PURIFICATION AND CHARACTERIZATION OF C-TERMINAL NUCLEOTIDE BINDING DOMAIN (NBD-2) OF Cdr1p, A MULTIDRUG ABC TRANSPORTER OF *CANDIDA ALBICANS*

Thesis submitted in partial fulfillment of the requirement for the award of degree of Master of Philosophy

BY

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CERTIFICATE

This is certify that this thesis titled " **Purification and Characterization of C-terminal Nucleotide Binding Domain (NBD-2) of Cdr1p, a Multidrug ABC transporter of *Candida albicans*** " submitted to Jawaharlal Nehru University, New Delhi in the fulfillment of the requirement for the award of the degree of Master of Philosophy, embodies original research work carried out by Antresh Kumar at School of Life Sciences, Jawaharlal Nehru University, New Delhi under our guidance and has been not submitted in part or full for any degree or diploma.

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DECLARATION

The research work entitled " **Purification and Characterization of C-terminal Nucleotide Binding Domain (NBD-2) of Cdr1p, a Multidrug ABC transporter of *Candida albicans*** " presented in this thesis embodies the results of original work carried out by me at the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work has not been submitted in part or full for any degree or diploma of this or any other university.

Antresh Kumar

(Candidate)

December, 2005

...To my senior

Sudhanshu Shukla

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Abbreviations

ABC	ATP binding cassette
AIDS	Acquired immunodeficiency syndrome
ALP	Adrenoleukodystrophy
CaMdr1p	*Candida albicans* multidrug resistance 1 protein
Cdr1p	Candida drug resistance 1 protein
DM	Demethylase
EF	Elongation Factor
FLU	Fluconazole
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency Virus
Kda	Kilo dalton
MDR	Multidrug resistance
MET	Multidrug efflux transporters
MFC	Major facilitator superfamily
MRP	Multidrug resistant associated protein
NBD	Nucleotide binding domain
$P450^{14\alpha dm}$	P450 14 α-demethylase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDR	Pleiotropic drug resistance
Pgp	P-glycoprotein
PMSF	Phenylmethylsulphonyl fluoride
RLI	RNase-L Inhibitor
SDS	Sodium dodecyl sulphate
TEMED	N, N, N, N'- tetramethylenediamine

Introduction

Introduction

The fungal infection in humans can be caused by a number of microbes like dermatophytes, hypomycetes and yeasts. Fungal infections may be divided into two broad categories: 1) nosocomial and 2) community acquired. All nosocomial fungal infections may be considered as opportunistic: because these fungi are normally commensal organisms but may cause life-threatening infection in immunocompromised patients. In contrast, community-acquired fungal infections encompass not only opportunistic but also endemic infection, in which susceptibility to the infection is acquired by living geographical area constituting the natural habitat of a pathogenic fungus. Falkow (1997) recognized two types of pathogenic microorganisms: 1) primary fungal pathogens that cause disease among a certain portion of healthy and normal individuals and such pathogen depends on its ability to replicate and transmitted in a particular host population and 2) opportunistic fungal pathogens that cause disease only in the immunocompromised patients.

Candida and Candidiasis

Candida species are true opportunistic pathogen that produces a wide spectrum of diseases ranging from superficial mucocutaneous diseases to invasive illnesses, such as hepatosplenic candidiasis, *Candida* peritonitis and systemic candidiasis (Prasad *et al,* 2002; Anaissie *et al.,* 2003). Candida species have been reported to be the sixth most commonly isolated and fourth most prevalent blood stream pathogen isolated. Till date, more than 150 species of *Candida* exist in nature; but only a few species are recognized as disease causing in humans. Only seven species in the genus of *Candida* are known opportunistic human pathogens that are listed in Table1. *C. albicans* accounts for approximately 50-60 % of candidiasis in humans. *C glabrata* recently has become important because of the increasing incidence of candidiasis world wide (Nedret *et al.*, 2002). It is also intrinsically more resistant to azoles and amphotericin B. Some of *Candida* species, *C. lusitaniae* and *C. guilliermondi* are important because of their resistance to fluconazole. Another important *Candida* species is *C. krusei,* since it has clinical significance dye to its intrinsic resistance to azole and its lower susceptibility to all the antifungals, including amophotericin B (Mukherjee *at el.,* 2005).

Table1: Species commonly causing candidiasis and its frequency

Medically significant *Candida* Species	% Abundance
Candida albicans	50-60 %
Candida glabrata	15-20 %
Candida parapsilasis	10-20 %
Candida tropicalis	6-12 %
Candida gulliermondi	<5 %
Candida lustaniae	<5 %
Candida krusei	1-3 %

Treatment of Candidiasis

The antifungal drugs, commonly available in the market are used for treatment of *Candida* infection. These antifungals are categorized into five classes on the basis of their structure and modes of action have been summarized in Table 2 or Fig 1. Azoles, the most commonly used drug, functions by inhibiting ergosterol biosynthesis by blocking 14α-lanosterol demethylase (*ERG11*). Allylamines block the first step of the ergosterol biosynthesis resulting in the accumulation of toxic squalene. Polyene derivatives of antifungal drugs directly bind to the membrane ergosterol, resulting in pore formation, which makes the cell membrane leaky and results in cell death. Candins inhibits cell wall synthesis by inhibiting the glucan synthesis. Pyrimidines, inhibit nucleic acid synthesis by inhibiting thymidylate synthase.

Multidrug Resistance (MDR)

In spite of the advancement of medical field and discovery of more drugs, combating fungal infection is a challenging task because the treatment of fungal infection is hampered by two commonly encountered problems: 1) availability of narrow spectrum dugs and toxicity associated with them, 2) development of resistance against the available antifungals. In the current scenario, the treatment of fungal infection has lagged behind bacterial chemotherapy due to its paucity of antifungals available. The reason behind this failure is that the compounds that inhibit fungal protein, RNA or DNA biosynthesis have

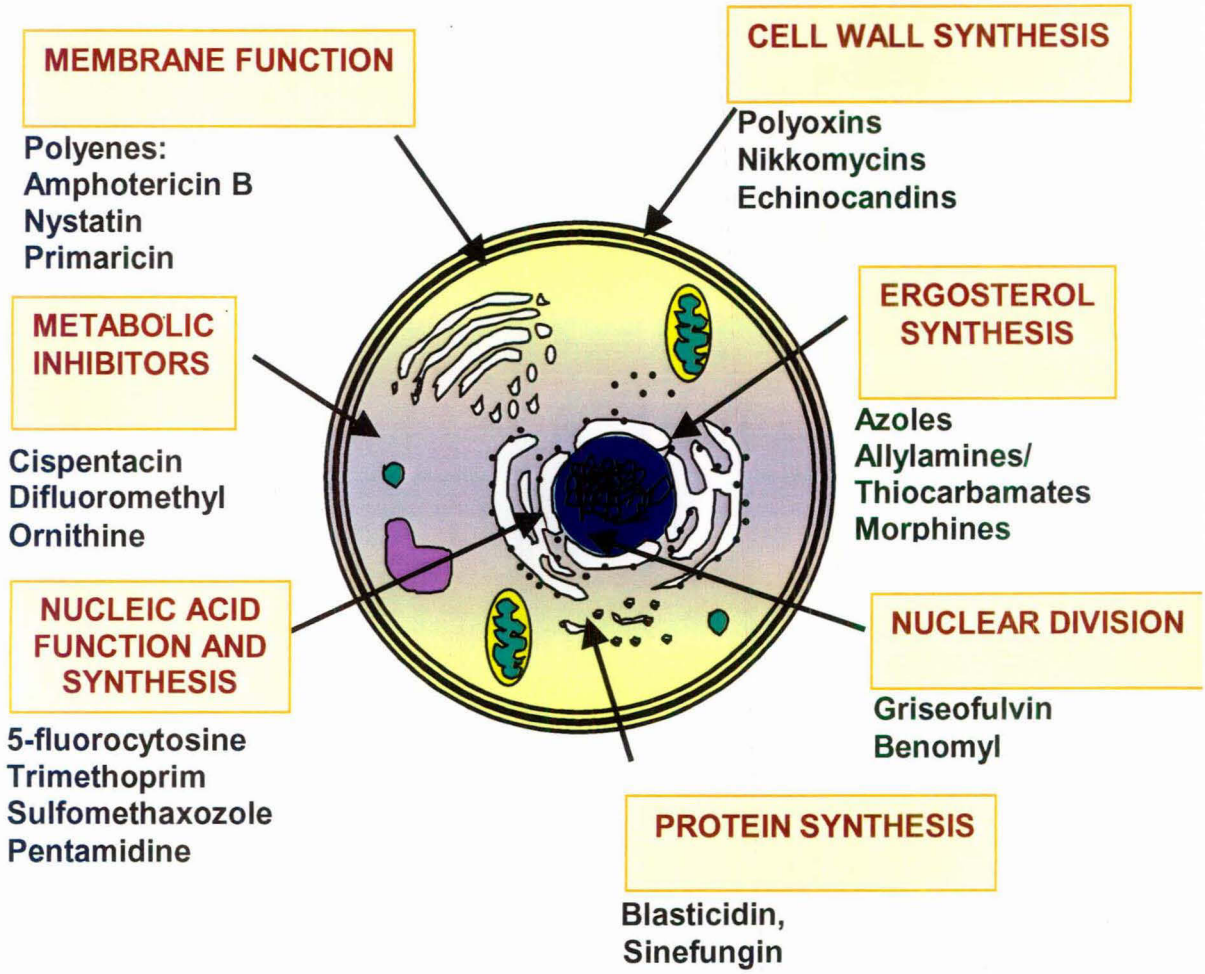


Fig. 1: Site and mechanism of action of different classes of drugs on a typical fungal cell. [Source: Adopted from Georgopapadakou and Walsh, 1994]

the same effect on the infected patients resulting in adverse side effects. A prime cause behind the failure in the treatment of fungal infection is also the emerging incidence of antifungal resistance or multidrug resistance. As similar to bacteria, resistance among fungi can be classified as primary and secondary level of resistance.

Primary or intrinsic, resistance refers to an organism's natural susceptibility to an antimicrobial agent. This innate level of susceptibility is thought to be a characteristic of the organism and is independent of drug exposure. It is a predictable trait. For instance, it is widely accepted that *Candida albicans* is more susceptible to fluconazole than *Candida krusei.* As a result, if *Candida krusei* is isolated, fluconazole is generally not selected for fungal treatment. When considering the larger picture, primary resistance is more

Table. 2: Structural representation of antifungals drugs of each class along with its target and mode of action in yeast Cell.

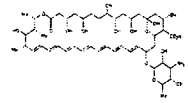
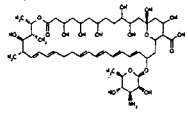
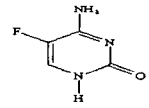
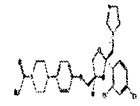
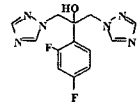
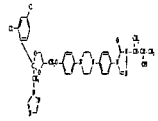
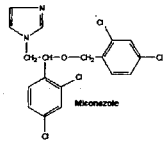
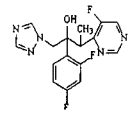
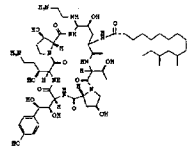
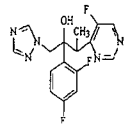
Class	Drug	Site of action	Target	Mode of action	Resistance organisms
Polynes	Amphotericin B 	Membrane	Membrane ergosterol	Binds to sterols, Mainly ergosterol, in cell membrane resulting in change in permeability of membrane and cell death	*Fusarium spp., Aspergillus nidulans, Candida spp., Trichosporon spp.,*
	Nystatin 				
Pyrimidines	5-Flucytosine 	Nucleic acid function and synthesis	Cytosine permease, Cytosine deminase, uracil: phosphoribo-syltransferase	It enters cells via cytosine permease and is deaminated to active form 5-FU is then converted to FUMP by phosphoribo-syltransferase. FUMP can be converted into FUTP or FdUMP, which inhibits protein synthesis or DNA synthesis, respectively.	*Candida spp., Cryptococcus neoformans, Aspergillus.*
Azoles	Ketoconazole 			Inhibition of Cytochrome P450 14α-demethylase; accumulation of lanosterol leading to perturbation of fungal cell membrane.	*Candida spp., Cryptococcus spp.,*
	Fluconazole 	Ergosterol synthesis	Cytochrome P450 14α-demethylase	More selective Inhibition of CytochromeP450 14α-demethylase	*Candida spp., Cryptococcus spp., Histoplasma capsutum*
	Itraconazole 			Inhibition of Cytochrome P450 14α-demethylase.	*Candida spp., Aspergillus spp.,*
	Micronazole 				

Table2 (*continued*)

Class	Drug	Site of action	Target	Mode of action	Resistance organisms
	Voriconazole 			More selective Inhibition of Cytochrome P450 14α-demethylase	*Candida spp., Muccor, Rhizomucor*
Candins	Echinocandins 	Cell wall Synthesis	β-1,3-Glucan synthase	Inhibition of cell-wall glucan synthesis leading to susceptibility of fungal cell to osmosis lysis.	*Candida spp,. Cryptococcus spp., Aspergillus spp.*
Allylamines	Terbinafine 	Ergosterol Synthesis	Squalene epoxidase	Inhibition of Squalene epoxidase; fungicidal effect may be due to accumulation of toxic squalene rather than deficiency of ergosterol.	*Saccharomyces cerevisiae, Ustilago maydis, Candida glabrata*

problematic. A single agent or class of drugs is widely administered because the fungus most commonly encountered is highly susceptible to it.

Secondary or acquired resistance is much less predictable and potentially more problematic than primary resistance under conditions of environmental stress like exposure to antifungal agents, a population of initially susceptible fungi may start showing resistance. This may result from expression of newly acquired genetic alterations, translation and expression of previously repressed metabolic pathways, or unmasking of an existing, less susceptible fungal subpopulation.

Mechanism of antifungal resistance

There is significant diversity in the molecular mechanism of conferring resistance to drugs. The drug resistance in *Candida is* a multifactorial phenomenon (Balkis *et al.*, 2002; Morschhauser, 2002) Fig. 2. Some of the well-known mechanisms of drug resistance in pathogenic *Candida* include: alteration in the ergosterol biosynthesis pathways by an overexpression of the *ERG11* gene, which encodes the drug target enzyme 14α-demethylase or by a point mutation in target enzymes, which leads to reduced affinity to fluconazole; reduced intracellular accumulation of drugs is another prominent mechanism of resistance in *Candida* cells where gene encoding drug efflux pump belonging to ABC transporter and

MFS superfamilies of proteins are overexpressed. The azole-resistant clinical isolates of *Candida* exhibit activation of genes encoding ABC (Cdr1p, Cdr2p) or MFS (CaMdr1p) proteins. Invariably, resistant *Candida* shows a simultaneous increase in efflux of drugs. Rapid efflux of incoming drug prevents cell from accumulating lethal concentration of azoles and enable them to survive (Prasad *et al.,* 1995; Sanglard *et al.,* 1995; White, 1997a). Drug inactivation, which is a very common mechanism in bacteria, has not been found in *Candida* cells. Recent gene profiling results already suggest even more complexities, which may affect and control the phenomenon of antifungal resistance (De Backer *et al.,* 2001; Kaur *et al.,* 2004; Krishnamurthy *et al.,* 2004; Rogers and Barker, 2002, 2003). Some of the well-known mechanisms of drug resistance are listed in Table. 3 and few of them are discussed below.

***ERG11* (14α-lanosterol demethylase)**

Azole resistance in *Candida albicans* primarily through *ERG11*, which encodes 14α-lanosterol demethylase (*CYP51*, also called P450 14DM) involved in sterol biosynthesis. Several lines of evidence suggest that the primary target of azoles is a 14α-lanosterol demethylase (heme protein), which is involved in the conversion of lanosterol to ergosterol. Azole derivatives interact with heme molecule in P450 DM where an unhindered nitrogen atom of the azole ring (N_3 in imidazole or N_4 in derivatives) binds to heme iron at its sixth co-ordination position. Azoles block the sixth co-ordination position, which is normally occupied by activated oxygen and thus prevents initiation of the hydroxylation reaction. Inhibition of 14α-lanosterol demethylase does not block the pathway at lanosterol, although lanosterol concentrations do increase and this inhibition leads to depletion of ergosterol. Instead, lanosterol with its 14- methyl group remains intact, but downstream enzymes become the drug targets to generate 14-methylated intermediates (lanosterol 4,14-dimethylzymosterol and 24-methylenedihydrolanosterol). These intermediates are toxic and responsible for cell growth inhibition (Robert, 2005). The entry of azole inside the cell still remains unsolved although it has been suggested that the hydrophobicity of this drug could facilitate its entry. Once the drug enters the cell its interaction with P450 DM can be affected in two ways to develop drug resistance:

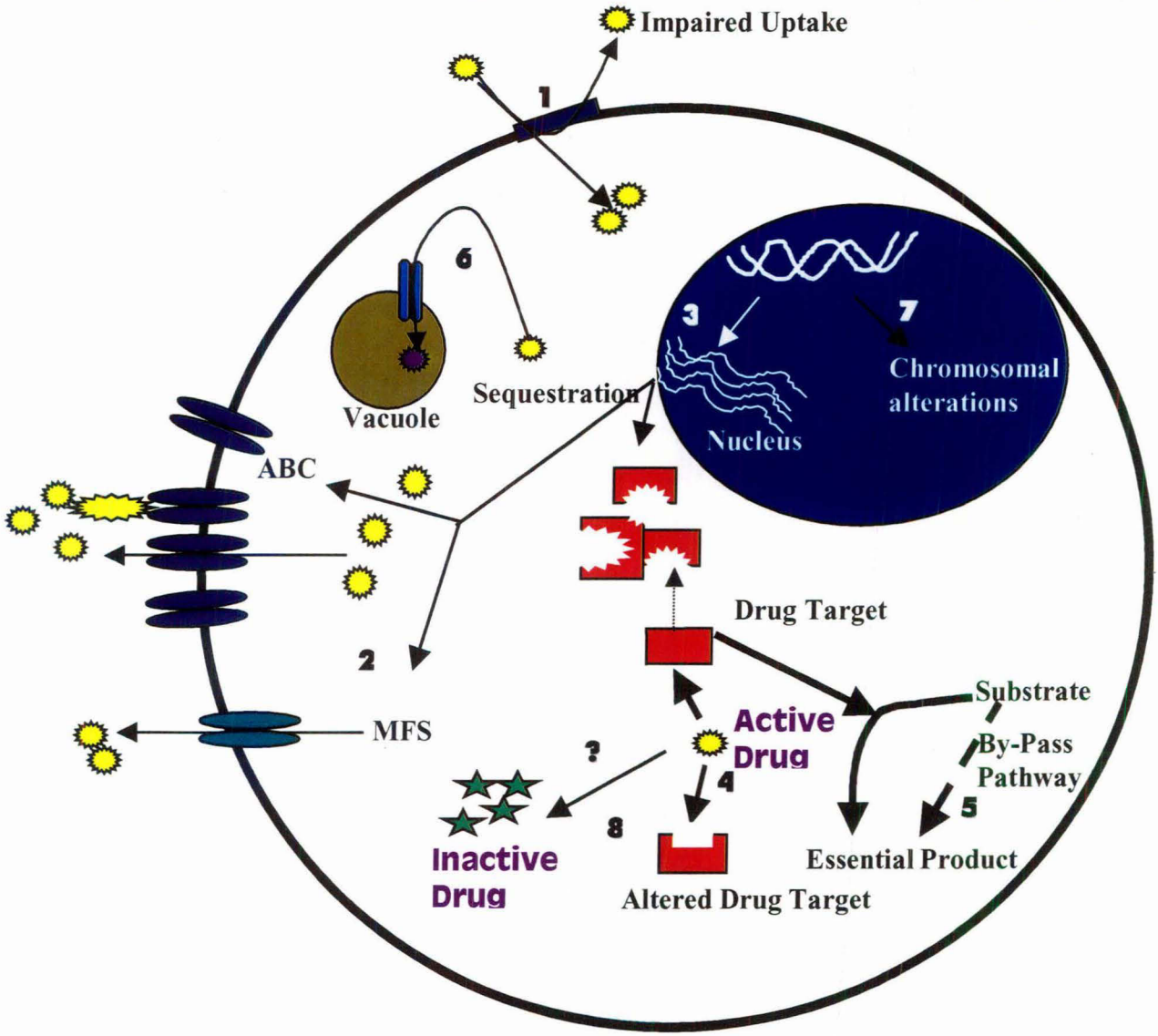


Fig. 2: Prominent drug resistance mechanisms in *Candida*. (1) Changes in cell wall / plasma membrane leading to impaired drug uptake. (2) Efflux of drugs mediated by the ABC or the MFS class of efflux pumps. (3) Overexpression of the drug target or of the efflux pumps. (4) Mutation in the drug target (P45014DM) does not allow the drug to bind due to low affinity. (5) Activation of alternate pathways such as $\Delta^{5,6}$-desaturase. (6) Sequestration of the drug into an organelle-like vacuole by organellar pump. (7) Chromosome alterations or changes in chromosome number as a means to maintain more copies of the required gene. (8) Modification of drug to an inactive form. [Source: modified from Prasad *et al.*, 2000].

