Purification and Characterization of the N-Terminal Nucleotide Binding Domain of an ABC Drug Transporter of *Candida albicans*: Uncommon Cysteine 193 of Walker A Is Critical for ATP Hydrolysis†

Sudhakar Jha,‡ Neerja Karnani,‡ Suman K. Dhar,§ Kasturi Mukhopadhayay,‡ Suneet Shukla,‡ Preeti Saini,‡ Gauranga Mukhopadhayay,§ and Rajendra Prasad*,‡

Membrane Biology Laboratory, School of Life Sciences, and Special Center for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India 110067

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ABSTRACT: The *Candida* drug resistance protein Cdr1p (~170 kDa) is a member of ATP binding cassette (ABC) superfamily of drug transporters, characterized by the presence of 2 nucleotide binding domains (NBD) and 12 transmembrane segments (TMS). NBDs of these transporters are the hub of ATP hydrolysis activity, and their sequence contains a conserved Walker A motif (GxxGxGKS/T). Mutations of the lysine residue within this motif abrogate the ability of NBDs to hydrolyze ATP. Interestingly, the sequence alignments of Cdr1p NBDs with other bacterial and eukaryotic transporters reveal that its N-terminal NBD contains an unusual Walker A sequence (GRPGAGCST), as the invariant lysine is replaced by a cysteine. In an attempt to understand the significance of this uncommon positioning of cysteine within the Walker A motif, we for the first time have purified and characterized the N-terminal NBD (encompassing first N-terminal 512 amino acids) of Cdr1p as well as its C193A mutant protein. The purified NBD-512 protein could exist as an independent functional general ribonucleoside triphosphatase with strong divalent cation dependence. It exhibited ATPase activity with an apparent K_m in the 0.8−1.0 mM range and V_{max} in the range of 147−160 nmol min^{-1} (mg of protein)$^{-1}$. NBD-512-associated ATPase activity was also sensitive to inhibitors such as vanadate, azide, and NEM. The Mut-NBD-512 protein (C193A) showed a severe impairment in its ability to hydrolyze ATP (95%); however, no significant effect on ATP (TNP-ATP) binding was observed. Our results show that C193 is critical for N-terminal NBD-mediated ATP hydrolysis and represents a unique feature distinguishing the ATP-dependent functionality of the ABC transporters of fungi from those found in bacteria and other eukaryotes.

One of the most clinically significant mechanisms of azole resistance in the pathogenic yeast *Candida albicans* is the overexpression of the multidrug transporter protein Cdr1p (*Candida* drug resistance), belonging to the ABC[1] (ATP binding cassette) superfamily of transporters (*1*−*3*). 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 (*4*−*6*). 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 homologues, i.e., P-glycoprotein (P-gp)/MDR1 and the multidrug resistance associated protein (MRP1) (*7*).

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 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 (*8*, *9*). This promiscuity toward substrates is a characteristic feature of most of the ABC-type drug transporters and hence makes their functionality all the more complex to understand (*2*, *9*). 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

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* Corresponding author. E-mail: rp47@hotmail.com. Telephone: 91-11-26704509. Fax: 91-11-26717081.

‡ Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University.

§ Special Center for Molecular Medicine, Jawaharlal Nehru University.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl 1-thio-β-D-galactoside; PMSF, phenylmethanesulfonyl fluoride; PEI, polyethylenimine; kDa, kilodalton(s); ABC, ATP binding cassette; NBD, nucleotide binding domain; TMD, transmembrane domain; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmleimide; TNP-ATP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; GST, glutathione *S*-transferase.

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Table 1: List of Plasmids and Strains Used in This Study

name	description	reference
plasmid		
pS12-35	plasmid carrying the complete *CDR1* gene	*13*
pGex2T-Z	modified pGex2T where in the *Bam*HI-*Eco*RI of pGex2T a oligo was inserted containing *FLAG* and *HMK* sequences and *Spe*I, *Sma*I, *Kpn*I, *Xho*I, and *Eco*RI sites	this study
pSJGN1-8	plasmid carrying NBD-512 cloned at the *Bam*HI site of pGex2T-Z	this study
pSJGN1MutC/A	plasmid pSJGN1-8 carrying the C193A mutation in NBD-512	this study
pSJGN1MutC/K	plasmid pSJGN1-8 carrying the C193K mutation in NBD-512	this study
bacterial strains		
BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ (r_B^- m_B) *gal dcm* (DE3)pLysS	Novagen
SJGN1-8P	BL21(DE)pLysS cells carrying the pSJGN1-8 plasmid	this study
SJGNMutCA-P	BL21(DE)pLysS cells carrying the pSJGN1MutC/A plasmid	this study
SJGNMutCK-P	BL21(DE)pLysS cells carrying the pSJGN1MutC/K plasmid	this study

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 (*10*).

NBD protein sequence(s) contain(s) certain conserved amino acid stretches, which are considered to be critical for this domain's functionality (*11*). 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 the 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 (*11*, *12*). Interestingly, though the N-terminal NBD of Cdr1p contains the conserved Walker A (GRPGAGCST) and B (IQCWD) motifs, and an ABC signature sequence (VSGGERKRVSIA) (*13*), the commonly conserved lysine residue within the Walker A motif is replaced by a cysteine. This replacement appears to be a unique feature of the N-terminal NBD of almost all the known fungal ABC-type transporters. However, the N-terminal NBD of Ste6p of *Saccharomyces cerevisiae* is an exception to this generalization (*14*). The significance of this replacement from a charged residue to a sulfhydryl amino acid is not apparent. Superimposed with these findings is the observation that Walker A of the C-terminal NBD of Cdr1p contains the commonly conserved lysine (GASGAGKT). The present work is an attempt to examine the independent functionality of the N-terminal NBD of Cdr1p and to ascertain the role of an unconventionally positioned cysteine within Walker A. For this, we have cloned, overexpressed, and purified the N-terminal NBD (NBD-512) protein of Cdr1p. Our study demonstrates that the purified NBD-512 protein elicits cation-dependent ATPase activity for which cysteine 193 within Walker A is critical.

EXPERIMENTAL PROCEDURES

Strains and Media. E. coli BL21(DE)pLysS cells as well as their transformants containing the plasmid constructs used in this study are listed in Table 1. These strains were grown in LB (Luria−Bertani) broth or on LB plates supplemented with ampicillin (0.1 mg mL^{-1}) when required.

Chemicals. Luria−Bertani medium was obtained from Difco. DNA-modifying enzymes were purchased from Roche Molecular Biochemicals. Ultrapure deoxyribonucleotides (dATP, dGTP, dCTP, dTTP) for PCR, glutathione−Sepharose 4B matrix, and the sequencing kit (Sequenase version 2.0 enzyme) were obtained from Amersham Biosciences Ltd. Na^+−K^+ plasma membrane ATPase, ribonucleotides (ATP, CTP, GTP, UTP), IPTG (isopropyl 1-thio-β-D-galactopyranoside), bacterial protease inhibitor cocktail, aprotinin, pepstatin A, leupeptin, antipain, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), polyethlenimine (PEI)−cellulose strips, and other molecular grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The *Pfu* DNA polymerase for PCR amplification was from Stratagene, Inc. (La Jolla, CA). The thrombin cleavage capture kit was purchased from Novagen (Madison, WI). Oligonucleotides listed in Table 2 were commercially procured from Integrated DNA Technologies, Inc..

