did not affect its localization.

3.1.2.8 NTPase assay of ΔCdr1p in insect cells
To check whether ΔCdr1p expressed in insect cells is capable of eliciting NTPase activity like Cdr1p in yeast (Fig 15a), NTPase assays were performed on membranes purified from vSKCDR1Δ infected insect cells. As shown in Fig. 22a, only recombinant virus infected membranes of Sf9 cells showed NTPase activity. Both ATPase and UTPase activities of recombinant virus were vanadate (50 μM) sensitive (Fig. 22b) while the activities of wild type AcNPV infected cells were largely insensitive to vanadate. The appearance of nucleotide phosphatase activities was closely linked to the expression of ΔCdr1p, which peaked around 72 hpi (Fig. 22c). No such peaks of activities were seen in controls. Similar to Cdr1p ATPase expressed in yeast, vSKCDR1Δ infected Sf9 cells also exhibited broad specificity towards other nucleotides (Fig. 22d).

3.1.2.9 Insect cell derived recombinant ΔCdr1p ATPase activity is stimulated by a number of drugs
Drug stimulatable ATPase activity by mammalian P-glycoprotein is well known. It has been demonstrated that different stimulatory effects of drugs on the ATPase activity is closely linked to the difference in drug transport activities (Gottesman, et al., 1996; Ambudkar, et al., 1992). However, such an effect has not been observed with their counterparts in yeast (Decottignies, et al., 1994, Fig 23). Since a selective loss in drug resistance in yeast expressing ΔCdr1p was
FIG. 21 Immunofluorescence localisation of ΔCdr1p in recombinant Baculovirus infected Sf9 cells. Immunofluorescence was performed on insect cells as described in "Methods". The cells were harvested after infection for 72 h. The left and the middle panel show the controls (uninfected and wild type infected) mentioned as Mock and AcNPV. The rightmost panel shows the Sf9 cells infected with recombinant virus (vSKCDR1Δ).
FIG. 22. Properties of ΔCdr1p expressed in insect cells. (a) NTPase activities of ΔCdr1p expressed in insect cells. Membranes were purified from Sf9 cells infected with recombinant virus and wild type AcNPV virus. NTPase assay was performed as described in “Methods”. (b) Percentage inhibition of NTPase activities by vanadate. NTPase assay was performed in the presence of 50 μM vanadate as described in “Methods”. (c) Time dependent NTPase activity of ΔCdr1p expressed in Sf9 cells. Sf9 cells were harvested at different time points (in hours post infection) indicated in the figure, after infection with wild type virus (AcNPV) and recombinant virus (vSKCDR1A). (d) Substrate specificity of ATPase activity of recombinant virus (vSKCDR1A). As indicated in "Methods", ATPase assay was performed in presence of indicated nucleotides.
FIG. 23. Drug stimulated ATPase activity of ΔCdr1p expressed in yeast. ATPase assay was carried out as described in "Methods". The indicated concentration of the drug (same as was used to check drug resistance, see Table XVI) was added to the membrane fraction before initiating the reaction by 5 mM ATP. All other assay conditions were same as of Fig. 17a. The values are mean of three independent experiments.
observed, it was important to ascertain if it was not due to poor interaction of drugs with the transporter. The functional expression of ΔCdr1p in baculovirus expression system provided an opportunity to monitor the interaction between various drugs and ATPase activity. Fig. 24 depicts the effect of various drugs on ATPase activity. The concentrations of drugs used were similar to what was used to check drug resistance in yeast cells expressing complete or ΔCdr1p. It is clear from Fig. 24 that most of the drugs were able to stimulate ATPase activity, but it was most noteworthy in case of cycloheximide, o-phenanthroline, anisomycin, 4-NQO and Rh123. Interestingly, in certain instances, a direct correlation between drug resistance and their ability to stimulate ATPase activity could not be established.

3.1.3 **DISCUSSION**

Results presented in this section demonstrates that the deletion of TM12 of yeast P-glycoprotein (Cdr1p in present case) leads to a selective loss in multidrug resistance. A deletion of 79 aa from C-terminal end of Cdr1p and its expression in yeast resulted in impaired resistance to cycloheximide, anisomycin, sulfomethuron methyl and nystatin while resistance to other drugs e.g. o-phenanthroline, 4-NQO, fluconazole, itraconazole, ketoconazole, miconazole, erythromycin, benomyl and to mitochondrial inhibitors e.g. oligomycin and chloramphenicol remained unaltered (Table XVI). The ability to efflux Rh 123 and [³H]-β-estradiol by Cdr1p and ΔCdr1p expressing yeast cells also did not change as a result of C-terminal deletion (Fig. 17a and b). It has been previously shown immunologically as well as by mutational studies that TM 11-12 and TM 5-6 of human Mdr1p form part of drug binding domains (Welker, et al., 1995; Greenberger, 1993; Zhang, et al., 1995; Safa, 1988; Rao and Scarborough, 1994). Any mutation in these regions leads to loss in drug resistance. It has been pointed out that a progressive replacements in TM 12 region of human Mdr1p resulted in impaired resistance to actinomycin D, vincristine and doxorubicin, however, resistance to colchicine did not change (Zhang, et al., 1995; Welker, et al., 1995). The results of present study show that similar to human Mdr1p and voltage-gated ion channel, drug binding domains of a yeast transporter are located in and around TM 12 (Welker, et al., 1995; Greenberger, 1993; Zhang, et al., 1995; Safa, 1988; Rao and Scarborough, 1994). This would imply that the mammalian and yeast P-
FIG. 24. Drug stimulated ATPase activity of ΔCdr1p expressed in Sf9 infected with recombinant virus for 72 h. ATPase assay was carried out as described in “Methods”. The indicated concentration of the drugs (same as was used to check drug resistance, see table XVI) was added to the membrane fraction before initiating the reaction by 5 mM ATP. All other assay conditions were same as of Fig. 15a. The values are mean of three independent experiments.
glycoproteins are not only structurally homologous molecules but their functional domains are also preserved across the evolutionary scale, which is of enormous significance. In view of the conserved homology with respect to human MDR1, other part of the Cdr1p viz., TM 5 and 6 are also likely to contain drug-binding sites.