Table.3: Different mechanisms of drug resistance developed toward common antifungals by *albicans* as well as Non-*albicans* species of *Candida*.

Fungi	Antifungals	Target gene	Target	Mechanism of Resistance	References
Candida albicans	Azoles	*ERG11*	Cytochrome P-450 14α-demethylase.	Point mutation in the target enzyme alters the affinity of the enzyme to azoles. Overexpression of target genes.	Lamb *et al.*, 1997; Sanglard *et al.*, 1995.
		CaCDR1 *CaCDR2* *CaMDR1*	ABC transporters	Overexpression of ABC and MFS family of multidrug transporters.	Prasad *et al.*, 1995; Sanglard *et al.*, 1995 ; White, 1997a.
		ERG3	$\Delta^{5,6}$- desaturase	Alteration of the sterol.	Kelly *et al.*, 1997a.
	Non-azoles				
	5-Flucytosine		Thymidylate synthase	Not reported.	Cuenca-Estrella *et al.*, 2001;
	Polyenes Amphotericin B		Membrane ergosterol	Increased membrane fluidity.	Kelly *et al.*, 1997 ;
	Nystatin		Membrane ergosterol	Alteration in membrane sterols.	Martin & Dinsdale, 1982.
Candida dubliniensis	Azoles	*ERG11*	Cytochrome P-450 14α-demethylase	Overexpression of target genes.	Pinjon *et al.*, 2003.
		CaCDR1 *CaCDR2* *CaMDR1*	ABC transporters	Overexpression of ABC and MFS family of multidrug transporters.	Moran *et al.*, 1998.
		ERG3	$\Delta^{5,6}$- desaturase	Defect in the sterol.	Pinjon *et al.*, 2003.
Candida krusei	Azoles	*ABC1*	ABC transporter	Overexpression of target gene.	Katiyar and Edlind, 2001; Orozco *et al.*, 1996
		ABC2	Cytochrome P-450 14α-demethylase	Reduced susceptibility of target enzyme to inhibition. Reduced accumulation of drug.	Venkateswarlu *et al.*, 1996

	Non-azoles				
	Amphotericin B			Decreased membrane ergosterol content.	Safe *et al.*, 1997
	Nystatin			Absence of membrane ergosterol.	Mas & Pina, 1980
	5-Flucytosine			Not reported	Pfaller *et al* ., 2002
Candida glabrata	Azoles	*ERG11*		Overexpression of target gene.	Hitchcock *et al.*, 1993 Miyazaki *et al.*, 1998 Redding *et al.*, 2003
		CaCDR1 *CaCDR2* *CaMDR1*	ABC transporters	Overexpression of efflux pumps.	
		PDH1		Overexpression of target gene Mitochondrial loss. Decreased membrane permeability.	
	Non-azoles				
	Amphotericin B			Decreased membrane ergosterol component.	Sterling *et al.*, 1996
	5-Flucytosine			Not reported	Cuenca-Estrella *et al.*, 2001
Candida tropicalis	Azoles	CtCDR1	ABC transporter	Overexpression of efflux pumps.	Barchiesi *et al.*, 2000; Jandourek *et al.*, 1999

target alteration and overexpression of *ERG11* (Ghannoum and Rice 1999; Marichal 1999; White, 1997b).

Alteration in *ERG11*

Since Erg11p is the target of azole derivatives, it can be expected that amino acid substitution could affect the affinity of the drug. Indeed, many studies have documented that point mutation in the P450 DM (*ERG11*) gene, which resulted in changes in the affinity of the azoles to its target protein leading to resistance (Favre *et al* ., 1999 ; Kakeya *et al* ., 2000; Kelly *et al.*, 1999; Perea *et al* ., 2001; Sangland *et al.*, 1998a; White *et al* ., 2002 ; Xiao *et al* .,2004). White's group have analyzed a series of *Candida albicans* strains isolated earlier by Redding *et al* (1994) from a single HIV patient over a period of two years and identified a

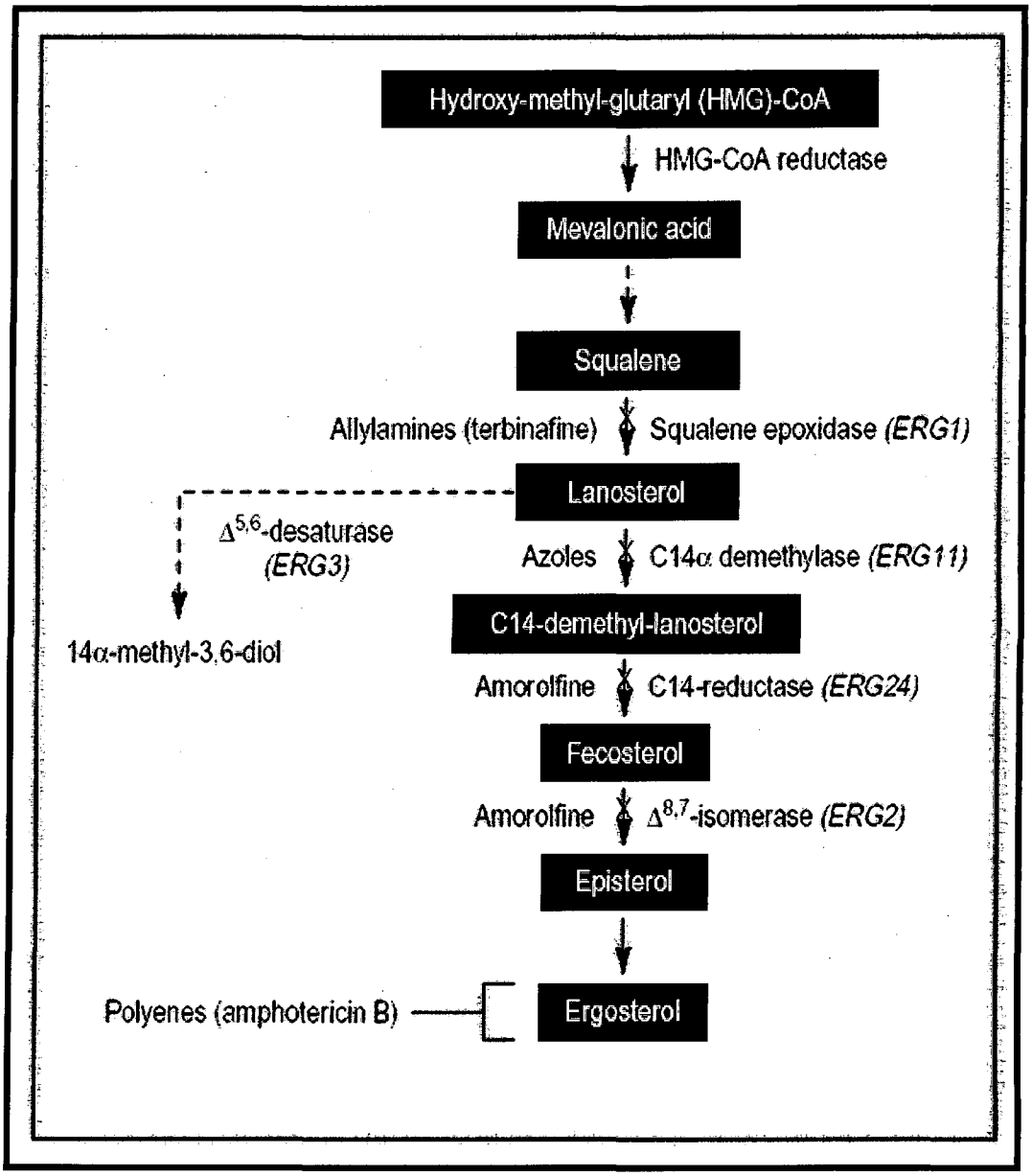


Fig. 3: Mechanism of action of antifungal drugs affecting the ergosterol biosynthetic pathway. The target enzymes are reported on the right with encoding genes in parentheses, whereas the antifungal drugs are reported on the left of the arrows indicating the sequential steps of sterol biosynthesis.

single amino acid substitution mutation *viz* R467K in *ERG11*. Since the location of this mutation is close to cysteine, which participates in the co-ordination of the heme iron in an Erg11p enzyme. It has been purposed that the mutation causes structural changes associated with heme. It has been further reported that R467K mutation alone can confer azole by

reducing the affinity of the enzyme for fluconazole (Lamb *et al.,* 2000). Using site directed mutagenesis, Lamb *et al* introduced the point mutation T315A and expressed the mutated protein (Erg11p) in *Saccharomyces cerevisiae,* which exhibited higher MIC values for fluconazole and ketoconazole. It showed reduced enzyme activity and affinity for azoles thus providing an example of a single amino acid change in the target protein leading to azoles resistance. Loffler *et al.* have identified seven mutations *viz* E266D, F105L, K287R, G448E, G450E G464S, and V488I in a set of fluconazole resistance isolates of these mutation, G464S and R467K in the heme binding domain of P450 14DM in clinical isolates have been shown to exhibit resistance by reduced affinity towards fluconazole. The mutation Y132H does not permit normal binding of fluconazole to protein (DM). Marichal *et al* observed that four mutations, D116E, K128T, E266D and G464S occurred with highest frequency whereas G464S was the only substitution, which was exclusively seen in azole-resistance isolates.

Overexpression of *ERG11*

Overexpression of 14α-demethylase has also been implicated in the mechanism of resistance to azoles antifungals. Resistance to fluconazole in many clinical isolates has often been associated with the overexpression of *ERG11.* (Harry *et al.,* 2002). However, it has been difficult to correlate the overexpression with the observed resistance, mainly because of the simultaneous existence of mutation in P450 14DM or overexpression of the efflux pumps in the same isolates.

Gene amplification is one of the common mechanisms of resistance in eukaryotic cells (Stark and Wahl, 1984; Vender Bleik *et al.,* 1988). However, over expression of *ERG11* in *C. albicans* has not been linked to gene amplification (Venden Bossche *et al.,* 1992.1994b; Marichal *et al.,* 1997). In a clinical isolate of *C. glabrate* increased level of P450 14DM was shown to be associated with the amplification of the *ERG11* gene. The amplification of the *ERG11* gene in this isolate was linked to chromosomal duplication, which in turn resulted in high levels of P450 14DM protein (Marichal *et al.,* 1997). That gene conversion or mitotic recombination could also play a role in fluconazole resistance in *C. albicans* was apparent from a study done by White's group where additional genetic variations in a clinical isolate of *C. albicans* with R467K substitution have been reported (White, 1997c). That gene

conversion or mitotic recombination could also play a role in fluconazole resistance in *C. albicans* was apparent from a study done by White *et al.* where additional genetic variations in a clinical isolate of *C. albicans* with R467K substitution have been reported. It was shown that the allelic differences present in sensitive isolates of *C. albicans* were eliminated in the resistant isolates from the region of *ERG11* promoter, ORF and terminator region and into the immediate downstream gene *THR1*, by gene conversion or mitotic recombination (White 1997a). The resulting strain has R467K mutation in both the copies of *ERG11* and was more resistant to azoles as compared to a strain with single allelic substitution.

***ERG3* ($\Delta^{5,6}$-desaturase)**

A defect in $\Delta^{5,6}$-desaturase, another enzyme of ergosterol biosynthesis pathway that encoded by *ERG3* gene. It has been also shown to contribute to azole resistance. A defective $\Delta^{5,6}$-desaturase leads to the accumulation of 14α-methylfecosterol instead of 14α-methylergosta-8, -dien-3β, 6-α diol. Accumulation of sufficient amounts of 14α-methylfecosterol compensates for ergosterol in the membranes and thus contributes to azole resistance in *C. albicans* (Ghannoum and Rice, 1999; Venden Bossche & Koymans, 1998; White *et al.,* 1998). The lethality of *S. cerevisiae* disruptant of *CYP51* (*ERG11*) can be suppressed by $\Delta^{5,6}$-desaturase. The depletion in ergosterol contents due to a defect in $\Delta^{5,6}$-desaturase in fluconazole resistant clinical isolates of *C. albicans* resulted in cross-resistance to Amphotericin B (Kelly *et al.,* 1997).

***ERG5* (Δ^{22}-desaturase)**

Another cytochrome P450, Δ^{22}-desaturase (CYP61 and also *ERG5*) has been purified from an *ERG11* (P45014DM) disrupted strain of *C. glabrata* (48). The purified enzyme showed desaturase activity in a reconstituted system. Δ^{22}-desaturase and its homologues have also been identified in *C. albicans* and *Schizosaccharomyces pombe*. The spectral analysis revealed that the azole antifungal compounds viz., ketoconazole, fluconazole and itraconazole in reconstituted Δ^{22}-desaturase are directly interact with the cytochrome heme (Lamb *et al.*, 1999).

***ERG6* (C-24 methyl transferase)**

It encodes C-24 methyl transferase, catalyzing a reaction not shared in cholesterol biosynthesis. Hence it is an attractive target for antifungals. Its disruption in *S. cerevisiae* confers pleiotrophic defects that include slow growth, poor mating, poor uptake of tryptophan, increased permeability, and increased cation antifungal susceptibilities, suggesting membrane increased permeability. Its disruption in *C. albicans* conferred hypersusceptibility to terbinafine, cycloheximide fenpropiomorph, and tridemorph, but not to azoles, and resistance to Amphotericin B. However, since no direct assays for this were reported, and deletion of *ScERG6* reduced activity of efflux pump Pdr5p (Kaur *et al.,* 1999), these conclusions are premature. Antifungal inhibitors that target *ERG6* can become potent synergens with existing antifungals, if it is shown that antifungal susceptibilities remain high in an *ERG6* disruptant that has overexpressed *CDR1* or *MDR1* and determined whether the increased efflux negates the benefits of *ERG6* disruption. Overexpressions of *ERG6* in transformants from our *C. albicans* library are resistant to azoles (unpublished observation).

Alteration in membrane composition

Ergosterol, which serves as bioregulator of membrane fluidity, asymmetry and consequently of membrane integrity in the fungal cells (Nozawa *et al.,* 1986) In addition to ergosterol, which mainly provides rigidity, stability and resistance to physical stresses, there are other membrane lipid components, which also affect drug susceptibilities of *Candida* cells (Loeffer *et al.,* 2000). It is well documented that clinical as well as adapted azole resistant isolates of *C. albicans* exhibit altered membrane phospholipids as well as sterol composition. The interaction between sterols and phospholipids in the cytoplasmic membrane affect membrane fluidity and asymmetry and consequently influence the transport of substrates across the membrane. A decrease in the amount of drug taken up by the fungal cell resulted from changes in the sterol and or the phospholipid composition of the fungal cell membrane. Using cerulenin as a lipid modulator, Mago & Khullar (1989) demonstrated that altered phospholipids and fatty acid profiles affected cell permeability of *C. albicans* and rendered the cells more resistant to miconazole. A recent study suggests that the interaction between ergosterol and sphingolipid, which appeared to be disrupted in *erg* mutants, were found to be critical for drug sensitivity of *C. albicans* cells. It is observed that

C. albicans cells, when grown in the presence of fumonisin B1 (specific inhibitor of sphingolipid synthesis), had lower sphingolipid content and similar to *erg* mutant cells, became hypersensitive to drugs (Mukhopadhayay *et al.,* 2004). Hitchcock *et al.,* (1993) showed that an azole and polyene resistant *C. albicans* mutants had a larger lipid content and lower lipid polar-lipid to neutral- lipid ratio than did strain susceptible to azoles. However, the most significant change in the lipid of the resistant strain was in the membrane sterol pattern, where ergosterol was replaced by methylated sterols such as methylfecosterol.

The lipid rafts localized within lipid bilayer are predominantly composed of sphingolipid and sterol is well documented in several systems. Liscovitch & Lavie (2000) hypothesize that the raft-dependent cholesterol efflux pathways may play a role in delivering drugs from various intracellular membranes to the plasma membrane from which drugs can be extruded from the cell by drug efflux. Interestingly, the acquisition of the MDR phenotype is also accompanied by the up regulation of lipids and proteins that constitute lipid rafts. Luker *et al* (2000) & others (Demule *et al.,* 2000) revealed that human P-gp (ABC transporter), is predominantly localized in cholesterol enriched membrane domains and depletion of cholesterol impairs human Pgp-mediated drug transport. The existence of ergosterol and sphingolipid rich microdomains (membrane raft) in *C. albicans* is recently established (Martin & Konapka, 2004). By employing isogenic *erg2, erg3, erg4, and erg6* mutants of *S. cerevisiae* strains, it was observed that due to defective ergosterol biosynthesis and accumulation of various intermediates therein, these mutants possessed high membrane fluidity. These mutants became sensitive to several tested drugs and elicited enhanced level of passive diffusion. This suggested that passive diffusion of drugs could contribute to hypersensitivity of *erg* mutants. However, when a membrane fluidizer benzyl alcohol enhanced membrane fluidity, it was observed that increment in fluidity alone did not affect the susceptibility of the tested drugs in *S. cerevisiae* cells. Thus, it appears that the change in membrane fluidity and increased diffusion therein alone is not sufficient to result in the observed higher susceptibility of *erg* mutants. In conclusion, the hypersensitivity of *erg* mutants of *S. cerevisiae* could be attributed to membrane permeability changes, which may involve changes in passive diffusion across the membrane or in active transport of these drugs. In support of the former, Van Den Hazel *et al* have shown that *S. cerevisiae* cells lacking *PDR16* and *PDR17* (encoding homologues of Sec14p) result in altered phospholipid

and sterol composition and renders cells hypersensitive to many drugs due to their increased passive diffusion. In support of the later possibility, ABC transporter Pdr5p of *S. cerevisiae* has been shown to function less efficiently in *erg6* deleted cells (Emter et al., 2002; Kaur & Bachhawat, 1999). Taken together, it is becoming apparent that changes in membrane lipid composition resulting in changes in membrane fluidity and interactions between membrane ergosterol and sphingolipid, particularly affect drug susceptibilities of *Candida* cells.

Chromosomal alterations

An alteration in chromosomal copy number in response to selective pressure, a regulatory principle of gene expression in lower fungi has also been recently discovered in *C. albicans*. Perepnikhatka *et al.* (1999) have shown that the exposure of *C. albicans* cells to fluconazole resulted in the non-disjunction of two specific chromosomes in drug resistant mutants. Drug exposure for different time periods led to the gain of one copy of chromosome 3 and in the loss of a homologue of chromosome 4. While at least two genes *CDR1* and *CDR2* are localized on chromosome 3, none of the genes associated with drug resistance are situated on chromosome 4. Interestingly, the mRNA levels of *CDR1*, *CDR2*, *ERG11* and *CaMDR1* in these mutants either remained same or were reduced . Therefore, chromosomal non-disjunction could represent another possible mechanism of drug resistance.