In Vitro Site-Directed Mutagenesis of the NBD-512. DNA sequence representing NBD-512 was amplified using the *CDR1* genomic clone pS12-35 as template (*13*). The primers (Table 2) used for this purpose allowed the introduction of *Bam*HI restriction sites at the 5′ and 3′ termini of the amplicon. The resultant 1545 bp amplicon was digested with *Bam*HI and ligated at the corresponding sites of the linearized pGex2T-Z vector. The resulting construct pSJGN1-8 was sequenced to confirm the in-frame insertion of NBD-512 in the pGex2T-Z vector and rule out any probable nucleotide changes in the sequence due to PCR. Site-directed mutagenesis was carried out using a mutagenesis kit (Stratagene). For this, the pSJGN1-8 construct was used as the template, and 16 cycles of PCR, each comprising 30 s at 95 °C, 30 s at 55 °C, and 13.2 min at 68 °C, were performed. The primers used (listed in Table 2) for this purpose were complementary oligonucleotides harboring cysteine to alanine/lysine mutation (C193A/K). The mutated construct was sequenced to confirm the incorporation of mutation at its target position.

Overexpression and Purification of the NBD-512 Protein and Its Mutant. E. coli BL21(DE)pLysS cells harboring the

Table 2: List of Oligonucleotides Used in This Study

oligonucleotide	sequence	purpose
NBD1F	5′ CGGGGATCCTCAGATTCTAAGATGTCG 3′	forward primer containing the *Bam*HI site for amplifying and cloning NBD-512 and sequencing
NBD1R	5′ CGCGGATCCCGACGGATCACCTTTCAT 3′	reverse primer containing the *Bam*HI site for amplifying and cloning NBD-512 and sequencing
C193A/F	5′ GGGAGACCCGGTGCTGGT**GCA**TCCACATTGTTAAAGACC 3′	forward primer for mutating C → A at amino acid position 193
C193A/R	5′ GGTCTTTAACAATGTGGA**TGC**ACCAGCACCGGGTCTCCC 3′	reverse primer for mutating C → A at amino acid position 193
C193K/F	5′ GGGGAGACCCGGTGCTGGT**AAA**TCCACATTGTTAAAGACC 3′	forward primer for mutating C → K at amino acid position 193
C193K/R	5′ GGTCTTTAACAATGTGGA**TTT**ACCAGCACCGGGTCTCCCC 3′	reverse primer for mutating C → K at amino acid position 193
NBD1F-DWA	5′ CGGGATCCAAGACCATTGCTGTTAAC 3′	for sequencing
NBD1R-DWB	5′ CGGAATTCTCTTTCACCACCGGAAAC 3′	for sequencing
CDF10	5′ CATTACCGTGGTAAAGTTATTTATTCTGC 3′	for sequencing
CDR10	5′ GCAGAATAAATAACTTTACCACGGTAATG 3′	for sequencing

required (i.e., pSJGN1-8, pSJGN1MutC/A, or pSJGN1MutC/K) construct were grown at 30 °C in LB medium containing 0.1 mg mL^{-1} ampicillin. On growing the culture to an OD_{600} of 0.4, IPTG was added to a final concentration of 0.5 mM, and the culture was further incubated for 3.30 h at 30 °C. Cells were harvested and resuspended in precooled PBS buffer containing 0.1% Triton-X-100, 10 mM DTT, 100 μM PMSF, and protease inhibitor cocktail. Cells were lysed, and the soluble fraction was incubated with glutathione−Sepharose 4B gel matrix (preequilibrated with PBS buffer containing 10 mM DTT and 100 μM PMSF) for 45 min and was then packed in a C-10 column (Amersham Biosciences) hooked to the Bio-Rad purification system (Biologic Duo-Flow). The column was washed with a wash buffer containing PBS buffer, 10 mM DTT, 500 mM NaCl, 100 μM PMSF, and protease inhibitor cocktail. The GST−NBD-512 fusion protein was eluted with elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH 8.0). To obtain NBD-512, the column containing the matrix-bound GST−NBD-512 fusion protein was cleaved with biotinylated thrombin. The thrombin-cleaved NBD-512 protein was collected and further incubated with streptavidin−agarose beads for 1 h. NBD-512 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 NBD-512 was measured by two methods: the continuous cycling assay and the end point P_i assays.

For the cycling assay, cycling components (3 mM phosphoenolpyruvate, 0.33 mM NADH, 10 units mL^{-1} pyruvate kinase, and 10 units mL^{-1} lactate dehydrogenase) were added to the ATPase assay buffer [60 mM Tris-HCl (pH 6.5) and 8 mM $MgCl_2$ supplemented with 5 mM ATP as and when required] to link the hydrolysis of ATP directly with NADH oxidation (*15*). Reactions with the Na^+-K^+ ATPase were carried out in the buffer specified by the supplier (Sigma Chemical Co.). Purified NBD-512 was preincubated at 30 °C in a temperature-controlled spectrophotometer (Varian, Cary 300). The reaction was initiated by addition of ATP, and the absorption was monitored at 340 nm for 5 min.

The radioactive γ-^{32}P release assay was carried out as described earlier (*16*). A standard 10 μL reaction mixture contained 60 mM Tris-HCl (pH 6.5), 8 mM $MgCl_2$, [γ-^{32}P]-ATP, 5 mM ATP, and purified NBD-512. Reactions were incubated at 30 °C for 30 min and terminated by addition of 2 μL of 200 mM EDTA followed by chilling on ice. Aliquots of 2 μL were applied to PEI−cellulose strips and air-dried. The released γ-^{32}P was separated from [γ-^{32}P]ATP by running the air-dried PEI−cellulose strips in solvent containing 0.5 M LiCl and 1 M formic acid. The autoradiograph was developed after exposing the dried TLC plates to X-ray film (Kodak). This autoradiograph was used to quantitate the γ-^{32}P release, by using the Image Quant software supplied by the manufacturer (BAS1800 Fuji Phosphoimager system).

For the end point P_i release colorimetry assay, the amount of inorganic phosphate (P_i) released over 30 min at 30 °C was measured as described previously (*17*). Briefly, a standard 100 μL reaction mixture containing the NBD-512 protein was incubated in ATPase buffer [60 mM Tris-HCl (pH 6.5) and 8 mM $MgCl_2$], supplemented with 5 mM ATP. The transfer of the mixture from ice to a water bath at 30 °C initiated the reaction. The reaction was stopped by addition of 1 mL of stop solution (0.5% SDS, 2% H_2SO_4, and 0.5% ammonium molybdate), followed by addition of 10 μL of freshly prepared coloring reagent (10% ascorbic acid), and absorbance was taken at 750 nm using a UV-2000 Shimadzu spectrophotometer. In the control set of reactions, the 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 standard curve for 0−100 nmol of inorganic phosphate.

RESULTS

Cloning, Expression, and Purification of NBD-512. 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 (Figure 1A) appears to be extrinsic with limited membrane interactions (*13*). NBD-512 investigated in this study contains the characteristic Walker A and B motifs, as well as the ABC signature (Figure 1B,C). We cloned the PCR amplicon representing NBD-512 of *CDR1* at the *Bam*HI site of the pGex2T-Z expression vector downstream to the glutathione *S*-transferase (GST) ORF (detailed in Experimental Procedures). The construct (pSJGN1-8) was transformed in *E. coli* BL21(DE)pLysS cells, and

