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Partial characterization of genes encoding the ATP-binding cassette proteins of *Cryptosporidium parvum*

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ABSTRACT. The present study aims to explore the possible mechanisms underlying the multidrug resistance characteristic of *Cryptosporidium parvum* by detecting the presence of ATP-binding cassette (ABC) protein encoding genes, especially one that shows high similarity to members belonging to the multidrug resistance protein (MDR) and multidrug resistance associated protein (MRP) subfamilies. PCR using ABC-specific degenerate primers successfully amplified two unique fragments, designated Cpnbd1 and Cpnbd2, from *C. parvum* genomic DNA. Cpnbd1 exhibited high degree of homology (99-100%) with the nucleotide-binding domains (NBDs) at the NH_2-terminal halves of two previously reported ABC proteins (CpABC and CpABC1) of human and bovine origin *C. parvum* isolates. It is likely that CpABC, CpABC1 and Cpnbd1 were encoded by homologous genes of a type of ABC transporter protein found in different *C. parvum* isolates. However, Cpnbd2 showed moderate levels of similarities (28-49%) to the NBDs of four ABC proteins characterised in *C. parvum* to date. Therefore, Cpnbd2 could be a novel member of an ABC superfamily of proteins in *C. parvum*. Phylogenetic analyses on a list of ABC transporters known to associate with MDR phenotype has significantly linked Cpnbd1 and Cpnbd2 to these transporters, thus suggesting that Cpnbd1 and Cpnbd2 proteins may contribute to the intrinsic multidrug resistance phenotype of *C. parvum*.

INTRODUCTION

Cryptosporidium parvum is an intestinal parasitic protozoa which causes self-limited diarrhoea in immunocompetent individuals and devastating and fulminant diarrhoea in immunocompromised patients (Griffiths, 1998). Despite extensive effort in searching for chemotherapeutic agents against cryptosporidiosis, it remains elusive. The major impediment in the use of a promising chemotherapeutic compound is the intrinsic multidrug resistance exhibited by *C. parvum* towards an array of drugs that is generally effective against similar organisms (Chen *et al.*, 2002). It is postulated that the intrinsic multidrug resistance of *C. parvum* is mediated by a general transporting mechanism, possibly by the ATP-binding cassette (ABC) superfamily of transporters.

The ABC superfamily of transporters, which consists of more than 1,000 members, is the largest and most widespread protein family known to date. The vast majority of its members are ATP driven transporters, which is responsible of translocating a wide variety of compounds across biological membranes (Higgins, 1992; Ambudkar *et al.*, 1999). Most ABC transporters share a similar architecture: a fundamental structure made up of four domains. Two of these domains are the hydrophobic membrane-spanning domains (MSDs), and two other domains are the hydrophilic nucleotide-binding domains (NBDs). NBDs are evolutionally conserved domains, which have considerable sequence homologies across the entire family, and the homologies range from 30% to 50%. Two NBDs within a transporter show higher similarity to each other than to the

respective domains from other member of transporter (Higgins, 1992; Ambudkar *et al.*, 1999).

In addition to their physiological functions, ABC proteins have enormous medical relevance. Members of the superfamily, the multidrug resistance protein (MDR) (Juliano & Ling, 1976) and multidrug resistance associated protein (MRP) (Cole & Deeley, 1993), are almost always associated with the multidrug resistance phenomenon exhibited by many intrinsic or acquired drug resistance cancer cell lines or human pathogens (Davidson, 2002). Transfection studies and investigation on the drug sensitive partial revertant cell lines clearly elaborated the correlation between MDR, MRP and multidrug resistance phenotypes (Gao *et al.*, 1998).

The present study aimed to explore and characterise the possible mechanisms underlying the MDR characteristic of *C. parvum* by detecting the presence of ABC transporter protein encoding genes, especially one that shows high similarity to members belonging to the MDR and MRP subfamilies of transporters.