Modification or degradation of the drug

Alterations in drug processing (modification or degradation or sequestration into membranes or organelles) are important drug resistance mechanism in a variety of cell types. To date little analysis of modification or degradation within a resistant cell has been performed for the medically important fungi. Until now, no evidence for degradation of an azole antifungal compound has been seen in fungal cells (Marichal 1999a).

Efflux of antifungals as resistance mechanism

In addition to an alteration or an overexpression of 14α-lanosterol demethylase involved in sterol biosynthesis, azole resistance in *C. albicans* is also elicited by other mechanisms like drug efflux, an important molecular mechanism of antifungal resistance.

Several azole resistant clinical isolates like *Candida albicans*, *A. fumigatus* and *Cryptococcus neoformans* exhibit transcriptional activation of efflux pump encoding gene and often show reduced intracellular accumulation of drugs (Nascimento *et al.*, 2003; Posteraro *et al.*, 2003; Semighini *et al.*, 2002; Slaven *et al.*, 2002; Thornewell *et al.*, 1997 & Tobin *et al.*, 1997). The azole resistant isolates mainly overexpress genes encoding multidrug efflux transporters (MET) belonging to two super families; the major facilitator superfamily (MFS) and ATP binding cassette (ABC) transporters.

Major facilitator (MFS) transporter

Efflux pumps/transporters, which are structurally quite similar to ABC pumps but do not contain ATP binding domains, are known as Major facilitators (MFS). The MFS has originally been defined as a superfamily of permeases that are characterized by two structural units of six transmembrane spanning α-helical segments, linked by a cytoplasmic loop. The structure function relationship of MFS have not been generalized in details due to the diversity in their nucleotide and amino acid sequences. But it has been postulated that the N-terminal halves of different major facilitator families share greater similarities than their C-terminal halves which suggest that C-terminal regions are involved in substrate recognition and N-terminal regions are involved in proton translocation (Paulsen *et al.*, 1996). In *S. cerevisiae*, 28 MFS proteins have been identified of which a few have been implicated to play a role in drug resistance. *FLR1* gene of *S. cerevisiae*, which encodes for a MFS protein, has been shown to cause resistance to cycloheximide, fluconazole, cadmium and H_2O_2. *CaMDR1*, its alleles and *FLU1* are shown to be the only drug transporters. *CaMDR1* was initially identified as a gene, which conferred resistance to the tubulin binding agent benomyl and tetrahydrofolate reductase inhibitor methotrexate (Ben-Yaacov *et al.*, 1994; Fling *et al.*, 1991). *CaMDR1* expression in *S. cerevisiae* confers resistance to several unrelated drugs and its overexpression has been linked to azole resistance in *C. albicans*. The expression of *CaMDR1* in *C. albicans* cells is enhanced by benomyl, methotrexate and several other unrelated drugs, and is found to be more pronounced in some of the azole-resistant clinical isolates (Becker *et al.*, 1995; Gupta *et al.*, 1998).

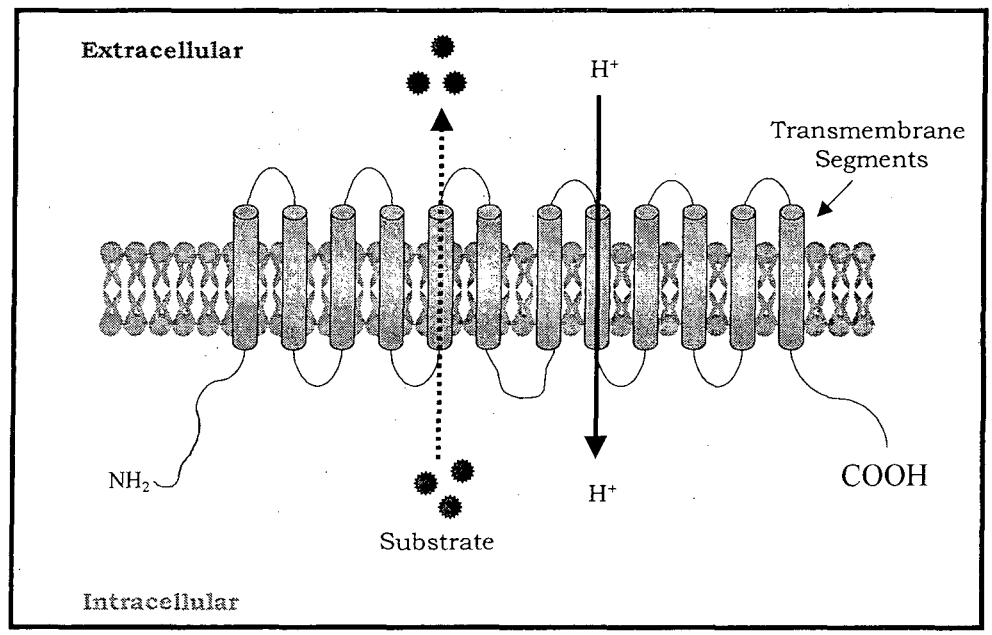


Fig. 4: A diagrammatic representation of MFS transporter of yeast.

It is well documented that seven polymorphic mutant alleles of *CaMDR1* (*CaMDR1-1* to *1-7)* have been identified. The complete sequencing of *CaMDR1* alleles revealed several in frame point mutations leading to changes in amino acid residues where insertion/ replacement of an aspartate residue in a stretch of serine-asparagine-aspartate rich domain was most noteworthy (White *et al.,* 1998). Interestingly, these alleles showed distinct drug resistance profile. The relevance of such alleles of *CaMDR1* in azole resistance in *C. albicans* remains to be ascertained. Recently *FLU1*, another gene encoding MFS protein of *C. albicans* was cloned by complementing strain of *S. cerevisiae,* which was hypersensitive to fluconazole. However, *FLU1* is not found to be involved in the development of fluconazole resistance in clinical isolates of *C. albicans*. Interestingly, studies revealed that preferred substrate of Flu1p is mycophenolic acid rather than fluconazole. Although more than two dozens of putative MFS genes are identified in *Candida* genome but except CaMdr1p, none of other proteins of this superfamily of MFS are reported to have any direct role in clinical fluconazole resistance. Recently, homologues of *CaMDR1* have been identified from *C. dubliniensis* and *C. glabrata* which are termed as *CdMDR1* and *CgMDR1*, respectively (Sanglard *et al.*, 1999; Moran *et al.,* 1998). It appears that increased expression

of *CdMDR1* is the main mechanism of fluconazole resistance involved in *C. dubliniensis* clinical isolates (Moran *et al.*, 1998). Since *CgMDR1* confers specific resistance to fluconazole, its constitutive expression in *C. glabrata* may be responsible for the intrinsically low susceptibility of this yeast species to fluconazole (Sanglard *et al.*, 1999). Interestingly, the Ser-Asn-Asp-rich domain present in CaMdr1p and its alleles was not found in CdMdr1p, Cyhrp (cycloheximide resistance protein from *C. maltosa*) and Flr1p from *S. cerevisiae* are 93, 57 and 46 % identical to CaMdr1p, respectively but do not confer resistance to benomyl (Gupta *et al.*, 1998).

ABC transporters

The rapidly growing ABC superfamily, also known as the 'ATPase trafficking', comprises an extremely diverse class of membrane transporter protein. Among several mechanisms that seem to contribute to MDR (Multi Drug Resistance) phenomenon, the overexpression of drug extrusion pump belonging to the ABC superfamily is the most frequent cause of resistance to herbicides, anticancer and cytotoxic drugs; overexpression of drug efflux pump encoding genes belonging to the ABC superfamily of transporters represents one of the prominent mechanisms by which the human pathogen *C. albicans* acquires MDR. Proteins belonging to the ABC superfamily are modular in nature. The presence of the nucleotide binding domains is a characteristic feature of these proteins (Walker *et al.*, 1982). The typical ABC transporter, also called a full size protein has two membrane domains comprising of six transmembrane segments (TM) each and two nucleotide-binding domains (NBD). On the other hand the half-size ABC proteins have only one membrane domain comprising of five to ten transmembrane segments and one nucleotide-binding domain. Some ABC proteins do not possess a transmembrane domain and are probably soluble proteins. The *PDR5* gene (also called *STS1*, *YDR5* and *LEM1*) was the first gene to be identified as a drug transporter in *S. cerevisiae* involved in multidrug resistance by several independent investigators (Kralli *et al.*, 1995). Overexpression of *PDR5* causes resistance to various structurally and functionally different drugs while its disruption leads to hypersensitivity to drugs. *PDR5* encoded protein has been partially purified and has been shown to carry out ATP hydrolysis, which in turn is linked to the transport of the drugs (Decottignies *et al.*, 1994).

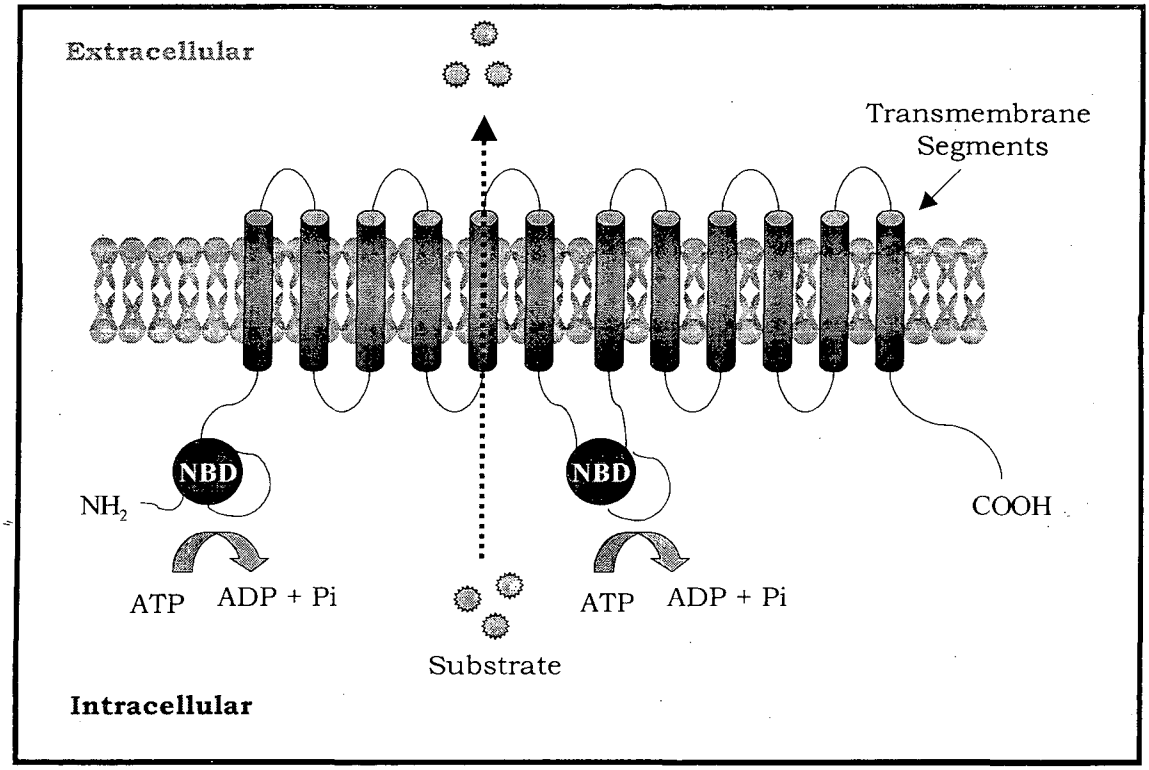


Fig. 5: A diagrammatic representation of an ABC transporter of yeast

The drug efflux pump encoded by *CDR1* of *C. albicans* was the first ABC efflux pump implicated in conferring resistance to cycloheximide in a *PDR5* disruptant hypersensitive strain of *S. cerevisiae*. *CDR1* encodes a 1501 amino acid long protein (169.9 kDa) whose predicted structural organization is characterized by two homologous halves, each comprising a hydrophobic region with a set of six transmembrane stretches, preceded by hydrophilic nucleotide binding fold (Fig. 5) The structure is identical to that of the *S. cerevisiae* ABC proteins Pdr5p and Snq2p. It mirrors the architecture of the yeast a-mating pheromone transporter *Ste6*, as well as mammalian drug resistance P-glycoprotein (Pgp or *MDR1*) and Cystic fibrosis factor CFTR. The significance of inversion of domains in some of the ABC drug transporters is not understood and may be related to their physiological functions.

Cdr1p is remarkably similar to Pdr5p of *S. cerevisiae*. The similarity is not limited to only the ATP binding motif but is conserved along the entire length of the protein. Despite the high homology between *CDR1* and *PDR5*, and their encoded products, some functional features tend to distinguish them. For example, while single or low copies of *CDR1* are sufficient to increase drug resistance in *S. cerevisiae*, multiple copies of *PDR5* are required to yield a similar level of drug resistance. In addition to differences in the efficiencies in conferring drug resistance to the same drugs, the spectrum of drugs to which *CDR1* and *PDR5* confer resistance is also partly distinct. For example, both genes share overlapping specificities for cycloheximide and chloramphenicol, but *CDR1* affects sensitivity to oligomycin while neither amplification nor disruption of *PDR5* alters susceptibilities to it. It is worth mentioning here that some of the close homologs of *CDR1* in *C. albicans* are also functionally distinct. That, efflux pumps other than *CDR1*, could be contributing to drug resistance in *C. albicans* became apparent after isolation of its close homologue *CDR2* (Sanglard *et al.,* 1997). Cdr2p exhibits 84% identity with Cdr1p and confers resistance to fluconazole and several other drugs. Since azole resistance appears to be a multifactorial phenomenon (discussed below), this led to the search for more homologues of *CDRs*. Using PCR based cloning, other homologues of *CDR1* and *CDR2* namely *CDR3*, *CDR4* and *CDR5* were identified (Franz *et al.,* 1998; Sanglard *et al.,* 1998). Cdr3p and Cdr4p show highest homology to Cdr1p and Cdr2p, however, as compared to Cdr1p and Cdr2p which are more than 90 % similar, Cdr3p and Cdr4p are only 75 % similar to Cdr1p and Cdr2p. Interestingly, overexpression of *CDR3* and deletion of *CDR3* and *CDR4* did not affect drug susceptibilities in *C. albicans* (Franz *et al.,* 1998).

Why some of the known Cdrps are unable to elicit multidrug resistance phenomenon is not yet clear at this point. The model of Cdr1p and Cdr3p has similar topological arrangements where hydrophilic domain containing nucleotide-binding motif precedes hydrophobic transmembrane stretches. The only apparent difference between the two proteins appears to be in C-terminal where Cdr3p has an extended loop connecting TM11 and TM12. In addition, the last 21 amino acids in the C-terminal of Cdr3p are totally different from Cdr1p. Keeping in view the importance of these regions in drug binding in human Mdr1p and *Candida* Cdr1p, these subtle differences in structure of these proteins could affect substrate specificity, which makes them unable to bind and transport drugs (Krishnamurthy *et al.,*

1998a; Loo and Clarke, 1994; Zhang *et al.,* 1995). A high-resolution three-dimensional protein structure of Cdrps would finally be able to resolve the molecular basis of drug transport by these transporters, however, mutational analysis of homologous and non-homologous regions between different Cdrps can provide valuable information. This situation is reminiscent of the observation that two types of P-glycoproteins are found in mammals: 1) which transports hydrophobic drugs, 2) others which can not identify some amino acid residues in the TM6 of *MDR1* and these residues are also sufficient to allow an *MDR2* backbone in the N-terminal half of P-gp to transport several *MDR1* substrates. These studies indicate a close relationship between *MDR1*, a multidrug transporter and *MDR2*, a phosphotidylcholine flippase. Since Cdr1p, Cdr2p and Cdr3p have similar domain structure, their substrate preferences are most likely to be determined by some non-identical amino acid residues.

S. cerevisiae contains a battery of ABC transporters, which are involved in drug resistance. The complete genome sequence of the baker's yeast revealed 31 distinct genes encoding ABC proteins of which many are MDR. Three of these families, PDR5, MRP/ CFTR and MDR, contain ABC transporter membranes that are known to cause drug resistance in a variety of systems. On the basis of domain organization and domain homology to ABC transporter proteins from other organisms the complete genome sequence of *Candida albicans* contains 28 distinct genes encoding ABC proteins which divided into six separate sub families, PDR (Pleiotropic Drug Resistance), MDR (Multi Drug Resistance), MRP (Multi Drug resistance-associated protein), ALP (Adrenoleukodystrophy), EF3 (Elongation Factor) and RLI (RNase-L Inhibitor) (Gaur *et al.,* 2005).

Recognition of the transported solute by the ABC protein/ domain

Protein-dependent uptake systems initially recognize their substrate (ligand to the respective binding protein), and ATP at opposite sides of the membrane. Consequently, this implies that the ABC subunits do not necessarily participate directly in substrate recognition. Consistent with this notion are the findings that MalK and UgpC from *E. coli*, involved in the transport of chemically distinct substrates, such as maltose and glycerol-3-phosphate, respectively, are exchangeable (Hekstra and Tommassen, 1993), and that the LacK protein of *Agrobacterium radiobacter*, participating in the uptake of lactose can substitute for MalK in

S. typhimurium and *E. coli* (Wilken *et al.,* 1996). Moreover, the ATPase activity of the purified MalK protein was neither stimulated nor inhibited by maltose (Morbasch *et al.,* 1993). Other groups reported differing results. While the ATPase activity of the purified PotA protein from *E. coli* was inhibited by millimolar concentrations of the transported substrate spermidine (Kashiwagi *et al.,* 1995), galactose stimulated the enzymatic activity of the MglA protein from *E. coli* (Richarme *et al.,* 1993). These effects might, however, not be specific and rather due to intrinsic properties of polyamines or due to a homogenous protein preparation in the latter case. In contrast, in bacterial or eukaryotic exporters, both the substrate to be translocated and ATP are targeted to the same (cytoplasmic) side of the transport system. Genetic and biochemical approaches provided some evidence supporting the notion that the ABC domains of bacterial polypeptide secretion systems might participate in substrate recognition. The ATPase activity of PrtD from *E. crysanthemi* was strongly inhibited by the C-terminal secretion signal of the secreted protease PrtG, while the corresponding fragment of HasA that is not a substrate of the Prt system exhibited only a minor effect (Delepelaire, 1994). Further analysis of the same system revealed an initial interaction of the ATPase subunit with the substrate as a prerequisite for the assembly of the transport complex (Létoffé *et al.,* 1996). By suppressor analysis, Sheps *et al.* found a single mutation in the ABC domain of HlyB, involved in the secretion of K-hemolysin (HlyA) in *E. coli* that corrected the transport defect of a C-terminal truncated variant of HlyA. This finding was interpreted in support of a direct interaction with either the substrate or the membrane spanning domains. On the other hand in contrast to the result obtained with PrtG, a peptide encompassing the C-terminal 200 amino acid residues of HlyA caused only marginal inhibition of the ATPase activity of the ABC domain from HlyB fused glutathione-S-transferase (Koranakis *et al.,* 1993). Biochemical evidence obtained with export systems that mediate resistance to the antibiotic oleandomycin in *Streptomyces antibioticus* (Buche *et al.,* 1997) or secretion of capsular polysaccharide in *E. coli* (Bliss and Silver, 1997) was also taken as evidence in favor of the ABC domains to be involved in binding of the substrate. However in both cases, a note of caution seems appropriate. Oleandomycin, at a concentration of 2 mM, changed the intrinsic fluorescence of a fusion of the N-terminal ABC domain of OleB to maltose binding protein by 20 % (Buche *et al.,* 1997), which might indicate a conformational change upon interaction with the substrate.