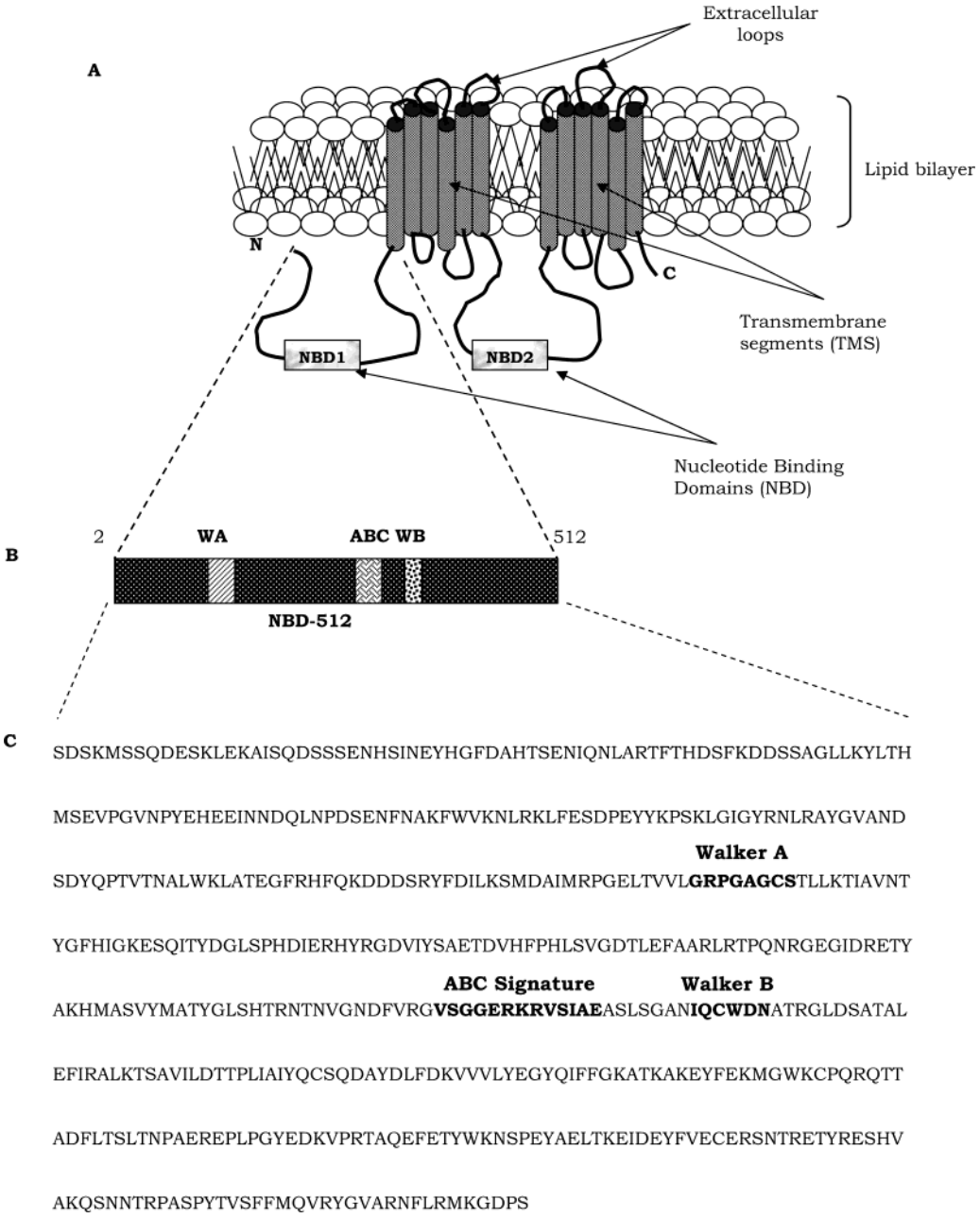

FIGURE 1: Predicted structural organization of Cdr1p domains and amino acid sequence of N-terminal NBD. (A) Topological model of Cdr1p showing two putative transmembrane domains (TMD) and two nucleotide binding domains (NBD). Each TMD comprises six α-helices spanning the lipid membrane. The cytoplasmic domains of Cdr1p, i.e., NBD1 and NBD2, are located at the N- and C-terminus, respectively. (B, C) Schematic representation and amino acid sequence of the N-terminal NBD of Cdr1p highlighting the positions of Walker A (WA) and Walker B (WB) motifs and the ABC signature.

the resulting transformant designated as SJGN1-8P was induced by IPTG to overexpress the GST−NBD-512 fusion protein. Figure 2A depicts the SDS−PAGE profile of the total bacterial lysate proteins wherein an overexpressed band of the GST−NBD-512 fusion protein, of estimated M_r 85 kDa, was observed. The bacterial cell lysate from IPTG-induced SJGN1-8P 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-512) was cleaved with biotinylated thrombin to generate unbound NBD-512 protein (~58 kDa). Purified NBD-512 hence obtained was >95% pure, as estimated from the Coomassie Blue stained SDS−PAGE gel, using Quantity One software (Bio-Rad) (Figure 2A).

Circular Dichroism Spectra of NBD-512. The circular dichroism (CD) spectra of the purified NBD-512 protein had a mean residue molar ellipticity, which exhibits a maximum at 194 nm and two minima: one at 209 nm and the other at 223 nm (Figure 2B). The CD spectra indicated NBD-512 protein to be structured and preferentially α-helix in nature.

Purified NBD-512 Elicits ATPase Activity. To unambiguously demonstrate the functionality of purified NBD-512 as an ATPase, two different methods of ATP hydrolysis were used; one was based on the formation of ADP as the end product, i.e., the continuous cycling assay linking ATP hydrolysis to NADH oxidation, and the second was based on the estimation of inorganic phosphate (P_i) release as a function of ATPase activity. The P_i release was measured