MATERIALS AND METHODS

C. parvum genomic DNA extraction

C. parvum oocysts (IOWA isolate, bovine genotype) used in this study were purchased from Waterborne Inc, USA. Genomic DNA was extracted from the oocysts by using the QIAamp DNA blood mini kit (QIAGEN, Germany).

Degenerate primers for PCR amplification of ABC transporter encoding genes

PCR approach using degenerate primers, was used to identify ABC transporter encoding genes in *C. parvum*. The sense and antisense primers corresponding to peptide GCGKST(L/I)(I/L) and (G/A)(V/S)KLSGGQ were selected based on the analysis of the highly conserved NBDs of several ABC transporter proteins (Dallagiovanna *et al.*, 1994). The degeneracy of the primers was adjusted to account for the codon bias found for *C. parvum* genes.

PCR amplification of ABC transporter encoding genes

The amplification of *C. parvum* ABC transporter protein genes involved two rounds of PCR, namely the primary and secondary amplifications. In primary amplification, degenerate primers were incubated with *C. parvum* genomic DNA, according to standard protocol, in a volume of 20µl. The optimum PCR annealing temperature was 55°C. The secondary PCR amplification condition was identical to the first round, except that 1 µl of 50X diluted primary PCR products was used as DNA template.

PCR product purification and cloning

The QIAquick gel extraction kit (QIAGEN, Germany) was used to extract DNA bands from agarose gels. Purified PCR products were cloned into pGEM®-T vector (Promega, USA), and transformed into *Escherichia coli* JM109 strain (Promega, USA).

Sequencing analyses

The resultant positive recombinant clones were sequenced in both directions on an ABI PRISM® 377 DNA sequencer (BST Techlab, Singapore). Sequencing was primed using T7 forward and SP6 reverse promoter primers encoded on the pGEM®-T.

Nucleotide and amino acid sequences were compared with the sequences deposited in National Center for Biotechnology Information (NCBI, National Institutes of Health, USA) databases via the BLAST server. Multiple alignments of DNA and protein sequences were performed using the ClustalW programme. Protein similarities were calculated using the GeneDoc sequence editor. Conservative amino acids are grouped as: (D,E,N,Q,H) (S,A,T) (K,R) (F,Y) (L,I,V,M). The aligned protein sequences were analysed by distance based neighbour-joining method, using the Njplot

programme. Bootstrap replicates of 1000 were set to assess the reliability of the tree. An unrooted tree was also constructed by using the Njplot.

RESULTS AND DISCUSSION

PCR using ABC-specific degenerate primers successfully amplified two unique fragments from *C. parvum* genomic DNA. Sequencing of the fragments revealed two partial open reading frames (ORFs). ORF1 was 279 base pairs in length, which encoded a peptide of 93 amino acids. ORF2 was 303 base pairs in length, and encoded a 101-amino acid peptide. Comparisons of ORF1 and ORF2 to the protein sequences in the NCBI databases revealed that both fragments shared high similarities with NBDs of a variety of ABC transporters. Thus, ORF1 and ORF2 were respectively designated *Cpnbd1* and *Cpnbd2*. For protein sequences, they were designated as *Cpnbd1* and *Cpnbd2* [Figures 1(a) and (b)].

Comparisons of Cpnbd1 and Cpnbd2 with the NBDs of *C. parvum* ABC proteins

At the amino acids level, Cpnbd1 was 100% identical to the NH_2-terminal half (_N) of CpABC1, an ABC protein characterised in *C. parvum* KSU-1 isolate (bovine genotype) (Zapata *et al.*, 2002). At the nucleotide level, both gene fragments showed 99% identity. The difference was due to a single nucleotide substitution at the third position of codon-33, which was a silent substitution, as both CAG^{99} in *Cpnbd1* and CAA^{99} in *CpABC1*_N codes for the amino acid glutamine.