Since the exact boundaries of an ABC domain are difficult to define, the results do not rule out the participation of residues not strictly belonging to the ABC domain in substrate binding. Bliss and Silver showed that KpsT, involved in the secretion of polysialic acid in *E. coli* can be co-immunoprecipitated with the substrate, but only to a minor extent and in the context of the complete system. Again, these results do not exclude the interaction of the substrate with other transport components. Taken together, further experimental data are required until a more uniform picture on how bacterial export systems recognize their substrates might emerge. In mammalian systems, the observation that the ATPase activity of P-glycoprotein is regulated by drugs that are transported is not conclusive since substrate binding sites have been demonstrated on the membrane-spanning domains but thus far not on the nucleotide-binding folds as deduced from their primary structures (Zhang *et al.*, 1995). Computational analysis suggested that these proteins are phylogenetically more related to each other than to other members of the family (Kuan *et al.*, 1995).

Functional necessity of both ABC domains

The structural organization of eukaryotic ABC transporters has provided an experimental advantage for studies on the functional necessity of both nucleotide binding domains. Analyses of mutations in either one of the two Walker A motifs in P-glycoprotein (Azzaria *et al.*, 1988), or the Ste6 protein from yeast (Berkower & Michaelis, 1991) unanimously revealed that two nucleotide binding folds matching the consensus sequence are indispensable for function. This conclusion was supported by results from bacterial uptake systems. In the oligopeptide permease of *S. typhimurium*, which comprises two ABC proteins encoded by individual genes, both were shown to be required for activity (Hiles *et al.*, 1987). The functional analysis of MalFGK2 complexes from *E. coli* containing a single mutated copy of MalK (Davidson & Sharma, 1997), and of covalently coupled wild-type and mutant MalK fusion proteins from *S. typhimurium* also indicated that two intact nucleotide-binding folds are indispensable for transport activity. These findings also correlate with a stoichiometry of nearly two ATPs hydrolyzed per substrate as the most realistic number measured for bacterial binding protein-dependent systems and the reconstituted P-glycoprotein (Eytan *et al.*, 1996). Moreover, studies from several groups that used different experimental approaches suggested that both nucleotide binding sites of P-

glycoprotein must interact (Senior *et al.,* 1995), although the failure to detect co-operativity between the two ATP sites with a purified preparation of P-glycoprotein in detergent solution was also reported (Sharom *et al.,* 1995). In contrast, cooperative interaction was also observed with the reconstituted maltose transport system of *E. coli.* Thus, the functional interplay of both sites might require a membrane-bound state of the protein. Members of a subgroup of bacterial ABC proteins involved in the uptake of certain sugars, such as ribose (Buckle *et al.,* 1986), arabinose (Scripture *et al.,* 1987), and L-methylgalactoside (Hogg *et al.,* 1991), respectively, from *E. coli* and ribose from *B. subtilis* (Woodson & Devine, 1994), appear to be natural dimmers present, the carboxy-terminal A-sites either contain an arginine in place of the invariant lysine (Buckle *et al.,* 1986; Scripture *et al.,* 1987; Woodson & Devine, 1994) or are even more degenerated (Woodson & Devine, 1994). Since other residues cannot substitute for the conserved lysine without loss of ATPase activity, the C-terminal nucleotide binding folds are most likely catalytically inactive. Consequently, a functional transport complex would have to contain a homodimer of these proteins. However, no experimental evidence in support of this view is available at present. Interestingly, several P-glycoprotein- like ABC exporters from yeast and fungi carry a cysteine for lysine substitution in the first nucleotide-binding fold. Again, the functionality of this site as well as the oligomeric state of the transport system in the membrane is currently unknown. The question whether both nucleotide binding folds in an ABC transport protein are functionally equivalent or energize distinct steps in the translocation process has been addressed in studies involving the CFTR protein. CFTR displays a gated Cl^- channel activity that can be monitored by electrophysiological means in membranes or planar lipid bilayers, thereby allowing the elucidation of individual steps of the translocation process. From such studies, most authors agree on a model that suggests distinct functions of both nucleotide-binding folds in channel gating, but otherwise the individual steps that are powered by ATP hydrolysis are discussed controversially. Hwang *et al.,* studying the effects of non-hydrolyzable ATP analogs, and Carson *et al.,* using variants which contained mutations in the conserved Walker A lysine residue in either one or both nucleotide-binding sites (Shyamala *et al.,* 1991), proposed that ATP hydrolysis at the N-terminal ABC domain is required to open the channel while closing is catalyzed by hydrolysis at the C-terminal site. Furthermore, Carson and collaborators deduced from biochemical studies that ATP

binding at both sites might occur simultaneously. In contrast, Hwang *et al.* interpreted their data by proposing the necessity of ATP hydrolysis at one site for the interaction.

Mechanism of ABC transporters

All ABC transporters catalyze vectorial transport across biological membranes but their substrate diversity is enormous. It ranges from small inorganic ions such as chloride, amino acids, sugars, peptides and anticancer drugs to large proteins. Regardless of the nature of the substrate, immediately proceeded by a highly conserved sequence motif (linker peptide, LSGGQQ/R/KQR) that is unique to ABC transport superfamily (signature sequence) has proven to be a useful tool in identifying putative new members of the family. But in case of Cdr1p, Walker A of NBD2 is conserved. It is not yet known at which stage of the transport cycle ATP is hydrolyzed or how the chemical energy is converted into "power stroke", which finally shuttles the substrate across the membrane; in other words, is the binding of ATP, its hydrolysis or the dissociation of the inorganic phosphate the triggering step? Studies on the functional exchangeability of the N- and C- terminals in the ABC domains of Pgp suggested that residues in the helical domain might participate in the signal transduction between the nucleotide binding site and the transmembrane domain or, alternatively might directly be involved in substrate recognition. This conclusion was based on the analysis of chimeras and mutants, which demonstrated that most of the amino-terminal ABC domain could be replaced by the corresponding carboxy-terminal residues without dramatic effects on protein function. In contrast, replacement of a short sequence motif within the helical domain of the N-terminal half of the protein by the corresponding C-terminal residues caused alterations of the drug resistance profile (Beaudet and Gros, 1995).

Another key question is the location and nature of the substrate- binding site. None of the crystal structures was obtained in the presence of ligand. Some ABC transporters, such as the maltose importer, hemolysis B and the histidine permease, are rather specific for certain substrates. In these so called 'traffic ATPases', however, the specificity arises from substrate-binding of protein, which, by definition, does not belong to the ABC transporter but is required for efficient translocation. On the other hand, ABC transporters such as *MDR1* or *TAP* are promiscuous. *MDR1* is able to expel nearly every known anticancer drug from the inner leaflet of the plasma membrane into the extracellular space. This process confers tumour

cells with resistance to chemotherapeutic drugs, which is one of the largest problems in modern cancer therapy. *TAP* is able to transport peptides ranging from 8 to 40 amino acids from the cytosol into the lumen of the endoplasmic reticulum (Schmitt *et al.,* 2000). Like *MDR1*, *TAP* recognizes a large diversity of substrates. This substrate diversity of *TAP* can be literally superimposed on the peptide binding principle of MHC class I molecules (Uebel *et al.,* 1997). Although the substrate-binding sites of *MDR1* and *TAP* have been mapped by cross-linking and other biochemical approaches, it still is a mystery that how a single protein can deal with a myriad of ligands without losing affinity, specificity or efficiency.

Even the mechanism of substrate transport remains controversial. In the case of *MDR1*, it is assumed that two ligand-binding sites, one with high affinity and one or more with low affinity, exist within the TMD. Both recently proposed models assume that the NBDs act in an alternating manner. In one model (Senior *et al.,* 1998), drug transport from the high affinity to the low-affinity binding site occurs on dissociation of inorganic phosphate from one of the NBDs. The dissociation represents a relaxation of the NBD from a high-energy to a low-energy level. The other model proposes that two drugs bind simultaneously and that ATP hydrolysis provides the energy necessary for drug dissociation (Shapiro & Ling, 1998). The laboratory of Ambudkar (Sauna & Ambudkar, 2001) has proposed a modified model in which ATP sites are recruited randomly. After ATP binding to one site, the affinity of the other ATP-binding site is reduced so that only one NBD acts at a time. ATP hydrolysis moves the substrate from the high affinity to the low-affinity binding site. After ADP dissociation, which restores high affinity to the other ATP binding site, ATP binds to the other NBD. Hydrolysis of this second ATP is used to restore the ground state of the transporter. It has been demonstrated that substrate binding and ATP hydrolysis are tightly coupled (Gorbulev *et al.,* 2001); however, the alternative model (Sauna & Ambudkar, 2001) implies that two ATP molecules are hydrolyzed per molecule of transported drug. Which of the proposed models of the catalytic cycle of *MDR1* and other ABC transporters is correct remains a subject of intensive research.

Aim and scope of work

Candida albicans is a diploid opportunistic fungal pathogen, which causes candidiasis in immunocompromised individuals. Candidiasis accounts for approximately 50- 60 % of fungal infection in human. The frequency of this infection has been raised drastically in the past decades due to the increasing number of the immunocompromised patients (HIV/AIDS) and debilitated patients. Prolonged and widespread usage of antifungals especially azoles has led to the overexpression of Cdr1p an ATP Binding Cassette (ABC) transporter, one of the most common mechanisms of multidrug resistance in *Candida albicans* (Prasad *et al.,* 2002.). This drug resistance in *Candida albicans* is a major hurdle to treatment of fungal infection in human. The ABC transporter Cdr1p harvests the energy present in cellular ATP to drive the translocation of a structurally diverse set of drugs against concentration gradients across the membrane barriers. The nature of Cdr1p substrates varies enormously as it includes structurally unrelated compounds such as azoles, lipids and steroids (Krishnamurty *et al.,* 1998; Dogra *et al.,* 1999).

Cdr1p is made up of two halves, each containing one NBD followed by a TMD. Each TMD comprises of six transmembrane segments (TMS), which are involved in substrate specificity. Each NBD contains a conserved ABC region, including the ATP binding motif known as Walker A and Walker B which are involved in ATP hydrolysis. The N-terminal NBD (NBD1) of Cdr1p contains conserved Walker A (GRPGAGCST), Walker B (IQCWD) motif & an ABC signature sequence (VSGGERKRVSIA) and the C-terminal NBD of Cdr1p contains conserved Walker A (GASGAGKST), Walker B (LLFLD) and Signature like sequences.

Nokaido *et al* (1997) have revealed that the lysine residue of Walker A motif binds to the β- and γ- phosphates of ribonucleotides and plays a critical role in ATP hydrolysis. Mutations of this lysine residue have been shown to reduce or abolish the hydrolysis activity and in some cases impair nucleotide binding. Interestingly, C-terminal NBD of Cdr1p contains the conserved Walker A (GRPGAGKST) and B (LLFLD) motifs, and an ABC signature like sequence (Prasad *et al.,* 1995). In prokaryotic ABC type transporters such as the histidine permease of *E. coli*, both NBDs are functionally identical and equally contribute to the protein activity. Inactivation of either one of these NBDs in the full protein

results in a transporter that has its activities reduced to 50 %. On the other hand, the NBDs of the eukaryotic transporters such as the human P-gp/ MDR1, CFTR (Cystic Fibrosis Transmembrane conductance Regulator) and MRP1, though highly conserved and similar in sequence, do not appear to be functionally complementary, as inactivation of either of them completely abolishes ATPase and transport activities of the protein (Azzaria *et al.,* 1988).

We have previously purified and characterized the biochemical properties of an individual catalytically active N-terminal NBD of Cdr1p (Jha *et al.,* 2003). By exploiting site directed mutagenesis and fluorescence spectroscopy, we have established that the uncommon Cys193 of Walker A and conserved Asp327 of Walker B play a major role in ATP hydrolysis whereas Trp326 of Walker B is important for ATP binding (Rai *et al.,* 2005). Jha *et al.* (2003b) have described the relative contribution of both the N-terminal and C-terminal NBDs (*in vivo* system) in terms of ATP binding, hydrolysis and transporter activity of native Cdr1p (full protein). These properties of Cdr1p were examined wherein an atypical Cys-193 of walker A of NBD1 (C193K) and a conserved Lys-901 (K901C) of Walker A of NBD2 were replaced. The drug resistance profile of Cdr1p mutant variant cells harboring C193K or K901C gave interesting insight into the functioning of the two NBDs. The cells expressing K901C were hypersensitive to drugs as compared to C193K variant or to native Cdr1p. This clearly establishes that the two NBDs respond asymmetrically to the substitution of conserved residues of their respective Walker A motifs. Recently in our lab, purified Cdr1p (full protein) also stated that ATP binding is not a prerequisite for the drug binding to Cdr1p and both the mechanisms of drug binding as well as ATP binding occur independent to each other (unpublished data).

The present work is an attempt to examine the independent functionality of C-terminal NBD of Cdr1p. For this, we have cloned, overexpressed and purified C-terminal NBD (NBD2) protein of Cdr1p. Our results have established that the purified C-terminal NBD of Cdr1p is catalytically active and exhibits divalent cation dependent ATPase activity (195 nmoles\mgprotein min^{-1}) with an apparent K_M in the range of (600- 800 μM) and V_{max} between.170- 200 nmoles/ mg protein min^{-1}. ATPase activity of C-terminal NBD showed an optimal pH at 7.5. Our study for first time provides a catalytically active C-terminal domain of Cdr1p, an ABC transporter purified from a fungal pathogen *Candida albicans*. The

availability of purified C-terminal domain protein will help to resolve the role(s) of two NBDs in ATP binding, hydrolysis and drug transport.

Materials and Methods

MATERIALS AND METHODS

Strains and Media

Escherichia coli cells BL21 (DE3) as well as their transformants containing the plasmid constructs used in this study. These strains were grown in LB (Luria-Bertani) broth or on LB plates, supplemented with either ampicillin (0.1mg ml^{-1}) when required.

Chemicals

Luria Bertani media was obtained from Difco (USA). Ultra pure deoxyribonucleotides (dATP, dGTP, dCTP, dTTP) for PCR, glutathione-sepharose 4B matrix were obtained from Amersham Biosciences Ltd. Ribonucleotides, IPTG (isopropyl 1-thio-β-D-galactopyranoside), bacterial protease inhibitor cocktail, aprotinin, pepstatin A, leupeptin, antipain, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT) and other molecular grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The *Taq* DNA polymerase for PCR amplification and *BamH*1 used for cloning purpose were purchased from New England Biolabs Inc. (USA). Thrombin cleavage capture kit was purchased from Novagen (Madison, WI).

Cloning Vector and Primer

Vectors used for cloning of C-terminal NBD were pS12-35, carrying complete *CDR1* gene (Prasad *et al.,* 1995) and pGex2T-Z (expression vector) that was obtained form Amersham Biosciences Ltd. Oligonucleotides were commercially procured from Integrated DNA Technologies, Inc. (Iowa USA). The sequences of oligonucleotides are as follows:

NBD2F 5'-CGG GAT CCA TGC AAA AGG GGG AAA TT-3'

NBD2R 5'-CGG GAT CCC CAA TCT TGA ACA ATA GT-3'

The underlined sequences at 5' end of forward and backward primer sequences showed *BamHI* restriction site.

Construction of expression vectors

DNA sequence representing C-terminal NBD was amplified using *CDR1* genomic clone pS12-35. The primers used for this purpose allowed the introduction of *BamHI* restriction sites at the 5' and 3' termini of the amplicon. The resultant 1206 bps amplicon was then digested with *BamHI* and ligated at the corresponding sites of the linearized bacterial expression vector (pGex2T-Z). The resulting constructs (pSSGN2) was then transformed in *E. coli* BL21 (DE3) cells and grown on LB agar plates supplemented with antibiotic selection marker ampicillin (0.1 mg ml^{-1}).

Expression and purification of GST-NBD2

The transformants (harboring pSSGN2 plasmid) cells were grown at 30 °C in LB medium containing 0.1 mg ml^{-1} ampicillin to an OD_{600} of 0.4, IPTG was added to a final concentration of 0.5 mM in the culture and the cells were incubated for 4 hrs at 30 °C with continuous shaking. Cells were harvested by centrifugation at 7000 rpm for 10 min at 4 °C. The harvested cells were then resuspended at 4 °C in 2 % of the original culture volume of lysis buffer containing 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 10 mM DTT, 2 mM $MgCl_2$, 0.1 mM ATP, 10 % Glycerol and protease inhibitors (bacterial protease inhibitor cocktail, 2 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and 1 μg ml^{-1} pepstatin A). Cell lysis was performed by first incubating the cells at 37 °C for 15 min with 0.1 mg ml^{-1} lysozyme, followed by sonication of 3 cycles (each comprising of a 30 sec of pulse at 4 W followed by incubation on ice for 1 min) using sonicator, 150 M SONIPREP. The lysed cell suspension was further incubated with 0.1 % Triton-X-100 with continuous rotation at 4 °C for 45 min. The soluble fraction was separated from insoluble fraction (cells debris) by centrifugation at 12000 rpm for 30 min at 4 °C. The soluble fraction was further incubated with glutathione-sepharose 4B gel matrix (pre-equilibrated with PBS buffer; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 4 °C for 90 min on continuous shaking. After binding the fusion protein to gel matrix, The matrix was washed three times with washing buffer containing 50 mM Tris-Cl (pH-7.5), 5 mM DTT, 600 mM NaCl, 100 μM PMSF and protease inhibitor cocktail (as mentioned earlier). GST-NBD2 fusion protein was eluted with two column volumes of elution buffer (10 mM glutathione in 50 mM Tris-HCl; pH 8.0) In order to

obtain C-terminal NBD (NBD2), the column containing the matrix-bound GST-NBD2 fusion protein was first equilibrated with five column volumes (10 ml) of 1X thrombin cleavage buffer (thrombin cleavage capture kit, Novagen) followed by thrombin cleavage. Thrombin cleavage was essentially performed at 4 °C for 12 hrs with continuous circulation of the 1X thrombin cleavage buffer. Thrombin cleaved NBD2 protein was collected and further incubated with streptavidin-agarose beads for 1 hour. NBD2 was separated from Thrombin (biotinylated) by streptavidin-agarose spin filters. The dialyzed protein was stored at –80 °C until further use.

ATPase assay

ATPase activity of purified NBD2 was measured by end point P_i release colorimetric method assay (Fiske & Subba Rao). For the end point Pi release colorimetric assay, the amount of inorganic phosphate released over 30 min at 30 °C was measured as described previously (Nakamura *et al.*, 2002). Briefly, a standard 100 µl reaction mixture containing NBD2 protein was incubated in ATPase buffer [60 mM Tris-HCl (pH 7.5) and 8 mM $MgCl_2$], supplemented with 5 mM ATP (from a 100 mM stock of Mg-ATP, brought to pH 7.5 with NaOH). The transfer of the mixture from ice to a water bath at 30 °C initiated the reaction. The reaction was stopped by adding 1 ml of stop solution (0.5 % SDS, 2 % H_2SO_4 and 0.5 % ammonium molybdate), followed by the addition of 10 µl of freshly prepared coloring reagent (10 % ascorbic acid). The assay reaction was further incubated at 30 °C for 30 min and absorbance was taken at 750 nm using UV-2000 Shimadzu spectrophotometer. In control set of reactions, NBD-512 protein was added after termination of the reaction (i.e. incubation at 30 °C for 30 min and addition of stop solution). ATPase activity was plotted using a 0-100 nmoles of inorganic phosphate standard curve.

Other procedures

Protein fractions were analyzed on both 8 % and 10 % SDS-polyacrylamide gels (SDS-PAGE) as described earlier (Laemmli, 1970). Protein concentration was determined by Bradford's reagent (Bradford *et al.*, 1976). Coomassie Brilliant blue-G250 protein staining, destaining and DNA cloning procedure were followed according to Sambrook *et al.* (1989).