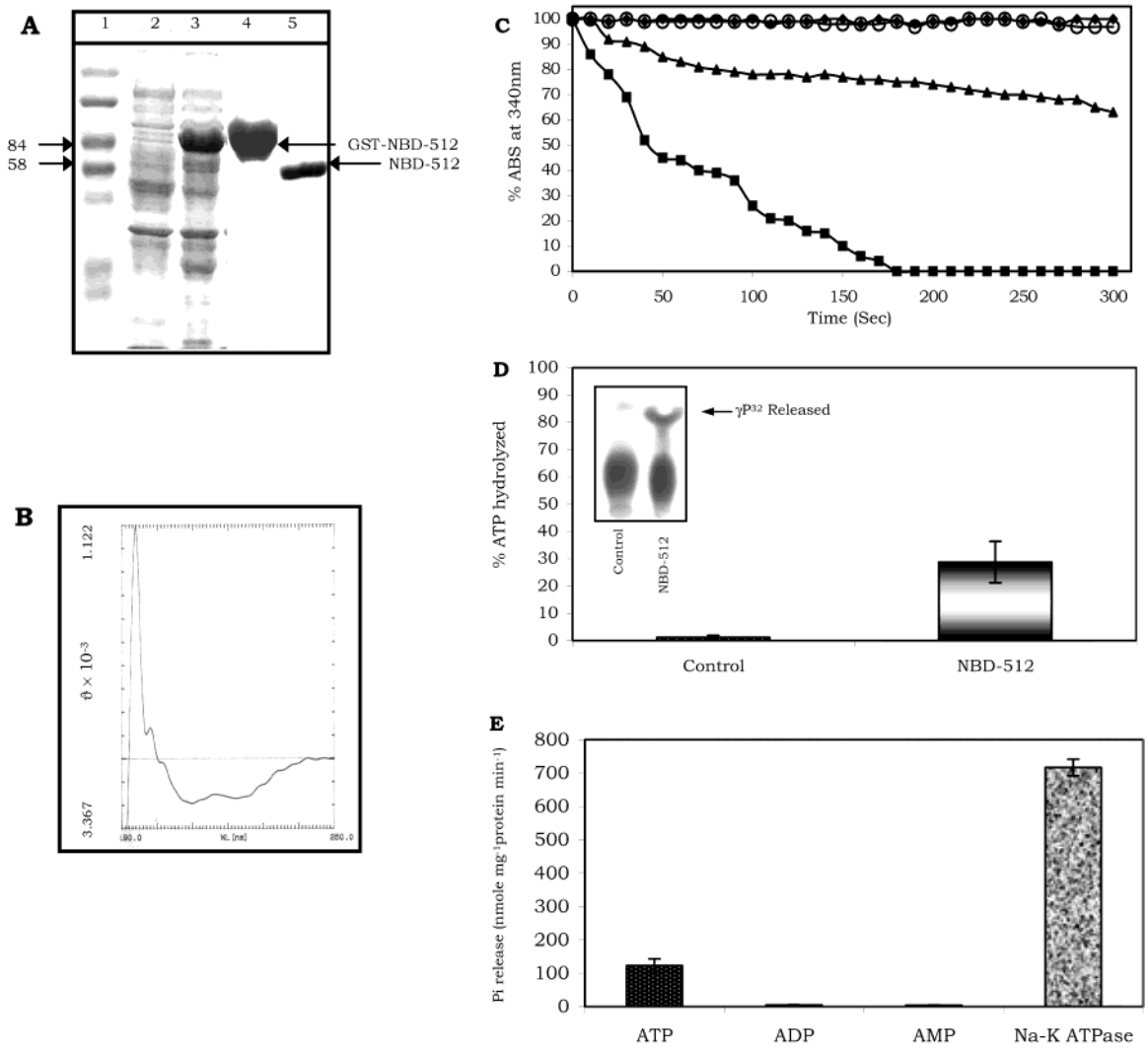

FIGURE 2: Purification and ATPase assay of purified NBD-512. (A) SDS−PAGE analysis of the overexpressed fusion protein and the purified NBD-512 protein. Lanes: 1, protein molecular mass marker; 2, uninduced SJGN1-8P cell lysate; 3, cell lysate from IPTG-induced SJGN1-8P cells (equal amounts of protein were loaded in lanes 2 and 3); 4, purified GST−NBD-512 fusion protein (30 μg); 5, purified NBD-512 (5 μg) after thrombin cleavage. As indicated with arrows, GST−NBD-512 and NBD-512 migrated with predicted molecular masses of ~85 and ~58 kDa, respectively. Protein fractions were analyzed by 10% SDS−PAGE (*32*) followed by Coomassie Blue R250 staining (*33*). Protein concentration was determined by both molar extension coefficient measurements (*34*) and Bradford's method (*35*). (B) Circular dichroism (CD) spectra of the NBD-512 protein. The mean residue molar ellipticity of the NBD-512 protein (1.74 μM) as a function of wavelength was monitored in 10 mM Tris-HCl, pH 7.5. The CD spectra were recorded between 190 and 250 nm at 25 °C in a 1 mm path-length cuvette using a Jasco J700 spectropolarimeter. (C) Continuous cycling assay. Each assay contained either 2 μg of NBD-512 protein (▲) or 0.01 unit of Na^+-K^+ ATPase (■) in the ATPase assay buffer containing 5 mM ATP and cycling components as detailed in Experimental Procedures. The negative control reactions for ATPase activity, i.e., in the absence of either ATP (○) or NBD-512 protein (◆), under similar assay conditions are also plotted. ATPase activity was measured by monitoring the NADH oxidation (decrease in absorption of NADH at OD_{340nm}). (D) End point assay. ATPase activity by NBD-512 was monitored by the release of radioactive γ-^{32}P from [γ-^{32}P]ATP. The radioactive substrate and products were separated using thin-layer chromatography (TLC). The autoradiograph (represented as an inset within the histogram) shows the γ-^{32}P released on [γ-^{32}P]ATP hydrolysis. The values plotted in the histogram were quantitated from the autoradiogram using the Image Quant software, supplied by the manufacturer (BAS1800 Fuji Phosphoimager system). (E) Relative capacity of NBD-512 to release P_i from ATP, ADP, and AMP. Inorganic phosphate (P_i) release was monitored colorimetrically (as detailed in Experimental Procedures) in an assay system containing 5 mM ATP, ADP, or AMP in ATPase buffer with either NBD-512 (0.5 μg) or Na^+-K^+ ATPase (0.01 unit) in the reaction mixture. All of the reactions were carried out in triplicate. The histogram hence generated (±SD) represents the average of three independent experiments.

by employing two different assay methods. In one, it was monitored by the liberation of γ-^{32}P by using radiolabeled [γ-^{32}P]ATP as the substrate, while in the other P_i release was estimated colorimetrically.

(A) Continuous Cycling Assay. In this coupling assay system, the ADP formed in the ATPase reaction was monitored by the decrease in the absorption of NADH when pyruvate is converted to lactate (*15*). The assay is illustrated in Figure 2C wherein a known ATPase (i.e., Na^+-K^+ plasma membrane ATPase) was used as a positive control to check the functionality of the assay. As is evident from Figure 2C, addition of Na^+-K^+ ATPase protein led to a rapid, time-dependent decrease in the absorption of NADH at 340 nm, thus confirming the functionality of the coupling assay method. In a similar setup the addition of purified NBD-512 protein also showed a steady drop in NADH absorption. This decline in NADH absorption was both protein (NBD-512) and substrate (ATP) dependent (data not shown). In

negative control experiments i.e., where protein or ATP alone was used in the reaction mixture, no decline in the absorption of NADH was observed (Figure 2C).

(B) Radioactive γ-^{32}P Release. To further validate the ATPase activity of the purified NBD-512, [γ-^{32}P]ATP was used as substrate in the reaction mixture. The released γ-^{32}P was separated from nonhydrolyzed [γ-^{32}P]ATP using thin-layer chromatograpy (TLC), as detailed in Experimental Procedures. The release of radiolabeled γ-^{32}P in the lane representing the reaction performed with NBD-512 protein was distinctively absent in the control lane (without protein) (Figure 2D inset). On quantitating the spots on the autoradiogram, we could calculate the NBD-512-mediated hydrolysis to be 30% of the starting amount of [γ-^{32}P]ATP (Figure 2D). The release of γ-^{32}P by NBD-512 was observed to be protein concentration and time dependent (data not shown).

(C) Colorimetric P_i Estimation. Results from the continuous cycling assay and radioactive γ-^{32}P release assay demonstrated that the purified NBD-512 is able to catalyze ATP hydrolysis. However, the above assay does not rule out the possibility of a contaminating protein, i.e., either a nonspecific phosphatase or another cellular ATPase, to be responsible for the observed activity. To exclude such a possibility, the activity was monitored by another assay in which ATPase activity was measured colorimetrically as a function of P_i release. Results presented in Figure 2E show that, in the presence of the NBD-512 protein, P_i release was observed only when ATP was used as substrate. The P_i release mediated by the NBD-512 protein with AMP or ADP would be expected only if nonspecific phosphatase(s) was (were) present as a contaminant in the NBD-512 preparation. That NBD-512 elicits true ATPase activity was further confirmed by the observation that the mutation C193A, in the Walker A nucleotide binding motif of NBD-512, almost completely abolished the ATPase activity of purified Mut-NBD-512 protein (discussed below). As a positive control for the experiment, P_i release was also estimated independently with purified Na^+-K^+ ATPase protein (Figure 2E).

Taken together, it is clear from three different assay approaches that the purified NBD-512 domain of Cdr1p is functionally active as an ATPase. Henceforth, in the following experiments ATPase activity was routinely monitored colorimetrically

Characterization of NBD-512 ATPase Activity. Protein titration of NBD-512-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 nonprotein contaminants (Figure 3A). To analyze if the ATPase activity of NBD-512 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 (Figure 3B). We also checked for the pH dependence on NBD-512 ATPase activity and found that the activity peaked over a broad pH range extending from 6.0 to 7.5, which is in agreement with the activity of full Cdr1p (*18*) (Figure 3C).

ATPase activity of the NBD-512 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-512 (Figure 3D). The order of divalent cation dependence was observed to be $Mg^{2+} > Mn^{2+} > Ca^{2+} > Co^{2+}$.