The physiological role of multidrug transporters has been the subject of intense research. The fact that Cdr1p can transport steroid hormones tempts us to speculate whether the human steroid could be the physiological substrate for yeast multidrug transporters. Recently it has been shown that Pdr5p/Snq2p of S. cerevisiae, both homologues of Cdr1p, transport β-estradiol (Mahe, et al., 1996; Kolaczkowski, et al., 1996). Earlier, estrogen and corticosterone binding proteins have been characterized both in C. albicans and S. cerevisiae (Skowronski and Feldman, 1989; Malloy et al., 1993; Feldman, 1996; Loose et al., 1983). This, together with the fact that human hormones like progesterone and β-estradiol can significantly enhance the transcription of CDR1 mRNA, strongly suggest the possibility of a steroid signaling pathway in C. albicans which would have considerable clinical significance.

While there was total loss of resistance to some drugs by strain carrying ΔCDR1, the NTPase activity was not lost completely and remained higher as compared to its host JG436. We have tested, whether a few drugs which are apparently being transported by Cdr1p, could stimulate ATPase activity, as observed in the case of mammalian P-glycoprotein. But none of the tested drugs could significantly stimulate ATPase activity of Cdr1p in yeast. The NTPase activity of Pdr5p could also not be stimulated by tested drugs after purification from overexpressing strain. The detergent used for solubilization of membrane protein probably interfered with drug binding and NTP hydrolysis in the above study (Decottignies et al., 1994). However, as discussed below, we could demonstrate the drug stimulated ATPase activity of ΔCdr1p expressed in the baculovirus expression system.

In order to functionally characterise the Cdr1p, its variant ΔCdr1p was overexpressed in baculovirus-insect cell expression system to obtain the protein in large quantities. Overexpression of several membrane proteins including human Mdr1p has been achieved by using baculovirus expression system (Germann, et al., 1990). Since insect cells are able to accomplish many of the higher eukaryotic post-translation modifications, a recombinant protein produced by this expression system is expected to be functionally active. Here it is reported that
ΔCdr1p, which had deletion of 79 aa from C-terminal end, was expressed in Sφ9 cells infected with the recombinant virus. The molecular size of Δ Cdr1p after accounting the deletion of 79 aa is about ~160 kDa, however, on SDS-PAGE it migrated to ~130 kDa. Human Mdr1p when expressed in baculovirus expression system also showed a lower band of 140 kDa which corresponded to its non-glycosylated precursor (Germann, et al., 1990). The ΔCdr1p though synthesised as a function of polyhedrin promoter activity, probably is also not appropriately glycosylated. The baculovirus expression system is capable of accomplishing glycosylation of recombinant protein but probably the hydrophobic nature and large size of multidrug transporter interferes with it. It is known that Ppolh (used in this study) which is a very late expressing promoter, expresses the protein at 36-72 hpi and by that time the cellular machinery is unable to completely glycosylate the synthesised protein (Hasnain, et al., 1994; Sridhar, et al., 1993; Theibaut, 1993; Jha, et al., 1992; Nakhai, et al., 1991; Nakhai, et al., 1991). Indeed, the lack of glycosylation of ΔCdr1p neither affected the functionality of the protein nor affected its intracellular localization. The immunofluorescence revealed that the ΔCdr1p is properly localised in plasma membrane.

The expression of ΔCdr1p in baculovirus-insect expression system generated a high drug stimulated plasma membrane bound ATPase activity. Drug stimulated ATPase activity, which is very well demonstrated for Mdr1p (Sarkadi, et al., 1991; Welker, et al., 1995), could not be observed in case of yeast transporter when expressed in yeast cells. Therefore, this observation acquires considerable significance. It is apparent that the C-terminal truncation of Cdr1p did not alter drug interaction with nucleotide binding domains (the drug stimulated ATPase activity in complete Cdr1p has not been compared). It has been demonstrated for human Mdr1p that different stimulatory effects of drugs on ATPase activity are closely related to the drug transport (resistance) activities (Gottesman, et al., 1995; Gottesman, et al., 1996). However, such a distinct correlation was not observed. Yeast cells expressing ΔCdr1p were completely sensitive to cycloheximide and anisomycin but recombinant protein overproduced in Sφ9 cells showed cycloheximide and anisomycin stimulated ATPase activity. It was a reverse situation in case of miconazole. While ΔCdr1p expression, in yeast could confer resistance to miconazole but the drug had no effect on ATPase activity. The fact that the ATPase activities of Cdr1p and Δ Cdr1p in yeast plasma membrane were close to each other would suggest that the truncation of
Results and Discussion

79 amino acid residues had no effect on nucleotide binding domains and its interaction with the drugs. While yeast multidrug transporter have been identified for few years but in this work for the first time it is demonstrated that the organisation of drug binding domains are preserved in a multidrug transporter of a pathogenic yeast. Since CDRI is a homologue of PDR5 and SNQ2, a similar domain organisation is expected in these transporters as well. Because of the relative ease with the genetics of yeast, it would be worthwhile to ascertain by site directed mutagenesis, the role of individual amino acids in such conserved domains.