Results

Results

Candida albicans is an asexual diploid fungus that causes opportunistic infection commonly seen in immunocompromised and debilitated patients. An estimated 33-50% of patients with human immunodeficiency virus infection and AIDS contract oropharyngeal candidiasis and the synthetic triazole fluconazole has been the mainstay of their treatment. The widespread usage of prolonged fluconazole therapy has increased the incidence of treatment failure to fluconazole resistance *Candida*. A number of studies have identified the major molecular mechanisms for azole resistance. These include overexpression of, or mutation in the drug target, 14α-sterol demethylase or other steps of the sterol biosynthesis pathway. The overexpression of gene encoding for the drug extrusion pumps belonging to the ATP binding Cassette (ABC) and the Major Facilitator Superfamily (MFS) of protein is the most common. The mechanism of survival is certainly not the forte of this pathogen alone; tumour cells also utilize it in order to resist chemotherapy through overexpression of P-glycoprotein (P-gp)/ MDR1 and the multidrug resistance associated protein (MRP1). The overexpression of ABC proteins in all the organisms ranging from prokaryotes to eukaryotes exhibit drugs extrusion and leads to multi drug resistance (MDR). Generation of MDR phenomenon in *Candida albicans* is due to the overexpression of ABC gene encoding proteins called *Candida* drug resistance proteins (Cdrps). Cdr1p is one of the ABC proteins of *Candida albicans* that harvest the energy present in cellular ATP and translocate structurally diverse groups of drugs across the plasma membrane. The topology of Cdr1p is made up of two highly hydrophobic transmembrane domains (TMDs) and two hydrophilic & cytoplasmically located nucleotide-binding domains (NBDs). In our previous study of the laboratory, Jha *et al* has purified and functionally characterized the catalytically active N-terminal NBD (NBD1) of Cdr1p and also found out the role of some of the conserved amino acids. In the present study, we have purified and characterized the C-terminal NBD (NBD2) to assign its role with respect to full protein (Cdr1p).

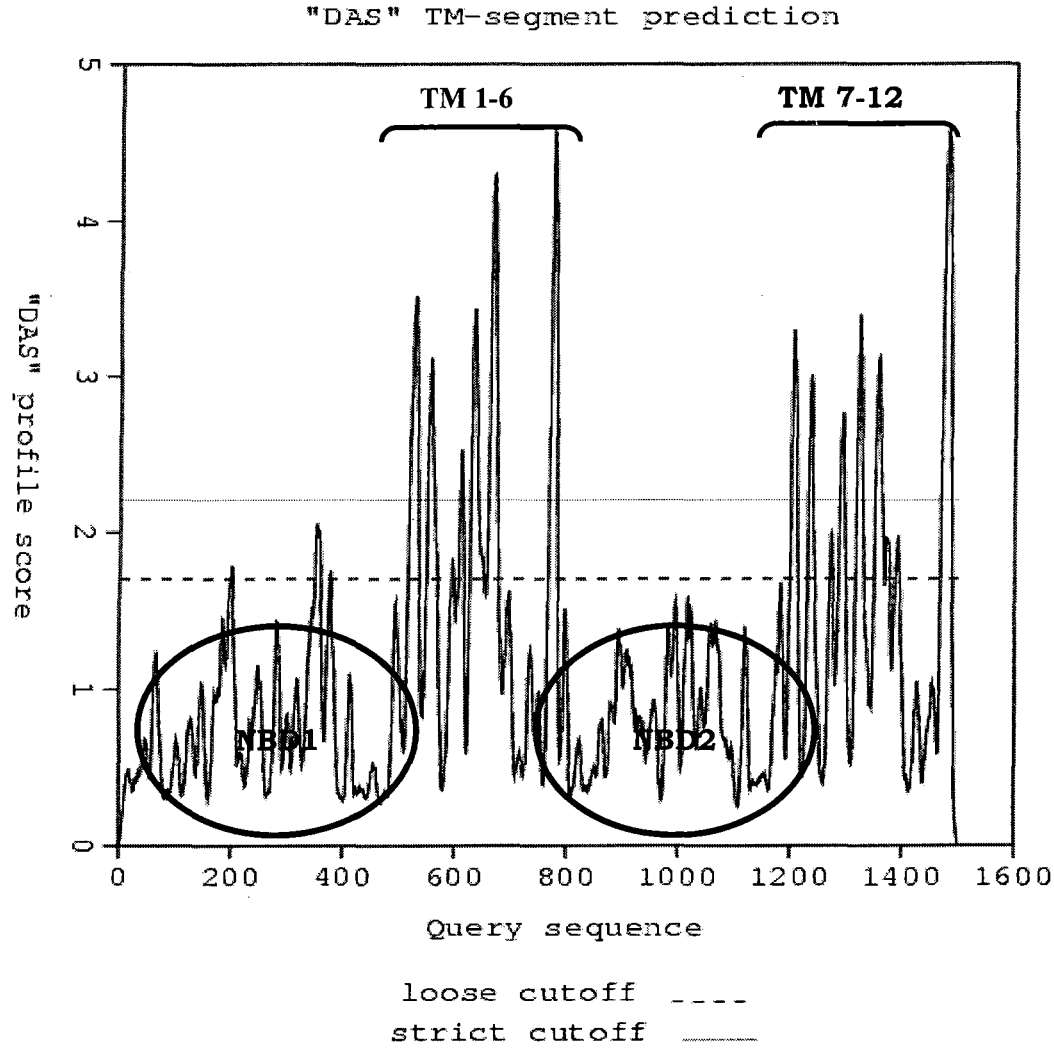


Fig. 6 : Transmembrane profile of Cdr1p. The translated protein sequence of Cdr1p was used a query sequence to predict the putative transmembrane domain and cytoplasmic nucleotide binding domain of Cdr1p using "DAS"- Transmembrane Prediction server (http://www.sbc.su.se/~miklos/DAS/).

***In silico* analysis of C-terminal NBD (NBD-2) of Cdr1p**

The first ABC transporter to be characterized in *Candida albicans*, designated as *CDR1*, was isolated as a gene implicated in conferring cycloheximide resistance in a *PDR5* disruptant hypersensitive strain of *S. cerevisiae* (Prasad *et al.*, 1995). The 4503 base pairs, the *CDR1* gene encodes for a protein of 1501 amino acids residues (169.9

kDa). By using DAS program, the hydropathy plots of Cdr1p showed that it has a modular structure of two hydrophilic and two hydrophobic domains which is topologically arranged in such a way that the hydrophilic domain precedes the hydrophobic transmembrane segments (Fig.6) (**http://www.sbc.su.se/~miklos/DAS**). ABC proteins have a modular structure wherein a typical 'full- length' protein of this superfamily comprises of two intra-cytoplasmic domains consisting of the nucleotide binding domain (NBDs) and two hydrophobic regions comprising of six transmembrane segments (TMS). The sequence of domain organization varies amongst the proteins of the superfamily. The NBDs contain the Walker A and Walker B motifs (Walker *et al.*, 1982) and a ABC signature or C motif consensus sequence, (L, V)-SGG- $(X)_3$-R-hydrophobic residue-X- hydrophobic residue-A that precedes the Walker B motif in these transporters (Michaelis and Berkower, 1995). In the Cdr1p, each hydrophilic domain (NBD) contains ATP-binding cassette domain. In the N-terminal NBD, the Walker A motif (187 GRPGAGCST) displays a cysteine (C), instead of the frequently conserved lysine (K), and a well conserved ABC signature (303 VSGGERKRVSIA), while in the Walker B region (323 IQCWD) the conserved aspartate (D) is present but not preceded by the typical four hydrophobic amino acids. However, strong conservation is observed in the sequence immediately following the Walker B aspartate. The LD residues placed five amino acids downstream from the Walker B aspartate are conserved in Cdr1p as well as in several other members of the ABC superfamily. In the C-terminal NBD, the Walker A motif (895 GASGAGKT) shows conserved amino acid lysine while in Walker B region (1022 LLFLD) the aspartic acid is well conserved like the N-terminal NBD but no typical ABC signature is present. The conserved amino acids that lie between Walker A and Walker B motifs of Cdr1p have been illustrated in Fig.7.

ATP binding domains of Cdr1p

An important property of the ABC transporter is that all ABC transporters harvest the energy by hydrolysis of cellular ATP to transport their substrates across the membrane barriers against the concentration gradients. The conserved NBDs located at the cytoplasmic periphery are the hub of such an activity. In prokaryotic ABC type transporters such as the histidine permease of *Escherichia coli*, both NBDs are functionally identical and equally contribute to the protein activity. Inactivation of either one of these NBDs in the full protein results in a transporter that has its activities reduced to 50 %. On the other hand, the NBDs of the eukaryotic transporters such as the human

P-gp/MDR1, CFTR (Cystic Fibrosis Transmembrane conductance Regulator) and stretches of the ABC proteins are highly conserved and similar in sequence and also do not appear to be functionally complementary. Since inactivation of either of them completely abolishes ATPase and transport activity of the protein. On the basis of fluorescence spectroscopy and mutational analysis, we have established that N-terminal NBD, uncommon Cysteine (C) 193 of Walker A motifs and conserved Aspartic acid (D) 327 of walker B motifs play an important role in the ATP hydrolysis (Jha *et al.*, 2003 & Rai *et al.*, 2005) whereas the tryptophan (W) 326 of walker B is involved in ATP binding (Rai *et al.*, 2005). We have recently demonstrated that the relative contribution of both the N-terminal and C-terminal NBDs in ATP binding, hydrolysis and drug transport activity of Cdr1p *in vivo*. For this we have swapped amino acid at the respective position within Walker A motifs *viz.* atypical Walker A Cysteine 193 of NBD1 (C193K) and / or conserved Lysine 901 (K901C) of Waker A of NBD2 of Cdr1p. The mutant variants of Cdr1p showed that replacement of C193K and K901C in Walker A of NBD1 and NBD2 abolished the ATPase activity in 78 % and 88 % respectively and the replacement K910C was found to be supersensitive to tested drugs. Thus the two NBDs of the *Candida albicans* play asymmetric role in function. (Jha *et al.*, 2004) Detailed analysis of the C-terminal nucleotide-binding domain revealed that it contains a well conserved Walker A and Walker B domain which are know to be important for nucleotide binding / hydrolysis (Fig.7).

Cloning of C-terminal nucleotide binding domain (NBD-2)

In order to characterize the biochemical properties of C-terminal nucleotide binding domain of Cdr1p, a major ABC transporter of fungal pathogen *Candida albicans*, we cloned the C-terminal NBD DNA sequence in a bacterial expression vector. pGex2T is one of the most widely used expression system for the cloning and expression of recombinant proteins in *E. coli*. It contains the *taq* promoter which is a strong hybrid promoter composed of the –35 region of the *trp* promoter and the –10 region (Pribnow box) of the *lacUV5* promoter/ operator. Multiple cloning site of the pGex2T vector was engineered by incorporating additional oligonucleotide in such a way that gave us more option as far as choice on restriction enzyme site was concerned. pGex2T vector was also

			Walker A	ABC signature	Walker B
A.					
C. albicans	Cdr1p(N)	187	GRPGAGCS---	303 VSGGERKRVSIAE--322	IQCWDNATRGLD
C. albicans	Cdr1p(C)	890	GASGAGKT---999	LNVEQRKRLTIGV--1019	LLLFLDEPTSGLD
C. albicans	Cdr2p(C)	893	GASGAGKT--1001	LNVEQRKRLTIGV--1021	LLLFLDEPTSGLD
C. albicans	Cdr3p(C)	876	GASGAGKT---982	LNVEQRKRLTIAV--1002	LLVFLDEPTSGLD
C. albicans	Cdr4p(C)	882	GASGAGKT---988	LNVEQRKRLSIGV-1008	LLVFLDEPTSGLD
S. cerevisiae	Pdr5p(C)	902	GASGAGKT--1010	LNVEQRKRLTIGV-1031	LLVFLDEPTSGLD
S. cerevisiae	Snq2p(C)	886	GESGAGKT---993	LNVEQRKKLSIGV--1013	LLLFLDEPTSGLD
C. galbrata	Phd1p(C)	918	GASGAGKT--1026	LNVEQRKRLTIGV--1046-	LLVFLDEPTSGLD
C. neoformans	CnAfr1p(C)	951	GASGAGKT--1059	LSVEARKVTIGVE--1080	LLLFLDEPTSGLD
A. nidulans	AtrBp(C)	828	GSSGAGKT---936	LSVEQRKRVTIGVE-- 956	ILIFLDEPTSGLD
A. fumigatus	AtrFp(C)	925	GASGAGKT--1029	LNVEQRKKLSIGV--1049	LLLFLDEPTSGLD
S. cerevisiae	Pdr15p (C)	920	GASGAGKT--1025	LNVEQRKRLTIGV--1045	LLVFLDEPTSGLD
B.					
C. albicans	Cdr1p (C)	890	GASGAGKT---999	LNVEQRKRLTIGV--1019	LLLFLDEPTSGLD
H. sapiens	Pgp (C)	1072	GSSGCGKS--1176	SGGQKQRIAIAR-- -1196	ILLLDEATSALD
H. sapiens	CFTR (C	1243	GRTGSGKS--1346	LSHGHKQLMCLA---1367	KILLLDEPSAHLD
S .cerevisiae	Ste6p (C)	1088	GESGTGKS--1190	LSGGQAQRLCIAR 1210	ILILDECTSALD
H. sapiens	Mdr1p (C)	1327	GRTGAGKS--1430	LSVGQRQLVCLAR--1450	ILVLDEATAAVD
H. sapiens	ABCA1p (C)	1946	GVNGAGK--2044N	----NKRKLSTAMA--2064	PVVFLDEPTTGMD
H. sapiens	ABCD1p (C)	507	GPNGCGKS-605	LSGGEKQRIGMAR---624	KYALLDECTSAVID

Fig. 7: Sequence alignment of conserved Walker A and B motifs of NBDs of ABC transporters of different organisms. The amino acid sequences have been aligned to generate three columns for conserved sequences representing the Walker A and B motifs respectively. N and C indicated in brackets after each protein depicts the N- and C-terminal NBDs of the respective proteins. The amino acid residue number for each protein is indicated at the beginning of each sequence. **(A)** Conservation of Lysine residue (in bold) in Walker A among C-terminal NBD of fungal ABC transporters. **(B)** Comparison of Cdr1p C-terminal NBD with other non-fungal ABC transporters where conserved Glycine, lysine of Walker A and Aspatic acid of Walker B are highlighted in bold.

engineered with an internal LacI^q gene. The LacI^q gene encodes a repressor protein that binds to the operator region of the *taq* promoter preventing expression until induction by IPTG (isopropylthio-β-D-galactoside), thus maintaining tight control over expression of the inserted target gene. pGex2T also contains a thrombin cleavage site in between GST domain and the multicloning site (MCS), which would be effective in order to cleave GST from fusion protein. In addition to that, a FLAG epitope was also engineered at the C-terminal end of the multiple cloning site of the vector, which would facilitate identification and purification if needed. This modified vector was named as pGex2T-Z and details of the modification are highlighted in Fig. 8.

An overview of the strategy employed for purification is schematically represented in Fig. 9. The primer used for the amplification allowed the introduction of *BamH* I restriction sites at the 5' and 3' termini of the amplicon (Fig.10) The resultant 1206 bps amplicon was then linearized with *Bam*HI and for confirmation of positive clone, the amplicon was digested with *Spe*I and *Sca*I and the *Bam*HI digested amplicon was ligated at the corresponding sites of the linearized pGEX2-T vector. The ligated mixture was used for transformations and colonies were screened for the required construct. The positive construct selected for this study was used only after confirming by restriction enzyme digestion analyses to check the correct orientation as well as in frame insertion of NBD-2 within the expression vector. This construct was designated as pSSGN2 (Fig. 11). The confirmed clone was used for transformation into the expression host BL21 (DE3) and the transformed stained was designated as SSGN2.

Expression and Purification of C-terminal NBD (NBD-2) as a GST fusion protein

In order to express the C-terminal NBD as a GST-fusion protein, the transformant cells (SSGN2) harbouring pSSGN2 plasmid construct were grown with continuous shaking at 37°C, and on reaching an OD_{600} of 0.4, the culture was induced with different concentrations of IPTG for 3 ½ hours. As a negative control, we used uninduced SSGN2 cells, 1mM IPTG induced mock transformed (Fig. 13, Lane 4 & 3) cells and mock transformed uninduced cells (Fig. 13, Lane 2). Fig. 13 depicts the SDS-PAGE profile of the total bacterial lysate proteins wherein an expression of GST-NBD2 fusion protein is more in 0.5 mM IPTG as compared to 1 mM IPTG induction.

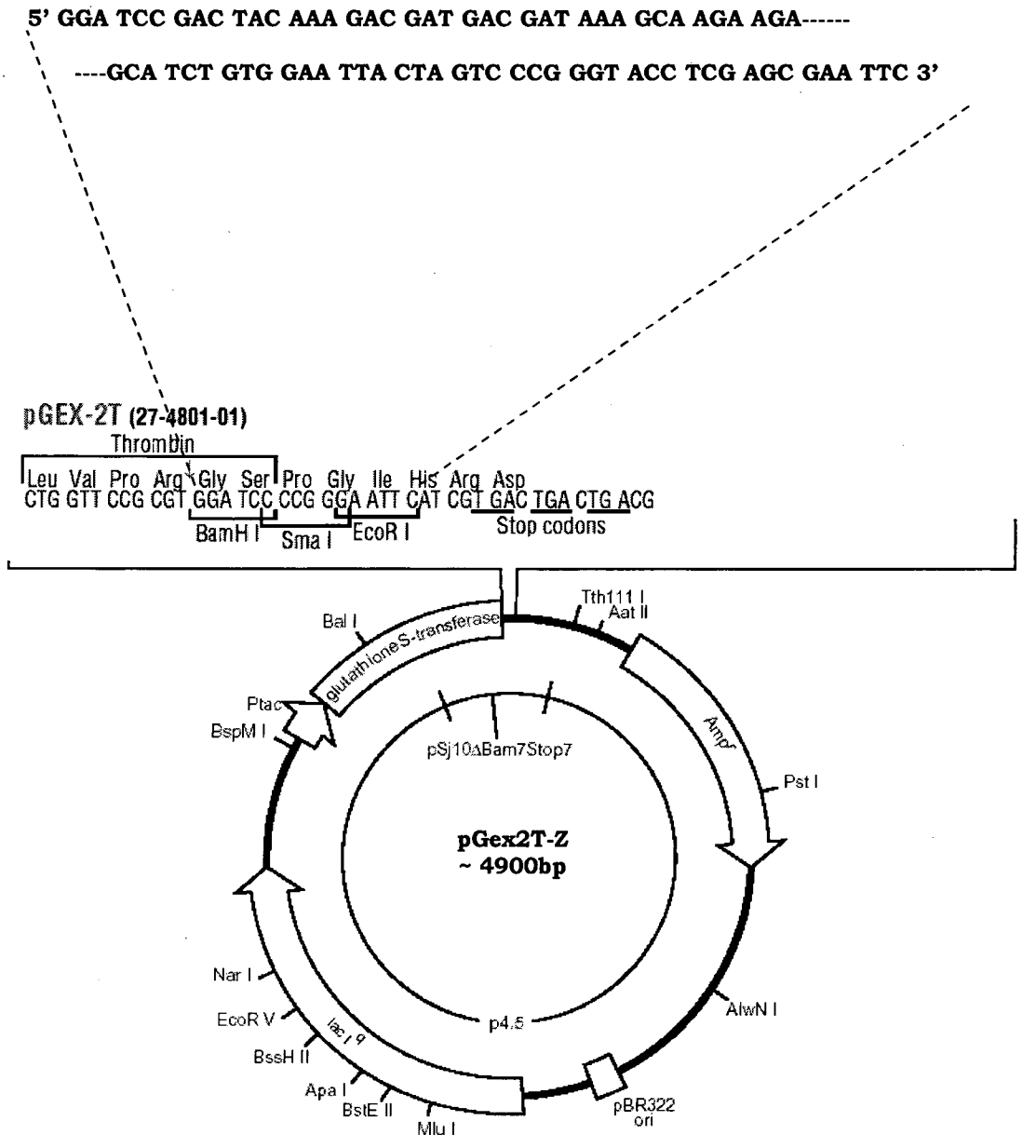


Fig. 8: Vector map of pGex2T-Z. Detailed vector map of pGex2T-Z showing the restriction enzyme site present in the multiple cloning and sequence of oligonucleotide incorporated between the *Bam*HI – *Eco*RI site of pGex2T. Diagrammatic representation of the *Ori*, *LacI*^{q}, glutathione-*S* transferase coding region, ampicillin resistance marker gene and thrombin recognition site are also illustrated.