Vanadate inhibits the ABC transporters by stabilizing a high-energy intermediate during the ATP hydrolysis cycle. It does so by replacing the terminal phosphate hydrolyzed by the ATPases in the occluded nucleotide state complex. Therefore, it is considered as a good indicator of ATPase activity of an ABC-type transporter (*19*). We checked for its influence on NBD-512 and found that 0.3 mM vanadate could inhibit ~50% of NBD-512 ATPase activity (Figure 3E). ATPase activity of NBD-512 was also inhibited by 0.1 mM sodium azide (~90%), a potent ATPase inhibitor.

NEM, being a specific modifier of cysteine residues, has been shown to affect the ATPase activity of ABC transporters containing cysteine residues, either in the nucleotide binding motifs or in the close proximity of the catalytic site of the proteins (*9*, *19*). In this study we observed that there was an ~60% reduction in the ATPase activity of NBD-512 when it was incubated with 0.3 mM NEM (Figure 3E).

NBD-512 Is a General Ribonucleoside Triphosphatase. We also analyzed the substrate specificity of NBD-512, and for this, we checked the versatility of this domain in hydrolyzing different ribonucleotides independently. We observed that NBD-512 is capable of hydrolyzing all of the four tested ribonucleotides, i.e., ATP, UTP, GTP, and CTP (Figure 3F). Analysis of ATPase activity of NBD-512 revealed simple Michaelis−Menten kinetics with an apparent K_m of 0.8−1.0 mM and V_{max} of 147−160 nmol min^{-1} (mg of protein)$^{-1}$ (data not shown). Kinetic analysis carried out with other NTPs indicated that NBD-512 has binding affinities in a similar range in the order ATP > UTP > GTP > CTP (data not shown).

NBD-512 Binds the Fluorescent Nucleotide Derivative TNP-ATP. The fluorescent derivative of ATP, i.e., TNP-ATP, is weakly fluorescent in aqueous solution. However, the quantum yield of this molecule is greatly enhanced in a hydrophobic environment such as that of NBD. In the present study, the binding of TNP-ATP to NBD-512 was monitored by changes in extrinsic fluorescence. Upon excitation at 408 nm, TNP-ATP in solution exhibited a characteristic fluorescence emission with a maximum at 559 nm. The addition of NBD-512 protein markedly increased the probe fluorescence and gave a blue shift with the peak at 552−553 nm (Figure 4A). The differential spectra corresponding to the fluorescence of bound TNP-ATP exhibited a maximum emission at 555 nm, indicating a 4 nm blue shift (data not shown). As a negative control GST protein was also checked independently for any TNP-ATP association, but no significant changes were observed in the differential spectra (data not shown). Figure 4B shows that the enhancement of TNP-ATP fluorescence at 555 nm (emission wavelength) depended on the probe concentration and allowed the determination of an apparent K_d for TNP-ATP of 2.0 μM. The TNP-ATP binding was efficiently competed with the addition of a molar excess of ATP as well as other NTPs (data not shown). Competition between ATP and its fluorescent analogue for the same binding site reconfirmed the specific interaction of the TNP-ATP with the protein. Also, competition with different NTPs confirmed broad substrate specificity of NBD-512.

The Cys193Ala Mutation Abolishes the ATPase Activity of NBD-512. Keeping in view the uncommon positioning

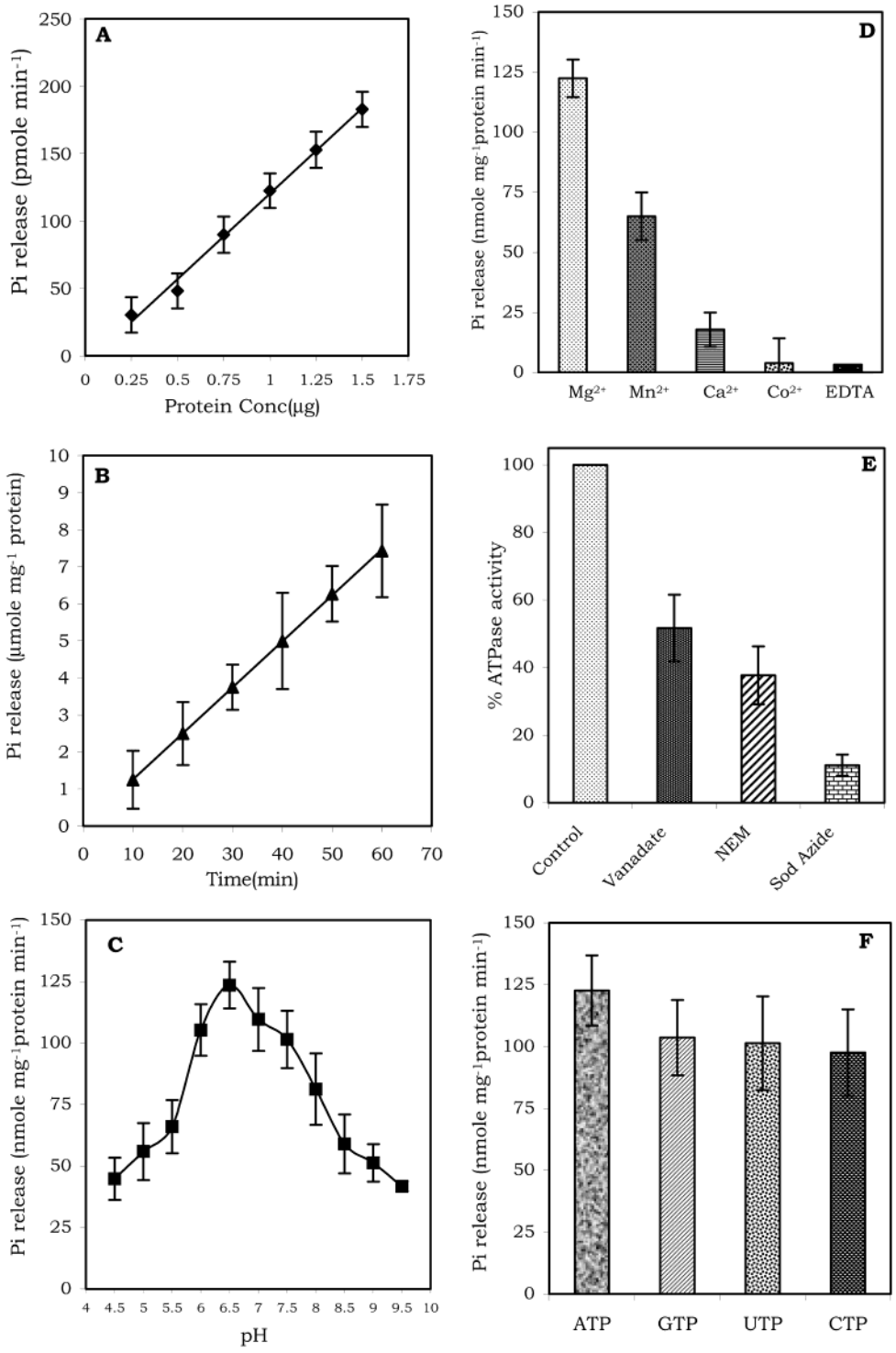

FIGURE 3: Biochemical characterization of the NBD-512-mediated ATPase activity. All of the experiments in this section have been performed in triplicate, and the values plotted (±SD) represent the average of three independent experiments. The assay conditions used for these experiments are similar to those described in Experimental Procedures except for the variations in biochemical parameters as follows. (A) ATPase activity of NBD-512 at increasing concentrations of purified NBD-512 protein. (B) ATPase activity of NBD-512 for varying lengths of time as indicated in the plotted graph. (C) pH profile of NBD-512 ATPase. The ATPase assay was performed in the pH ranges of 4.5−6.0 (created by using 60 mM MES) and 6.5−9.5 (created by using 60 mM Tris-HCl). (D) Divalent cation dependent ATPase activity of NBD-512. ATPase activity was assayed on addition of various metal chloride salts separately in independent reaction sets. (E) Effects of ATPase inhibitors. The ATPase activity of NBD-512 was measured in the presence of various inhibitors; e.g., sodium orthovanadate (0.1 mM) [it was prepared by boiling 50 mM solution in water for 3 min, and the concentration was determined by molar absorbance ($\lambda_{268nm} = 3600\ M^{-1}$) (*36*)], *N*-ethylmaleimide (0.3 mM) [chemical modification of the protein by NEM was done by incubating the protein (0.86 μM) for 10 min at 25 °C in 100 μL of reaction mixture containing ATPase assay buffer (*19*)], and sodium azide (5 mM) were added before initiation of the reaction. The bars represent the relative percentage decrease in the total ATPase calculated by taking the ATPase function activity in the absence of inhibitors as 100%. (F) Substrate specificity of NBD-512. The ATPase assay was performed in the presence of 5 mM each NTP used in independent sets of the assay mixture.

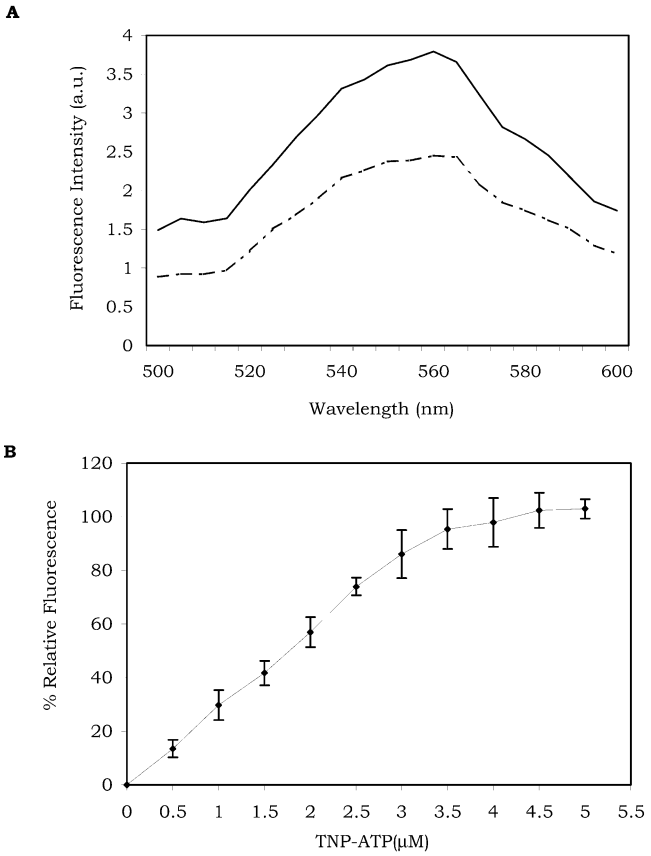

FIGURE 4: TNP-ATP binding with purified NBD-512 protein monitored by extrinsic fluorescence. Enhancement in fluorescence of the ATP analogue TNP-ATP was measured using a spectrofluorometer (Varian, Cary Eclipse). Samples containing 0.86 μM protein in ATPase buffer were incubated at 30 °C and excited at 408 nm (slit width 5 nm). The emission spectra were measured from 500 to 600 nm (slit width 5 nm). (A) Fluorescence emission spectra of TNP-ATP upon excitation at 408 nm. The extrinsic fluorescence of 5 μM TNP-ATP was measured both in the absence (dashed line) and in the presence (solid line) of 0.86 μM purified NBD-512 protein. The emission spectra were repeated several times. (B) Concentration-dependent binding of TNP-ATP. Increasing concentrations of TNP-ATP were incubated with 0.86 μM purified NBD-512 protein. Fluorescence was recorded at 555 nm. The graph shows the relative change in fluorescence calculated by subtracting the fluorescence of TNP-ATP used in the buffer from that observed with binding of TNP-ATP to NBD-512 protein. The experiment was done in triplicate, and the values (±SD) represent the average of three independent experiments.

of cysteine in Walker A (Figure 5) and the sensitivity of NBD-512 to NEM (Figure 3E), we performed site-directed mutagenesis of cysteine 193 to replace it with alanine. The mutated protein, designated as "Mut-NBD-512" (C193A), was overexpressed and purified as the wild-type NBD-512 (data not shown). The purified Mut-NBD-512 migrated as a single homogeneous band on SDS−PAGE with an expected M_r of 58 kDa (inset of Figure 6A). The CD spectra of Mut-NBD-512 confirmed it to be a structured protein, similar to the wild-type NBD-512 protein (data not shown). Interestingly, C193A mutation resulted in a 95% reduction in the ATPase activity of the wild-type protein (Figure 6A). Notably, this result also correlates with our observation that NEM treatment of NBD-512 impairs the ATPase activity (Figure 6B).

By exploiting TNP-ATP binding, we again attempted to examine whether the replacement of cysteine to alanine (C193A) in NBD-512 had any effect on ATP binding. We observed that the addition of the Mut-NBD-512 protein increased the TNP-ATP fluorescence to an extent that was similar to that of the wild-type protein (Figure 6C). Figure 6D shows that the enhancement of TNP-ATP fluorescence at 555 nm (emission wavelength) was dependent on the probe concentration with an apparent binding constant (K_d = 2.5 μM) similar to the that of the wild-type NBD-512 protein.

To check whether NEM influences TNP-ATP binding, the binding assays were performed in the presence of this inhibitor. It is evident from Figure 6E that preincubation of either wild-type NBD-512 or Mut-NBD-512 protein with NEM did not affect TNP-ATP binding, as their emission spectra did not show any significant change in fluorescence. This was further confirmed upon comparing their fluorescence at λ_{max} in the presence/absence of NEM (Figure 6F). This essentially suggests that NEM does not significantly influence the binding of the nucleotide to NBD-512 but rather affects the catalytic function, wherein cysteine in Walker A of NBD-512 (GRPGAGCS) appears to be an important residue.

DISCUSSION

The ABC-type drug transporter Cdr1p of the pathogenic yeast *C. albicans* is a large and complex membrane protein comprising 2 nucleotide binding domains (NBD) and 12 transmembrane helices (TMS). Due to its complex organization and highly hydrophobic nature, it has been difficult to understand the mechanism of energy transduction employed by it to efflux drugs.

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 the differential purification strategy adopted for both N- and C-terminal NBDs of mouse P-gp, with the former being soluble with a histidine tag and the latter needing a fusion with GST for attaining solubility (*20*, *21*). Also, Wang et al. have reported that the high-level expression of NBD1MLD (NBD1 of human P-gp/MDR1 with its linker region) in the 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 (*22*). The exact reasons for the low solubility of recombinant NBDs as well as their variable behavior during purification are not yet known, but certainly their purification from different transporters follows a tricky path. We also in our initial efforts tried to purify NBD-512 of Cdr1p as an independent moiety along with the histidine tag but found it to be constantly directed to the inclusion bodies, which also despite several alterations in growth conditions (e.g., temperature) and IPTG induction concentrations could not improve its expression in the soluble fraction (data not shown). However, when we expressed NBD-512 as a GST-tagged fusion protein, its expression, which constituted approximately 25% of the total cellular protein, resulted in moderate solubility and facilitated its purification. The use of thrombin and our two-step affinity chromatography