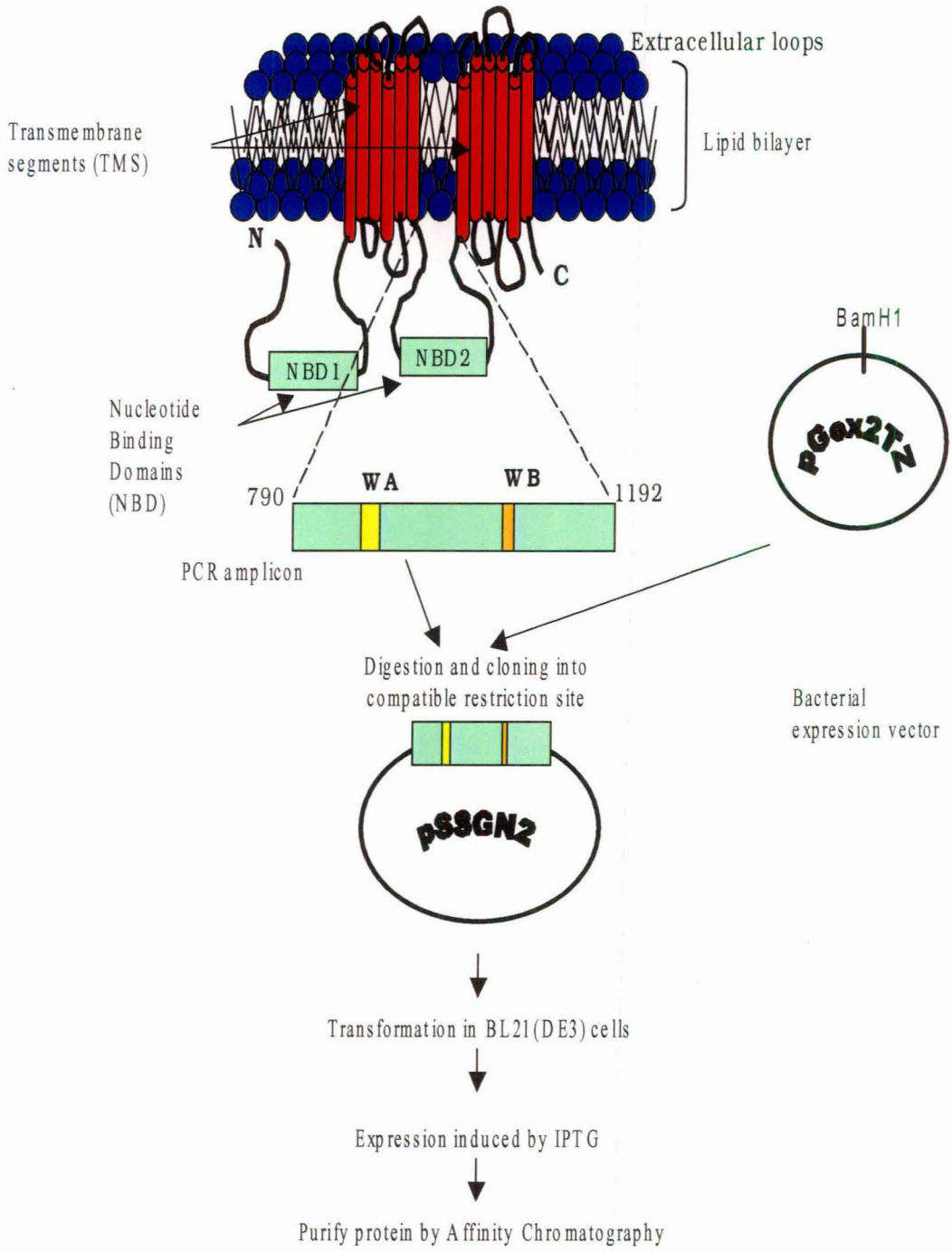


Fig. 9: Schematic representation of purification of the C-terminal NBD (NBD-2) of Cdr1p.

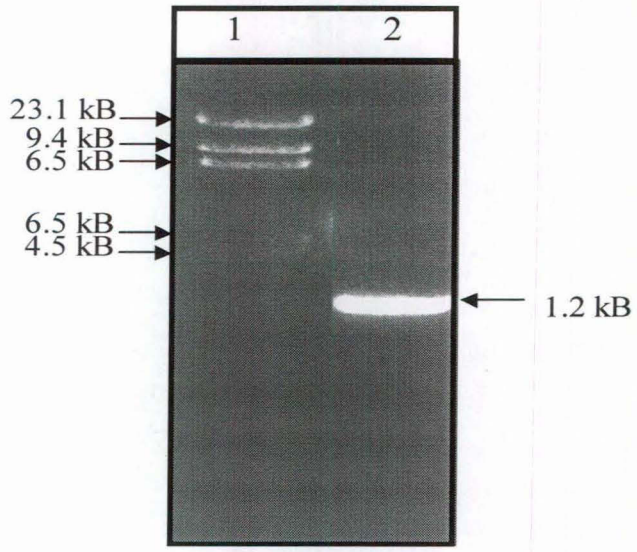


Fig.10: PCR amplicon of NBD-2. The plasmid pS12-35 which, contains genomic DNA sequences of CDR1, was used for PCR as a template. Lane 1, λ DNA digested with *Hin*dIII; and Lane 2, aliquot of samples where primers corresponding for NBD-2 amplification were used.

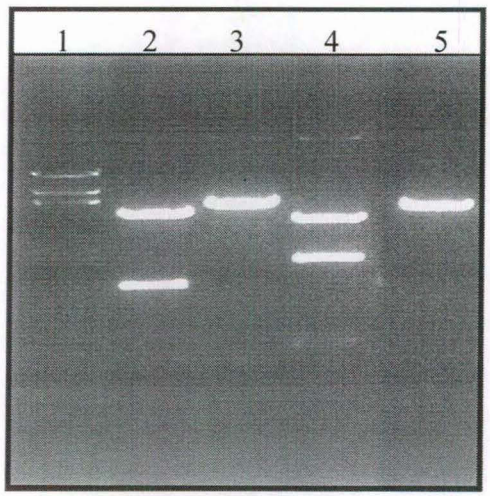


Fig.11: Confirmation of positive clone. pSSGN2 DNA was digested by multiple enzymes and loaded on 1% agarose gel. Lanes 1 Marker; 2, pSSGN2 digested with *Bam*HI; 3, pSSGN2 digested with *Spe*I; 4 pSSGN2 digested with *Sca*I; 5, pGEX2T-Z digested with *Bam*HI.

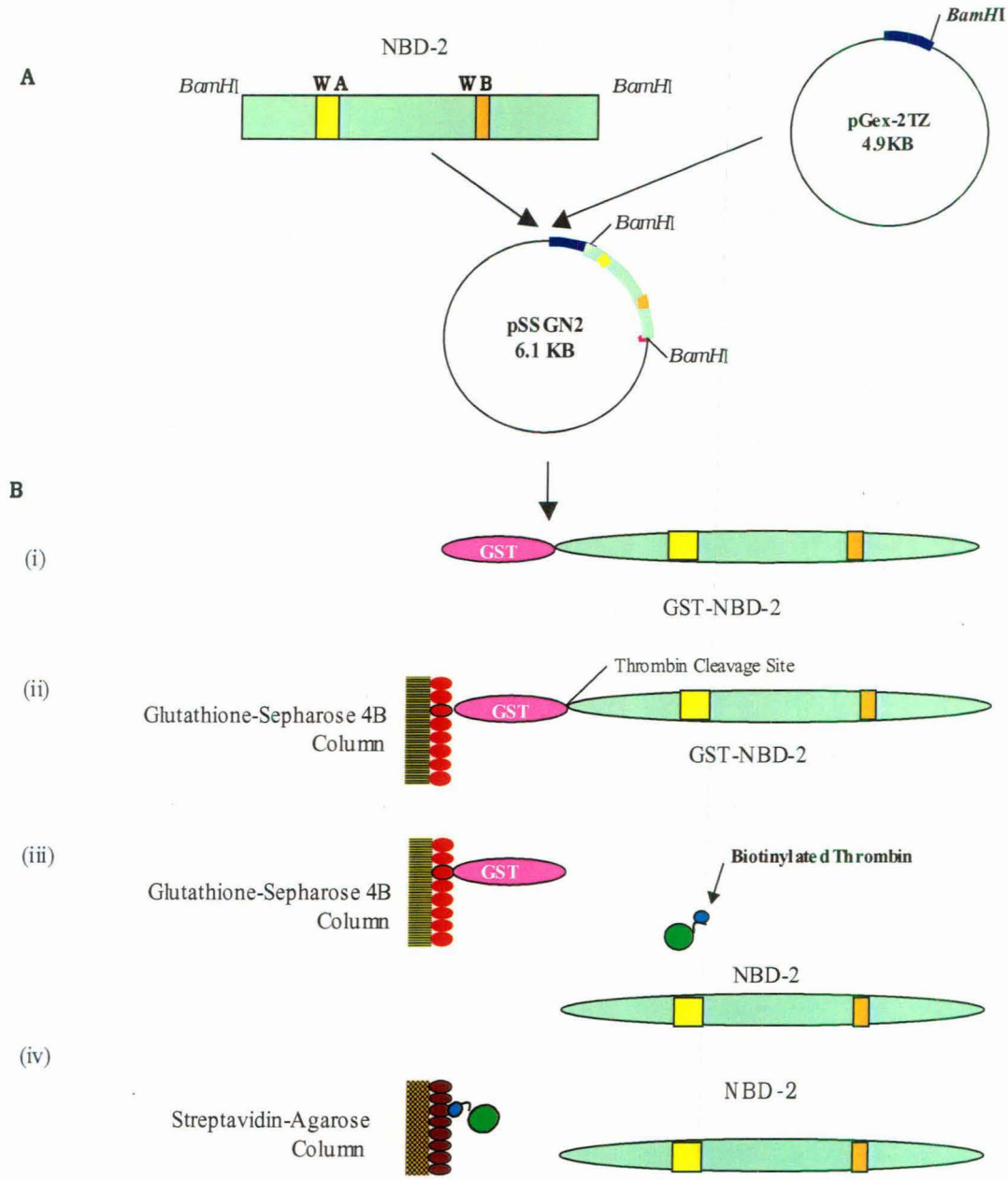


Fig. 12: Schematic representation of cloning, expression and purification of NBD-2. A. The *CDR1* ORF fragment representing NBD-2 was amplified using primers (detailed under "Materials and Methods") with *BamHI* overhangs to generate a ~1.2 kB amplicon. This amplicon was cloned at the *BamHI* site of pGex2TZ expression vector, immediately downstream of the Glutathione-*S*-transferase (GST) ORF. **B. (i)** Expression of the fusion protein GST-NBD-2 was performed by IPTG induction of SSGN2 cells. **(ii)** The expressed fusion protein was purified from bacterial lysate using Glutathione-Sephrose 4B column based affinity chromatography. **(iii)** Thrombin (biotinylated) cleavage of the fusion protein bound to the Glutathione-Sephrose 4B column resulted in the release of NBD-2 from the GST-NBD-2 recombinant protein. **(iv)** Eluent hence obtained through affinity chromatography was further loaded onto the streptavidin agarose column to segregate the biotinylated thrombin from NBD-2. Henceforth, all the experiments were performed with this purified NBD-2.

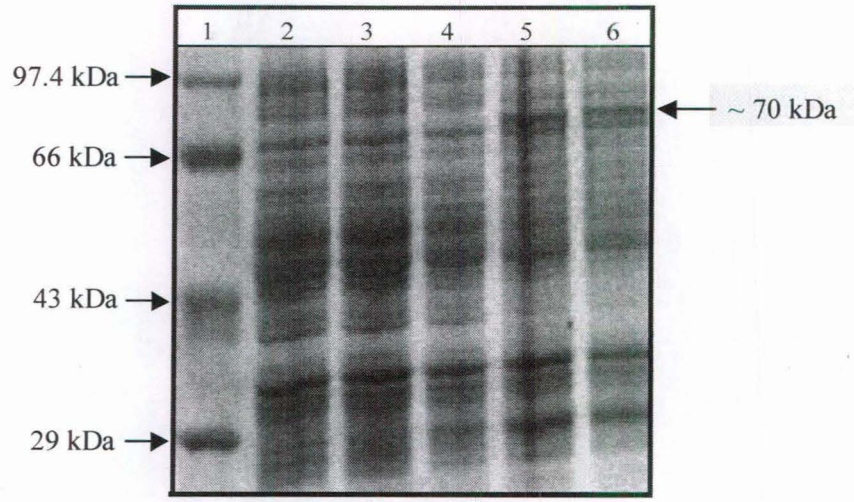


Fig. 13: Expression of GST-NBD 2. Cells were grown at 37 °C till the OD_{600} reached 0.4 followed by addition of different IPTG concentration. Cells were harvested after 3½ hours of induction and resuspended in SDS-loading dye and separated on 10 % SDS-PAGE. Lane 1, Protein molecular marker (Gennei); Lane 2, whole cell lysate of mock transformed uninduced BL21 (DE3) cells; Lane 3, whole cell lysate of mock transformed 1 mM IPTG induced BL21 (DE3) cells; Lane 4, whole cell lysate from uninduced SSGN2 cells; Lanes 5 & 6, whole cell lysate from SSGN2 cells induced with 0.5 and 1 mM IPTG respectively.

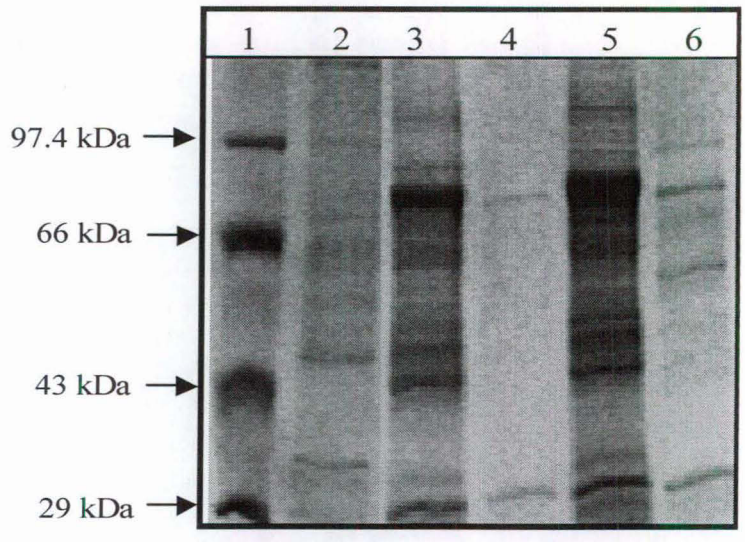


Fig. 14: Effect of various induction periods on solubility of GST-NBD2. SSGN2 cells were grown at temperature 37 °C till the OD_{600} reached 0.4 followed by addition of IPTG to final concentration of 0.5 mM. The sample were collected after 3 hours and 3½ hours of induction and then cells were harvested and resuspended in lysis buffer followed by sonication to lyse the cells. The samples were centrifuged to separate the insoluble fraction from the soluble. The samples were then boiled with SDS-loading dye and separated on 8 % SDS-PAGE. Lane 1, Protein molecular marker; Lane 2, whole cell lysate from uninduced SSGN2 cells; Lanes 3 & 4, insoluble and soluble fractions respectively obtained after lysis and centrifugation of lysate from cells with 3 hours of induction. Lanes 5 & 6, insoluble and soluble fractions respectively obtained after lysis and centrifugation of 3½ hours of

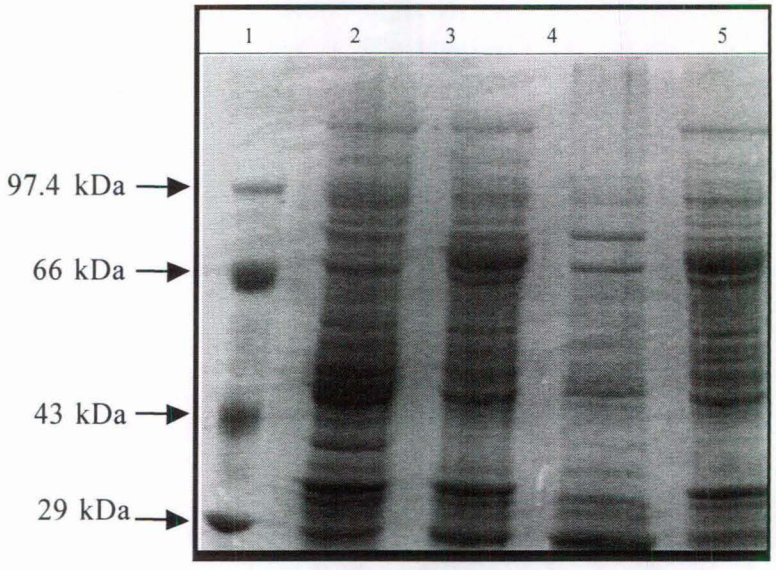


Fig. 15: Effect of temperature on solubility of GST-NBD2. SSGN2 cells were grown at temperature 37 °C till the OD_{600} reached 0.4 followed by addition of IPTG to final concentration of 0.5 mM. The cells were harvested after 3 ½ hours and resuspended in lysis buffer followed by sonication to lyse the cells. The samples were centrifuged to separate the insoluble fraction from the soluble. The samples were then boiled with SDS-loading dye and separated on 8 % SDS-PAGE. Lanes 1, Protein molecular marker; Lane 2, whole cell lysate from uninduced SSGN2 cells; Lane 3, whole cell lysate from induced SSGN2 cells; Lanes 4 & 5, soluble and insoluble fractions respectively obtained after lysis and centrifugation.

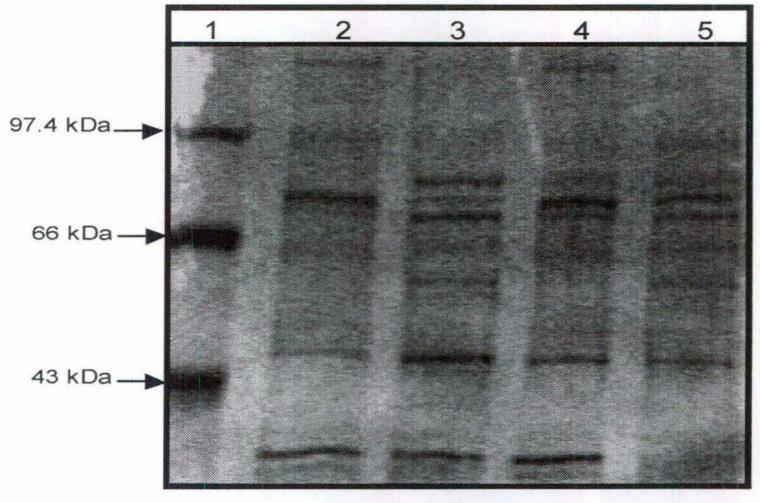


Fig. 16: Effect of temperature on solubility of GST-NBD2. SSGN2 cells were grown at temperature 30°C till the OD_{600} reached 0.4 followed by addition of IPTG to final concentration of 0.5 mM. The sample were collected after 3 hours and 4 hours of induction and then cells were harvested and resuspended in lysis buffer followed by sonication to lyse the cells. The samples were centrifuged to separate the insoluble fraction from the soluble. The samples were then boiled with SDS-loading dye and separated on 8 % SDS-PAGE. Lane 1, Protein molecular marker; Lanes 2 & 3, soluble and insoluble fractions respectively obtained after lysis and centrifugation of lysates from cells with 3 hours of induction. Lanes 4 & 5, soluble and insoluble fractions respectively obtained after lysis and centrifugation of lysates from cells with 4 hours of induction.