			Walker A	ABC signature	Walker B
A	*C. albicans*	Cdr1p(N)	187 GRPGAG**C**S---	303 VSGGERKRVSIAE--	322 IQCWDNATRGLD
	C. albicans	Cdr2p(N)	185 GRPGAG**C**S---	301 VSGGERKRVSIAE--	319 IQCWDNATRGLD
	C. albicans	Cdr3p(N)	178 GRPGAG**C**S---	294 ISGGERKRLSIAE---	313 IQCWDNSTRGLD
	C. albicans	Cdr4p(N)	190 GRPGAG**C**S---	306 VSGGERKRVSIAE--	325 VQCWDNSTRGLD
	S.cerevisiae	Pdr5p(N)	193 GRPGSG**C**T---	309 VSGGERKRVSIAE--	329 FQCWDNATRGLD
	S.cerevisiae	Snq2p(N)	192 GRPGAG**C**S---	309 VSGGERKRVSIAE--	329 IYCWDNATRGLD
	C. galbrata	Phd1p(N)	186 GRPGSG**C**T---	308 VSGGERKRVSIAE--	328 FQCWDNATRGLD
	C. neoformans	CnAfr1p(N)	258 GRPGAG**C**T---	373 VSGGERKRVSIAE--	393 VCSWDNSTRGLD
	A. nidulans	AtrBp(N)	136 GRPGSG**C**T---	151 VSGGERKRVSIIE---	171 VFCWDNSTRGLD
	A. fumigatus	AtrFp(N)	229 GRPGAG**C**S---	338 VSGGERKRVSIAE--	358 VVCWDNSTRGLD
B	*C. albicans*	Cdr1p(N)	187 GRPGAG**C**S---	303 VSGGERKRVSIAE---	322 IQCWDNATRGLD
	H. sapiens	Pgp(N)	427 GNSGCG**K**S---	531 LSGGQRIAIARAL----	551 ILLLDEATSALD
	S.cerevisiae	Ste6p(N)	392 GKSGSG**K**S---	506 LSGGQQQRVAIAR--	526 ILFLDEAVSALD
	H. sapiens	CFTR(N)	458 GSTGAG**K**T---	548 LSGGQRARISLAR---	568 LYLLDSPFGYLD
	H. sapiens	TAP1(N)	538 GPNGSG**K**S---	543 LSGGQRQAVALAR--	663 VLILDDATSALD
	H. sapiens	TAP2(N)	503 GPNGSG**K**S---	607 LAAGQKQRLALAR--	627 VLILDEATSALD
	E.coli	HlyB(N)	502 GRSGSG**K**S---	606 LSGGQRQRIAIAR---	626 LLIFDEATSALD
	S.typhimurium	HisP(N)	39 GSSGSG**K**S---	154 LSGGQQQRVSIAR--	174 VLLFDEPTSALD
	A. radiobacter	LacK(N)	36 GPSGCG**K**S---	134 LSGGQRQRVAIGR--	154 VFLFDEPLSNLD
	S.typhimurium	MalK(N)	36 GPSGCG**K**S---	134 LSGGQRQRVAIGR--	154 VFLLDEPLSNLD
C	*C. albicans*	Cdr1p(N)	187 GRPGAG**C**S---	303 VSGGERKRVSIA---	322 IQCWDNATRGLD
	C. albicans	Cdr1p(C)	890 GASGAG**K**T---	999 LNVEQRKRLTIGV--	1019 LLLFLDEPTSGLD
	C. albicans	Cdr2p(C)	893 GASGAG**K**T---	1001LNVEQRKRLTIGV--	1021 LLLFLDEPTSGLD
	C. albicans	Cdr3p(C)	876 GASGAG**K**T---	982 LNVEQRKRLTIAV--	1002 LLVFLDEPTSGLD
	C. albicans	Cdr4p(C)	882 GASGAG**K**T---	988 LNVEQRKRLSIGV--	1008 LLVFLDEPTSGLD
	S.cerevisiae	Pdr5p(C)	902 GASGAG**K**T---	1010 LNVEQRKRLTIGV--	1031 LLVFLDEPTSGLD
	S.cerevisiae	Snq2p(C)	886 GESGAG**K**T---	993 LNVEQRKKLSIGV--	1013 LLLFLDEPTSGLD
	C. galbrata	Phd1p(C)	918 GASGAG**K**T---	1026 LNVEQRKRLTIGV--	1046 LLVFLDEPTSGLD
	C. neoformans	CnAfr1p(C)	951 GASGAG**K**T---	1059 LSVEARKVTIGVE--	1080 LLLFLDEPTSGLD
	A. nidulans	AtrBp(C)	828 GSSGAG**K**T---	936 LSVEQRKRVTIGVE-	956 ILIFLDEPTSGLD
	A. fumigatus	AtrFp(C)	925 GASGAG**K**T---	1029 LNVEQRKKLSIGV--	1049 LLLFLDEPTSGLD

FIGURE 5: Sequence alignment of conserved Walker A and B motifs and ABC signature of NBDs of ABC transporters from different organisms. The amino acid sequences have been aligned to generate three columns of sequence conservations representing the Walker A and B motifs and ABC signature, respectively. N and C indicated in parentheses after each protein depict 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 the cysteine residue (in bold) in Walker A among N-terminal NBDs of fungal ABC transporters. (B) Comparison of the Cdr1p N-terminal NBD with other nonfungal ABC transporters where the conserved lysine of Walker A is highlighted in bold. (C) Amino acid sequence alignment of the C-terminal NBD of Cdr1p with other fungal ABC-type transporters highlighting (in bold) the conservation of the lysine residue in Walker A.

strategy helped us to study NBD-512 in isolation from GST and hence ruled out the disadvantages of using a fusion protein while drawing conclusions from functional characterization assays.

With the availability of highly purified preparations of NBD-512, it was possible to investigate the question, whether this domain alone was enzymatically active as an ATPase. Several possibilities exist in this context which include the following: Can NBD-512 alone act as a functional ATPase? Does NBD-512 function as only a "regulatory/cooperative" nucleotide binding site, and not as a hydrolysis site, and is a physical interaction of NBD1 and NBD2 required for ATP hydrolysis (in such a case no enzymatic activity may be detected)? Our results clearly demonstrate that the purified NBD-512 was not only able to hydrolyze ATP but other nucleotides such as CTP, UTP, and GTP. Thus NBD-512 elicits a general ribonucleoside triphosphatase activity. The observed broad specificity of NBD-512 with nucleotides matches well with the ATPase activity of full Cdr1p protein (*17*, *18*, *23*). It is not uncommon for human ABC proteins such as CFTR and P-gp/MDR1 to have wider specificity toward ribonucleotides. The broad nucleotide specificity has also been reported for yeast ABC proteins such as Pdr5p of *S. cerevisiae* (*24*) and CgCdr1p of *Candida glabrata* (*18*). Taken together, this is the first report to provide direct evidence that NBD of a yeast drug transporter Cdr1p can alone function as an active ATPase. Of note, the ATPase activity of NBD-512 was 2−3-fold less than that observed with overexpressed Cdr1p in insect/yeast cells (*18*, *23*). This observation is commonly observed with purified NBDs from human P-gp/MDR1 (*20*, *21*, *25*), CFTR (*26*, *27*), and MRP1 (*11*, *19*, *28*−*30*). Nonetheless, we observed the basic properties of NBD-512, such as cation dependence, pH, and sensitivity to NEM and to other ATPase inhibitors, to be similar to that reported for the native Cdr1p (*18*, *23*). It should be pointed out that drugs could not stimulate NBD-512 ATPase activity (data not shown). In fact, to date the direct interaction of drugs with isolated NBDs has not yet been established (*19*).