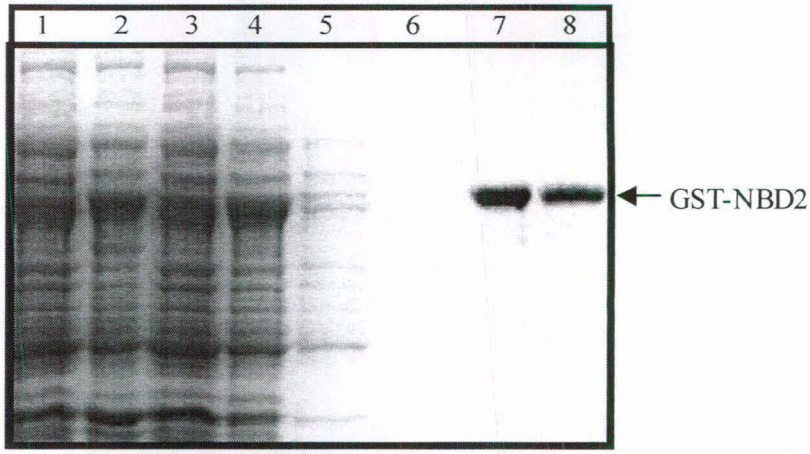


Fig. 17: Purification of GST-NBD2. SSGN2 cells were grown at 30 °C till the OD_{600} reached 0.4 followed by addition of IPTG to final concentration of 0.5 mM. Cells were harvested after 4 hours of induction and various fractions were resuspended in SDS-loading dye and separated on 8 % SDS-PAGE. Lane 1, whole cell lysate from SSGN2 cells induced with 0.5 mM IPTG; Lanes 2 & 3, insoluble and soluble fraction respectively obtained after lysis and centrifugation of lysate from cells grown at 30 °C; Lane 4, unbound protein sample after incubation with glutathaione-sepharose-4B matrix for 45 min; Lane 5, sample from the first wash; Lane 6, sample from the final wash; Lane 7, purified GST-NBD-2 eluted by 10 mM glutathione and Lane 8, bead sample after elution.

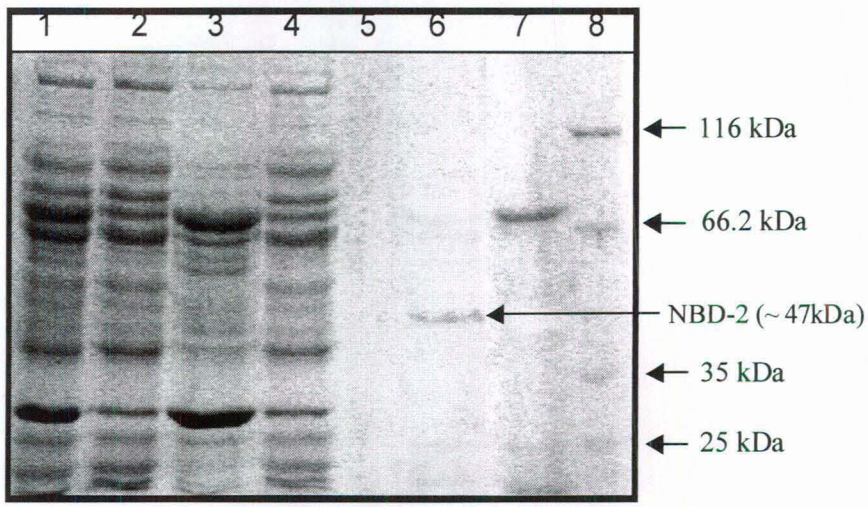


Fig. 18: Purification of NBD-2. SDS-PAGE analysis of the overexpressed fusion protein and the purified NBD-2 protein. Lane 1, cell lysate from IPTG induced SSGN2 cells (equal amounts of protein were loaded in lanes 1 and 4); Lanes 2 & 3, soluble and insoluble fractions obtained after lysis and centrifugation; Lane 4, unbound protein sample after incubation with glutathaione-sepharose-4B matrix for 45 min (Flow through); Lane 5, sample from the final wash; Lane 6, purified NBD-2 after thrombin cleavage; Lane 7, purified GST-NBD2 fusion protein and Lane 8, protein molecular marker (Fermantas). As indicated by arrows, GST-NBD-2 and NBD-2 migrated with predicted molecular weight of ~ 70 and ~ 45 kDa, respectively.

For further experiments we used 0.5 mM IPTG to induce the cells. The estimated M_r (relative molecular weight) of overexpressed GST-NBD-2 fusion protein was observed ~70 kDa. We also checked various IPTG induction periods like 3 hours and 3 ½ hours in respect to GST-NBD2 overexpression. At 3½ hours induction period, the expression of GST-NBD2 protein is more as compared to 3 hours induction (Fig. 14). However, the over expressed fusion protein was predominantly insoluble when expressed under these conditions as can be seen from Fig. 15.

To increase the yield of the soluble fraction of fusion protein, we tried variation in the growth temperature of culture as well as IPTG induction periods. And of the tried variations, maximum yield could be obtained in the soluble fraction when transformant cells were grown at 30 °C and induced with 0.5 mM IPTG for 4 hours (Fig.16). After optimisation of growing culture and purification conditions the C-terminal NBD (NBD-2) protein was purified from the soluble fractions of bacterial cell lysate by using a two-step affinity-chromatography procedure. The protein purification of GST-NBD2 has been shown in Fig. 1 and also described in Materials and Methods.

Purification of NBD-2 from GST-NBD2 after Thrombin cleavage

In order to purify NBD2 in isolation with GST domain we utilized the unique thrombin site, just proximal to the N-terminal of NBD2 of the GST-NBD2 fusion product. This helped in releasing the GST from NBD2 and gave us an advantage of analysing its function without the interference of any fusion moiety. GST-NBD2 fusion protein was cleaved with biotinylated thrombin (0.01 units μg^{-1} of protein; thrombin cleavage kit, Novagen) to generate unbound NBD-2 protein (~ 45 kDa) and separate it from GST (~ 27 k Da) still bound to the matrix. Selection of biotinylated thrombin was done to facilitate the second step of chromatography i.e., to remove thrombin contamination from NBD-2 on completion of the cleavage reaction (Fig. 12). Purified NBD-2 hence obtained was >95 % pure, as estimated on quantitation of the Coomassie blue stained SDS-PAGE gel (Fig. 18). This purified NBD-2 was used for further the biochemical characterization of the protein.

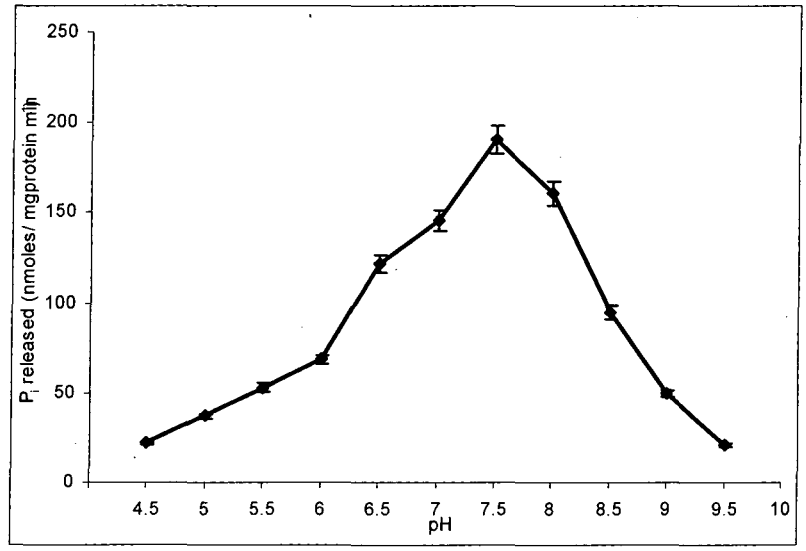


Fig. 19: pH profile of NBD-2 ATPase activity. The ATPase assay was performed in addition of 5 mM ATP with different reaction buffer at different pH ranges of 4.5 to 6.0 (created by using 60 mM MES) and 6.5-9.5 (created by using 60 mM Tris-HCl).

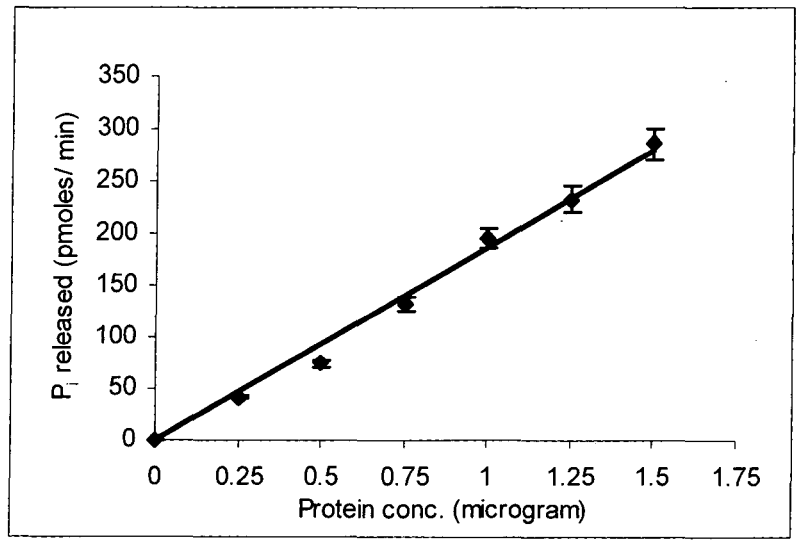


Fig. 20: ATPase activity of NBD-2 at increasing concentration of purified NBD-2 protein. . ATPase activity was assayed on addition of 5 mM of ATP and different amount of NBD-2 and reaction was performed for 30 min.

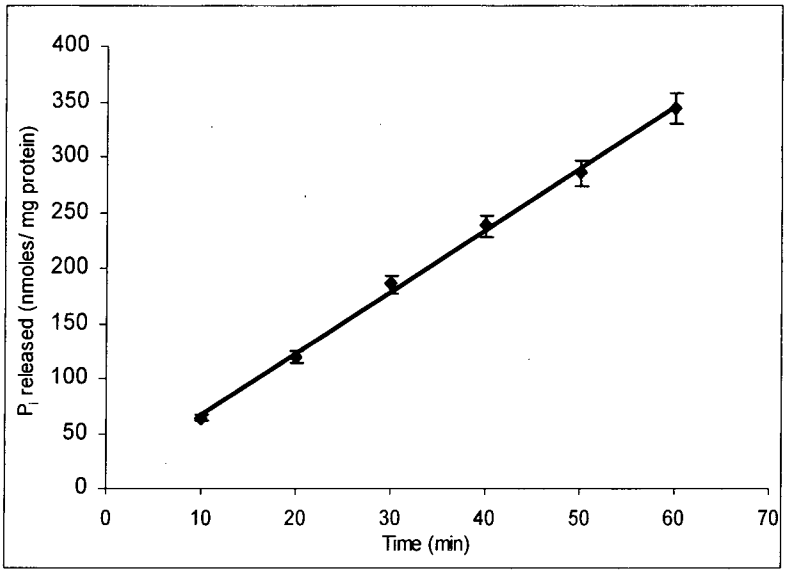


Fig. 21: ATPase activity of NBD-2 for varying length of time. ATPase activity was assayed on addition of 5 mM of ATP and reaction was performed for varying time periods.

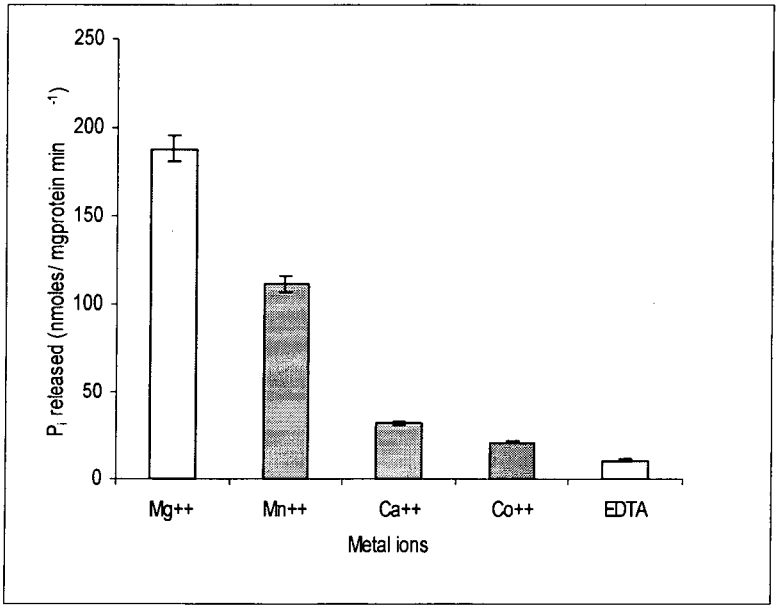


Fig. 22: Divalent cation dependent ATPase activity of NBD-2. ATPase activity was assayed on addition of various metal chloride salts separately in independent reaction sets.

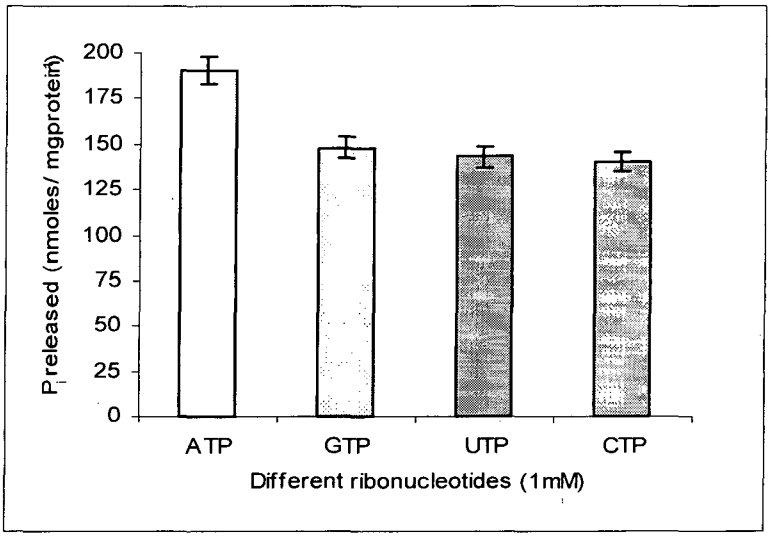


Fig. 23: Substrate specificity of NBD-2. ATPase assay was performed in the presence of 5 mM of each NTP used in independent sets of assay mixture.

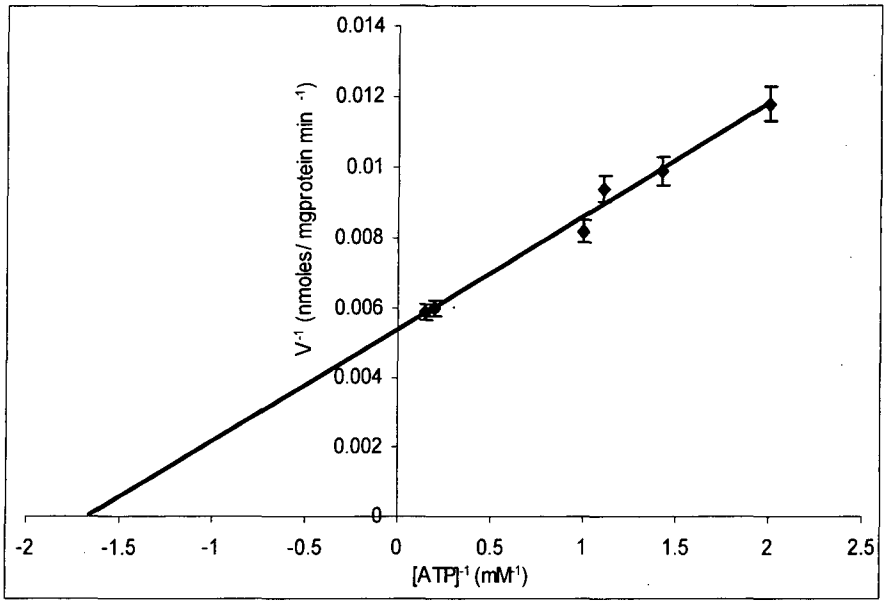


Fig.24: Lineweaver-Burk plot showing K_M and V_{max} of purified NBD-2.

Purified NBD-2 elicits ATPase activity

As described earlier, C-terminal NBD (NBD2) contains both the conserved Walker A and B motifs (Fig. 7). In order to resolve some of the questions like (i) whether NBD-2 domain contains an ATPase catalytic site, (ii) whether NBD2 also place role in ATP hydrolysis by Cdr1p either independently or in co-operation with other functional NBD1 of Cdr1p. and (iii) what is the relative contribution of the multiple nucleotide binding motifs to the overall ATP hydrolysis of Cdr1p, it was essential to establish that the purified protein was enzymatically active. To unequivocally demonstrate if NBD-2 has a capacity to catalyze the hydrolysis of ATP, inorganic phosphate released (P_i) was determined for checking ATP hydrolysis (Senior *et al.,* 1998) this was estimated colorimetrically (Nakamura *et al.*, 2002).

Characterization of NBD-2 ATPase activity

- **Effect of pH on ATPase activity of purified NBD-2:** First, we checked the effect of different pH on ATPase activity of purified NBD-2. For this we used MES (2-Morpholinoenanesulfonic acid) and Tris-Cl buffer whose buffering capacities lie between 4.5 to 6.0 and 6.5 to 9.5 respectively. For measurement of activity at lower pH (4.5 and 5.5), we used MES buffer and for activity at higher pH used Tris-Cl. It was found that the ATPase activity of C-terminal NBD (NBD-2) peaked over a broad pH range extending from pH 7 to pH 8 and the optimal activity was measured at pH 7.5 which is similar to the ATPase activity shown by full Cdr1p. The optimal activity found was ~ 195 nmoles/ mg protein min^{-1}. Fig.19.
- **ATPase activity of C-terminal NBD (NBD-2) is linear function of protein concentration:** To analyse the protein dependent ATPase activity of NBD-2, we used increasing concentrations of protein and monitored Pi released. The protein titration of NBD-2 mediated ATPase activity revealed that the increase in the rate of ATP hydrolysis is a linear function of protein concentration. This finding again ensures that the increased activity was not due to any non-protein contaminants (Fig. 20).
- **ATPase activity of NBD-2 is a linear function of time:** To analyse if the ATPase activity of NBD-2 is time dependent, a time course analysis was performed. We observed that the rate of ATP hydrolysis is linear over a time period of 60 min (Fig. 21).

- **NBD-2 activity is divalent metal ion dependent:** ATPase activity of NBD-2 protein was also strongly dependent on the presence of divalent cations since in their absence as well as in the presence of their chelator i.e. EDTA, there was a significant inhibition in the activity of NBD-2 This figure shows that the four cation investigated in this study have differential influence on the activity however, the order of divalent cation dependence was observed to be $Mg^{2+}>Mn^{2+}>Ca^{2+}>Co^{2+}$ (Fig. 22). Therefore, we observed that the Mg^{++} is the essential cofactor of NBD-2.

- **NBD-2 is a general ribonucleotide triphosphatase:** We also analysed the substrate specificity of NBD-2 and for this we checked the versatility of this domain in hydrolyzing different ribonucleotides independently. We observed that NBD-2 is capable of hydrolyzing all the four tested ribonucleotides i.e. ATP, UTP, GTP and CTP (Fig. 23). All ribonucleotides UTP, GTP and CTP exhibited almost same activity, which was lower as compared to ATP. The optimal activity was measured by using ATP that observed ~ 190 nmoles / mg protein min^{-1}. Therefore we observed that ATP is the main substrate of NBD-2, although it is capable of hydrolyzing all four ribonucleotides. Analysis of ATPase activity of NBD-2 revealed simple Michaelis-Menten kinetics with the K_M value ranging from 600 to 800 μM and V_{max} between 170to 200 nmoles/ mg protein min^{-1} (Fig. 24).

Taken together, our study provides the first direct evidence for involvement of the C-terminal NBD of Cdr1p in hydrolysing different ribonucleotides similar to N-terminal NBD (NBD-1). However some other biochemical properties (like pH dependence ATPase activity, K_M value) of the C-terminal NBD (NBD-2) are different in N-terminal NBD as shown in Table 4.

Table.4: Comparative analysis of biochemical characterizations of NBD1 and NBD2 of Cdr1p.

Properties	**NBD-1**	**NBD-2**
Nature	Ribonucleotide triphosphatase	Ribonucleotide triphosphatase
pH	6.5	7.5
Specific ATPase activity	136 nmoles/mg protein/ min	195 nmole/mg protein/ min
Metal ion dependence	Mg^{++}	Mg^{++}
K_M	120-125 μM	600-800 μM

Discussion

Discussion

The ATP-binding cassette (ABC) transporter constitutes one of the largest superfamily of proteins. These proteins were discovered almost two decades ago in bacteria as high affinity nutrient transporters (Dassa *et al.*, 1999). The majority of these proteins are involved in the energy dependent transport of substrates across cellular membranes. The total numbers of the ABC transporters found in *Candida albicans* genome are 28 (Gaur *et al.*, 2005). On the basis of domain organization, sequence based analysis and self organization map clustering, these ABC transporters of *Candida albicans* were put into six distinct subfamilies. The major ABC drug transporter, Cdr1p of pathogenic yeast *C. albicans* is a large and complex membrane protein comprising of two cytoplasmically localized nucleotide binding domains (NBDs) and twelve transmembrane helices (TMS). Due to its highly hydrophobic nature, it has been difficult to understand the mechanism of energy transduction employed by it to efflux drugs. The NBDs of the ABC transporter is the site of ATP hydrolysis and hence the hub of energy generation for drug efflux. Cdr1p, an ABC transporter of *Candida albicans* is made up of two halves, each containing one NBD followed by a TMD. Each NBD of Cdr1p contains a conserved ABC signature sequence, including the ATP binding and other motifs known as Walker A and Walker B which are involved in ATP hydrolysis. The N-terminal NBD (NBD1) of Cdr1p contains conserved Walker A (GRPGAGCST), Walker B (IQCWD) motif and an ABC signature sequence (VSGGERKRVSIA) and the C-terminal NBD of Cdr1p contains conserved Walker A (GASGAGKST), Walker B (LLFLD) and Signature like sequences. Each TMD comprises of six transmembrane segments (TMS), which involved in substrate specificity.

To understand the molecular mechanism of Nucleotide Binding Domains with respect to ATP binding and hydrolysis which are associated with drug efflux by Cdr1p, a major ABC transporter of *Candida albicans*, Jha *et al.* (2003) purified and characterized the N-terminal nucleotide binding domain (NBD-1) and demonstrated that the Cysteine (C) 193 of Walker A plays an important role in ATP hydrolysis. On the basis of structural modeling of NBD-1 of Cdr1p, they concluded that the γ phosphate (PO_4) moiety of ATP binds to Cysteine 193 of Walker A and hence is capable of severely impairing the ATPase activity if covalently modified or mutated. Tryptophan (W) 326 and Aspartic acid (D) 327 of Walker B of NBD-1 have also been found to be involved in ATP binding and ATP hydrolysis respectively (Rai *et al.*, 2005). Jha *et al* (2003b)

described the relative contribution of both the N-terminal and C-terminal NBDs (*in vivo* system) in terms of ATP binding, hydrolysis and transport activity of native Cdr1p (full protein). These properties of Cdr1p were examined wherein an atypical Cysteine 193 of walker A of NBD-1 (C193K) and a conserved Lysine 901 (K901C) of Walker A of NBD-2 were replaced. The drug resistance profile of Cdr1p mutant variant cells harboring C193K or K901C gave interesting insight into the functioning of the two NBDs. The cells expressing K901C were hypersensitive to drugs as compared to C193K variant or to native Cdr1p. This clearly establishes that the two NBDs of the Cdr1p play an asymmetric role in terms of substrate transport.

To further explore the role of C-terminal domain (NBD-2) and its contribution to full protein, we purified the C-terminal domain (NBD-2) of Cdr1p, an ABC transporter of *Candida albicans* by affinity chromatography using the GST Sepharose column. The GST fusion protein (GST-NBD-2) is bounded specifically to the Glutathione-sepharose column. On thrombin treatment, NBD-2 is released from the GST-NBD-2 fusion protein because thrombin cleavage site is present between GST and NBD-2 and released NBD-2 exhibited ATPase activity (~ 195 nmole/ mgprotein min^{-1}). However GST fused NBD2 (GST-NBD2) was observed catalytically inactive and did not exhibit ATPase activity. We could hypothesize that the GST protein fused with NBD-2 was somewhere masking the active site of the NBD-2. When GST removed by thrombin treatment the catalytically active site of NBD-2 is exposed and exhibited ATPase activity. This GST cleaved NBD-2 was further used for its biochemical characterizations. The maximum ATPase activity of NBD-2 was observed at pH 7.5, which was strongly dependent on Mg^{++}. Mg^{++} dependent ATPase activity was found to be maximum also for the full protein, Cdr1p at pH 7.5 (unpublished results) whereas NBD-1 exhibited maximum ATPase activity at pH 6.5 (Jha *et al*, 2003). We can therefore conclude that NBD-2 is a major player in the ATPase activity of Cdr1p full protein at pH 7.5. NBD-2 also showed broad substrate specificity and was capable of hydrolyzing all the four ribonucleotides i.e. ATP, UTP, GTP and CTP. All ribonucleotides UTP, GTP and CTP exhibited almost same activity, which was observed to be slightly lower as compared to ATP. We could therefore infer that ATP is the main substrate of NBD-2, although it is capable of hydrolyzing all the four ribonucleotides. The protein titration of NBD-2 mediated ATPase activity revealed that the increase in the rate of ATP hydrolysis is a linear function of protein

concentration. All these biochemical properties were found to be almost identical to the full protein Cdr1p.

Purification of NBDs of ABC transporters (e.g. P-gp/MRP/CFTR) has always been a matter of concern since they are often associated with low solubility and hence sequestering of the expressed form within the inclusion bodies. All these problems associated with purification eventually have led to low yields of the desired protein and hence given way to the expression of NBDs as fusion proteins. An example of this observation is of the differential purification strategy adopted for both N- and C-terminal NBDs of mouse P-gp, with the former being soluble with histidine tag and the latter needing a fusion with GST for attaining solubility (Baubichon-Cortay *et al.*, 1994; Dayan *et al.*, 1996). Also, Wang *et al.* have reported that the high level expression of NBD1MLD (NBD-1 of human P-gp/MDR1 with its linker region) in soluble fraction was possible, only when it was expressed as a fusion protein with maltose binding protein (MBP) and not when fused with GST or thioredoxin. The exact reasons for the low solubility of recombinant NBDs as well as their variable behaviour during purification are not yet known, but certainly their purification from different transporters follows a tricky path. Biswas *et al.* have cloned and purified the second nucleotide-binding domain (NBD-2) of the human ABCR protein by the S-protein agarose affinity chromatography and this expressed protein appeared to be moderately soluble and migrated as monomeric unit. This purified NBD-2 of ABCR is functionally active and exhibits ATPase activity.

In our study, we purified NBD-2 of Cdr1p as an independent moiety along with GST tag, but found that large amount of protein was directed to the inclusion bodies. So several alterations in growth conditions like temperature, IPTG induction concentrations and induction periods had to be done in order to improve its expression in the soluble fraction, thereby facilitating its subsequent purification. The use of thrombin and our two-step affinity chromatography strategy helped us to study NBD-2 in isolation from GST.

With the availability of highly purified preparations of NBD-2, it was possible to investigate the question, whether this domain alone was enzymatically active as an ATPase. Does NBD-2 function as only a "regulatory/ cooperative" nucleotide-binding site, and not as a hydrolysis site? Our results clearly demonstrate that the purified NBD-2 was not only able to hydrolyze ATP but also other nucleotides like CTP, UTP and GTP.

Thus NBD-2 elicits a general ribonucleoside triphosphatase activity. The observed broad specificity of NBD-2 with nucleotides matches well with the ATPase activity of full Cdr1p protein (Nakamura *et al.,* 2002; Wada *et al.,* 2002; Krisnamurthy *et al.,* 1998). The broad nucleotide specificity has also been reported for yeast ABC proteins like Pdr5p of *S. cerevisiae* (Egner *et al.,* 1998) and CgCdr1p of *C. glabrata* (Wada *et al.,* 2002). Such broad nucleotide specificity was also observed in the NBD-2 which is similar to that reported in the NBD-1 as well as full protein Cdr1p. Of note the ATPase activity of NBD-2 was slightly higher (~195 nmoles/ mg protein min^{-1}) than that observed with over expressed N-terminal NBD (NBD-1). The rate of ATP hydrolysis of the second domain (C-terminal domain/ NBD-2) of the MRP1 was reported lower than the N-terminal domain (NBD-1). The NBD-2 activity of Cdr1p is fully dependent on the presence of Mg^{++}, which is similar to the MRP1-NBD2 (Ramaen *et al.* 2005), since no/ very low activity of Cdr1p-NBD2 could be detected in the presence of EDTA. We observed that the basic biochemical properties of NBD-2, such as cation dependence, time course profile and substrate specificity are similar to that reported for the NBD-1 as well as native Cdr1p (Jha *et al.,* 2003). The optimal activity of NBD-2 was measured at pH 7.5, which is similar to the full protein Cdr1p. But the highest activity of N-terminal NBD (NBD-1) was reported at pH 6.5. Nokaido *et al* (1997) have reported that the lysine residue of Walker A motif binds to the β- and γ- phosphates of ribonucleotides and plays a critical role in ATP hydrolysis. Mutations of this lysine residue have been shown to reduce or abolish the hydrolysis activity and in some cases impair nucleotide binding. It is predicted that the optimal activity of NBD-2 observed at pH 7.5 is due to the presence of highly conserved lysine (pI 9.74) of Walker A motif, which is involved in ATP hydrolysis.

Taken together, our study provides the first direct evidence for involvement of the C-terminal NBD of Cdr1p in hydrolyzing different ribonucleotides similar to N-terminal NBD (NBD-1). In this study, we could demonstrate that the C-terminal NBD of Cdr1p containing the usual, highly conserved lysine within its Walker A motif (GASGAGKT) but lacking a typical ABC signature sequence (a fact almost universal to all fungal ABC transporters), exhibited independent ATPase activity like N-terminal NBD. This is indeed an interesting observation. However, the significance of both the catalytic sites (NBDs) of Cdr1p in ATP binding, hydrolysis and drug transport remains an exciting

area of further investigation. The cross talk between the two NBDs remains to be explored.

Summary

Summary

Candida species are true opportunistic pathogen that produces a wide spectrum of diseases ranging from superficial mucocutaneous diseases to invasive illnesses, such as hepatosplenic candidiasis, *Candida* peritonitis and systemic candidiasis (Prasad *et al,* 2002; Anaissie *et al.,* 2003). Candida species have been reported to be the sixth most commonly isolated and fourth most prevalent blood stream pathogen isolated. The abundance of candidiasis accounts for approximately 50 – 60 % in human. The frequency of this infection has risen drastically in the past decades due to the increasing number of the immunocompromised patients (HIV/AIDS). Prolonged and widespread usage of antifungals especially azoles has led to the overexpression of Cdr1p (*Candida* Drug Resistance) an ATP Binding Cassette (ABC) transporter, is one of the most common mechanism of multidrug resistance in *Candida albicans*. (Prasad *et al.,* 1996). This is well established with the reports from fluconazole resistant clinical isolates of *C. albicans* where the enhanced expression of Cdr1p has been shown to help the pathogen efflux this therapeutic azole and hence facilitate its own survival (Sanglard *et al,* 1995; Sanglard *et al.,* 1997; White, 1997). This mechanism of survival is certainly not the forte of this pathogen alone; tumor cells also utilize it in order to resist chemotherapy through overexpression of the Cdr1p homologs, i.e. P-glycoprotein (P-gp)/MDR1 and the multidrug resistance associated protein (MRP1) (Ambudkar *et al.,* 1999). This drug resistance in *Candida albicans* is a major hurdle to treatment of fungal infection in human..

Typically, the predicted topology of Cdr1p exhibits the characteristic features of an ABC transporter; it contains two highly hydrophobic transmembrane domains (TMD) and two cytoplasmically localized nucleotide binding domains (NBD). Each TMD comprises of six transmembrane segments (TMS), which are envisaged to confer substrate specificity to Cdr1p. The nature of Cdr1p substrates varies enormously as it includes structurally unrelated compounds such as azoles, lipids and steroids (Krishnamurty *et al.,* 1998; Dogra *et al.,* 1999). This promiscuity towards substrates is a characteristic feature of most of the ABC type drug transporters and hence makes their functionality all the more complex to understand (Prasad *et al.*, 2002; Dogra *et al.,* 1999). An important characteristic feature of these drug transporters is that they all utilize the energy of nucleotide hydrolysis to transport these substrates across the plasma membrane against the concentration gradient. The conserved NBDs located at the cytoplasmic periphery are the hub of such an activity. In prokaryotic ABC type transporters such as the histidine permease of *Escherichia coli*, both

NBDs are functionally identical and equally contributive to the protein's activity. Inactivation of either one of these NBDs in the full protein results in a transporter that has its activities reduced to 50%. On the other hand, the NBDs of the eukaryotic transporters such as the human P-gp/MDR1, CFTR (Cystic Fibrosis Transmembrane conductance Regulator) and MRP1, though highly conserved and similar in sequence, do not appear to be functionally complementary, as inactivation of either of them completely abolishes ATPase and transport activities of the protein (Azzaria *et al.,* 1988).

NBD(s) protein sequence(s) contains certain conserved amino acid stretches, which are considered to be critical for this domain's functionality. These include; the Walker A, with a consensus sequence GxxGxGKS/T, where 'x' represents any amino acid, the Walker B motif i.e. hhhhD, where 'h' represents any aliphatic residue, and an ABC signature, LSGGQQ/R/KQR. Structural and biochemical analyses of NBDs show that the lysine residue of Walker A motif binds to the β- and γ- phosphates of ribonucleotides and plays a critical role in ATP hydrolysis. Mutations of this lysine residue have been shown to reduce or abolish the hydrolysis activity and in some case impair nucleotide binding (Nikaido *et al.,* 1997). Interestingly, though N-terminal NBD of Cdr1p contains the conserved Walker A (GRPGAGCST), Walker B (IQCWD) motifs, and an ABC signature sequence (VSGGERKRVSIA) (Prasad *et al.,* 1995), the commonly conserved lysine residue within the Walker A motif is replaced by a cysteine. This replacement appears to be a unique feature of N-terminal NBD of almost all the known fungal ABC-type transporters. However, N-terminal NBD of Ste6p of *Saccharomyces cerevisiae* is an exception to this generalization (Decottignies and Goffeau, 1997). And the C-terminal NBD contains the conserved Walker A (GASGAGKT) , Walker B (LLFLD) and signature like sequence .

The present work is an attempt to examine the independent functionality of C-terminal NBD (NBD-2) of Cdr1p. For this, we have cloned, overexpressed and purified C-terminal NBD (NBD-2) protein of Cdr1p and have functionally characterize purified NBD-2 protein.

- The primary sequence and hydrophobicity profile predictions revealed that the N-terminal NBD (2-512 amino acid residues) as well as the C-terminal NBD (786-1195 amino acid residues) of Cdr1p appears to be extrinsic with limited membrane interactions. We cloned the PCR amplicon representing NBD-2 of *CDR1,* at the *Bam*HI site of pGex2T-Z expression vector downstream to the glutathione-*S*-

transferase (GST) ORF. The construct (pSSGN2) was transformed in *E. coli* BL21 (DE3) cells and the resulting transformant designated as SSGN2 was induced by IPTG to overexpress the GST-NBD-2 fusion protein. The bacterial cell lysate from IPTG induced SSGN2 cells was incubated with glutathione-sepharose 4B gel matrix to immobilize the fusion protein. After an extensive wash the matrix bound fusion protein (GST-NBD-2) was cleaved with biotinylated thrombin to generate unbound NBD-2 protein (~47 kDa).

- We checked the effect of different pH on ATPase activity of purified NBD-2. It was found that the ATPase activity of C-terminal NBD (NBD-2) peaked over a broad pH range extending from pH 7 to pH 8 and the optimal activity was measured at pH 7.5 which is similar to the ATPase activity shown by full Cdr1p. The optimal activity found was ~ 195 nmoles/ mg protein min^{-1}.
- Our results clearly demonstrate that the purified NBD-2 was not only able to hydrolyze ATP but other nucleotides like CTP, UTP and GTP. Thus NBD-2 elicits a general ribonucleoside triphosphatase activity.
- We also checked the protein titration of NBD-2 mediated ATPase activity and revealed that the increase in the rate of ATP hydrolysis was a linear function of protein concentration.
- We observed that the rate of ATP hydrolysis of NBD-2 was linear function of time.
- We also checked that ATPase activity of NBD-2 protein was also strongly dependent on Mg^{++} and essential cofactor of NBD-2.

In conclusion our study provides the first direct evidence that the predicted C-terminal NBD of Cdr1p can bind as well as hydrolyze ribonucleotides. However, the significance of both the catalytic sites (NBDs) of Cdr1p in ATP binding, hydrolysis and drug transport remains an exciting area of further investigation. The cross talk between the two NBDs remains to be explored.

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Appendices

MEDIUM AND BUFFERS:

Bacterial culture medium

Component	Composition (%)
Sodium chloride	1
Yeast extract	0.5
Tryptone	0.5

1X PBS buffer (pH-7.4)

Component	Concentration
Sodium chloride	140 mM
Potassium chloride	2.7 mM
Disodium hydrogen phosphate	10 mM
Potassium dihydrogen phosphate	1.8 mM

1X ATPase buffer

Component	Concentration
Tris-Cl (pH 7.5)	60 mM
Magnesium chloride	8 mM

Stop solution

Component	Composition (%)
Ammonium molybdate	0.5 %
SDS	0.5 %
Sulphuric Acid	2 %

Colouring reagent

Component	Composition (%)
Ascorbic Acid	10 %

SDS-PAGE Running Buffer:

Component	g/ litre
Glycine	14
Tris-Base	3
SDS	1

SDS-PAGE gel solutions:

Resolving solution (8%)

Component	Volume (in ml)
30 % Acrylamide mix	1.3
1.5 M Tris-Cl (pH- 8.8)	1.3
Autoclave Water	2.3
10 % SDS	0.05
10 % APS	0.05
TEMED	0.01

Stacking solution (5%)

Component	Volume (in ml)
30 % Acrylamide Mix	0.5
1 M Tris-Cl (pH- 6.8)	0.38
Autoclave Water	2.1
10 % SDS	0.03
10 % APS	0.01
TEMED	