The available structural data suggest that the invariant lysine residue within the Walker A motif of NBDs of ABC transporters is an important residue for binding to the β- and γ-phosphates of the nucleotides (*11*). Detailed mutational analysis performed with numerous ABC transporters confirms that any change of lysine within Walker A is generally not tolerated by the protein. For example, studies with N-terminal NBDs of CFTR and human P-gp/MDR1 protein confirmed that replacement of lysine of the Walker A motif with histidine/leucine (K464H, K464L) and methionine (K433M), respectively, abrogated ATP hydrolysis (*10*, *26*). In another study with MalK protein (the ATP-hydrolyzing component of the ABC transporter for maltose) from *Salmonella typhimurium*, the substitution of the invariant lysine by arginine also led to a reduced hydrolysis of ATP (*31*). Keeping in view the significance of lysine in ATP hydrolysis and its absence from the Walker A motif of NBD-512, we site directly mutagenized the uncommon cysteine residue to alanine (C193A). The purified Mut-NBD-512

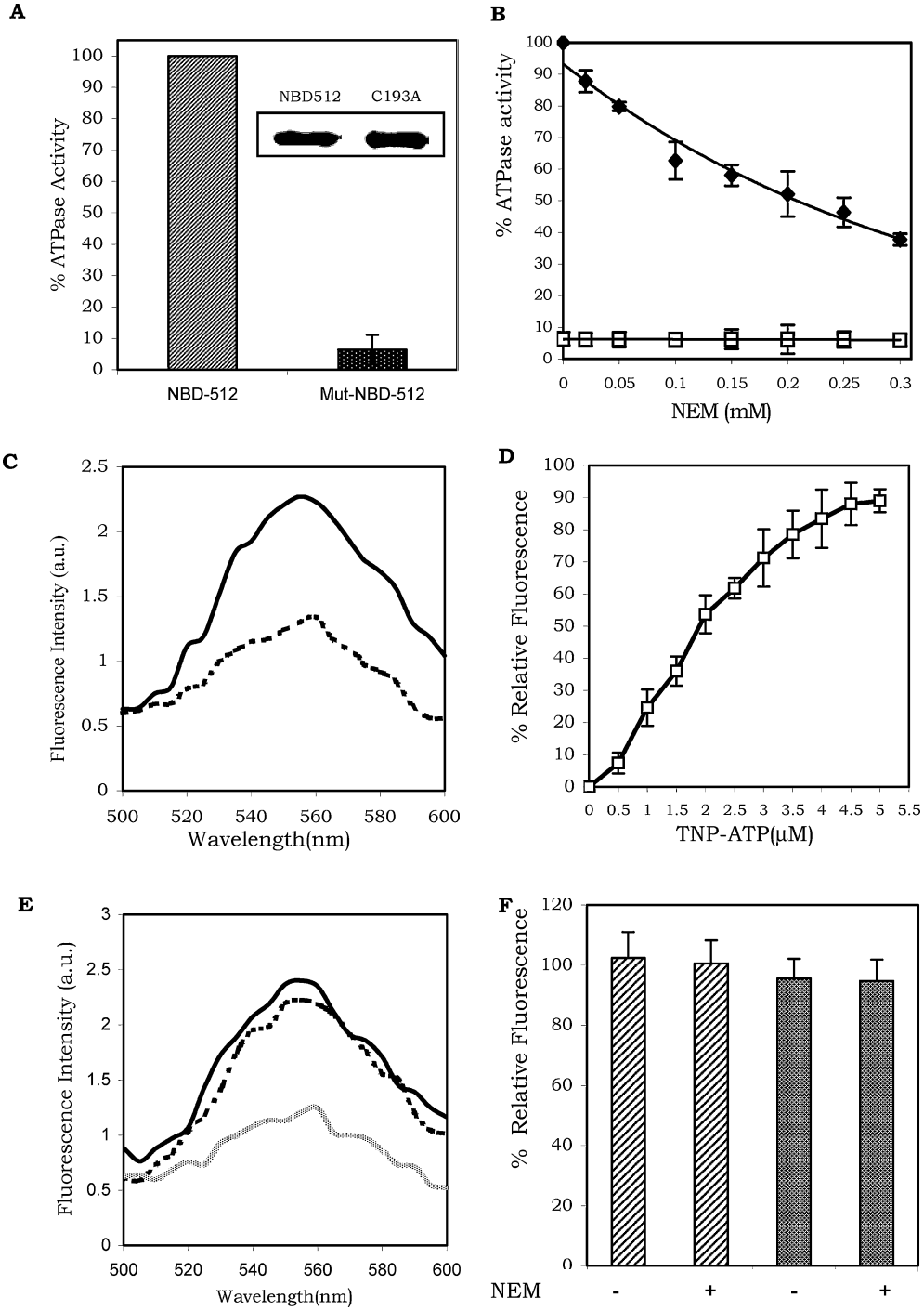

FIGURE 6: (A) Characterization of Mut-NBD-512. Comparison of the ATPase activity of NBD-512 and Mut-NBD-512. The ATPase assay was performed in the presence of an equal amount of either purified NBD-512 or purified Mut-NBD-512 proteins in ATPase assay buffer as described in Experimental Procedures. Both of the assays were carried out in triplicate, and the values (±SD) represent the average of three independent experiments. The inset shows the SDS−PAGE profile of the purified wild-type (NBD-512) and mutant (Mut-NBD-512) proteins. (B) NEM sensitivity versus ATPase activity of NBD-512 and Mut-NBD-512. The ATPase assay with different concentrations of NEM as indicated in the plotted graph was carried out in the presence of an equal amount of either purified NBD-512 (◆) or Mut-NBD-512 (□). (C) Fluorescence emission spectra of TNP-ATP upon excitation at 408 nm. The extrinsic fluorescence of 5 μM TNP-ATP was measured both in the absence (dashed line) and in the presence (solid line) of 0.86 μM purified Mut-NBD-512 protein. The emission spectra were repeated several times. (D) Concentration-dependent binding of TNP-ATP. Increasing concentrations of TNP-ATP were incubated with 0.86 μM purified Mut-NBD-512 protein. Fluorescence was recorded at 555 nm. The graph shows the change in relative fluorescence calculated by subtracting the fluorescence of TNP-ATP used in buffer from that observed with TNP-ATP bound to the NBD-512 protein. The experiment was done in triplicate, and the values (±SD) represent the average of three independent experiments. (E) Fluorescence emission spectra of TNP-ATP in the presence of NEM. The extrinsic fluorescence of 5 μM TNP-ATP was measured in the presence of 0.3 mM NEM without any protein (dotted line) or with an equal amount of NBD-512 protein (solid line) or Mut-NBD-512 protein (dashed line). (F) Binding of TNP-ATP to NBD-512 and Mut-NBD-512 in the presence of NEM. TNP-ATP (5 μM) binding was performed in the presence of NEM as mentioned in panel E. Slashed and solid bars represent TNP-ATP binding assays in the presence of NBD-512 and Mut-NBD-512, respectively. All of the reactions were carried out in triplicate, and the histogram (±SD) represents average of three independent experiments.

protein (C193A) showed a complete loss of ATPase activity (~95%). Our further analyses with the fluorescent derivative of ATP (TNP-ATP) revealed that the C193A mutation did not affect the TNP-ATP binding kinetics of the wild-type protein. Interestingly, the replacement of cysteine 193 with the commonly conserved lysine (C193K) resulted in an unstable protein due to unexplained reasons and thus could not be purified.

In conclusion, our study provides the first direct evidence that the predicted N-terminal NBD of Cdr1p can bind as well as hydrolyze ribonucleotides. Further, the mutational analysis within the Walker A region reveals the importance of an uncommon cysteine in ATP hydrolysis mediated by the N-terminal NBD of Cdr1p. How a cysteine residue can mimic the function of lysine remains an open question and needs to be investegated in the near future. Considering the fact that the C-terminal NBD of Cdr1p contains the usual lysine within its Walker A motif (GASGAGKT) and lacks a typical ABC signature sequence (a fact almost universal to all fungal ABC transporters), the demonstration of independent ATPase activity by the N-terminal NBD in this study is an interesting observation. However, the significance of both the catalytic sites (NBDs) of Cdr1p in catalysis and transduction of energy for drug efflux remains an exciting area to explore, wherein investigations related to the interaction of both the catalytic sites are an integral and necessary facet of ATP hydrolysis needing to be resolved.

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