Cpnbd1 also showed high similarity to the NBD at the NH_2-terminal of CpABC, the gene encoding the ABC protein of *C. parvum* SFGH1, an isolate of human genotype (Perkin *et al.*, 1999). Both proteins were 97% and 98% identical at amino acid and nucleotide levels, respectively, and the differences were attributed to four nucleotide substitutions. Two of the nucleotide substitutions resulted in silent changes: CAG^{99} at codon-33 of *Cpnbd1* and CAA^{99} of *CpABC*_N, which codes for glutamine, and GAT^{174} at codon-58 of *Cpnbd1* and GAC^{174} of *CpABC*_N, which codes for aspartic acid. Two other nucleotide substitutions, which occurred at the first position of the corresponding codons, resulted in amino acid changes. These were the ^{64}ATT at codon-22 of *Cpnbd1*, which codes for isoleucine, and the corresponding codon ^{64}GGT at *CpABC*_N, which codes for valine. Whereas at codon-42, ^{124}GTT of *Cpnbd1* coding for valine while ^{124}ATT of *CpABC*_N coding for isoleucine. The high degree of similarities between Cpnbd1, CpABC and CpABC1 implied that they could be encoded by homologous genes of a type of ABC transporter protein found in different *C. parvum* isolates.

As for Cpnbd2, it showed moderate similarities (28 – 49 %) to the NBDs at both NH_2- and COOH-terminal halves of ABC proteins characterised in *C. parvum* to date (Table 1). Therefore, Cpnbd2 could be a novel member of an ABC superfamily of proteins in *C. parvum.*

Comparisons of Cpnbd1 and Cpnbd2 with the NBDs of ABC proteins of some drug resistance cells

Multiple alignment (Figures 2 and 3) and phylogenetic (Figure 4) analyses on a list of ABC transporters known to associate with MDR phenotype (EhPGP 5: *Entamoeba histolytica* P-gp-like transporters 5; EhPGP 6: *E. histolytica* P-gp-like transporters 6; HuABCB1: *Homo sapien* ATP-binding cassette B1; HuABCC1: *H. sapien* ATP-binding cassette C1; HuABCC2: *H. sapien* ATP-binding cassette C2; LaMDR1: *Leishmania amazonensis* multidrug resistance protein 1; Ldmdr1: *L. donovan*i Multidrug resistance protein 1; LeMDR1: *L. enrietti* multidrug resistance protein-like 1; TbMRPA: *Trypanosoma brucei* multidrug resistance protein A; and TbMRPE: *T. brucei* multidrug resistance protein E) has strongly linked Cpnbd1 to HuABCC1 and HuABCC2.

HuABCC1 is a human MRP transporter identified in the H69AR doxorubicin-selected human lung carcinoma cell line (Mirski *et al.*, 1987). In normal tissue, HuABCC1 is found ubiquitously expressed at low levels. Certain normal tissues, however, do highly express HuABCC1 at cellular levels. In human cancer cells, HuABCC1 has been found overexpressed in various cancer types. In certain cancer types, HuABCC1 expression is an important predictor of treatment outcome. The expression of HuABCC1 is usually highest in tumours that derived from tissues that normally express the transporter protein, and tumours arising from these tissues are known to be intrinsically resistant against chemo-therapeutic treatment (Cole *et al.*, 1994; Hipfner *et al.*, 1999).

HuABCC2 is the second member identified in the MRP subfamily of transporter in human. Taniguchi *et al.* (1996) successfully isolated the HuABCC2 encoding gene from a cisplatin-resistant human cancer cell line. The research group also reported good correlation between cisplatin resistance and the up-regulation expression of HuABCC2. HuABCC2 has been detected in the liver, kidneys, intestine, placenta and the brain at normal level. HuABCC2 is believed to play an important role in eliminating endogenous toxic compounds and xenobiotics from the body. In hepatocytes, the expression of HuABCC2 is markedly increased by various chemical carcinogen and chemopreventive agents (Payen *et al.*, 2002). Meanwhile, intestinal HuABCC2 may play a role in reducing the level of 2-amino-1-methyl-6-phenylimidazo [4,5-6] pyridine; the most abundant food derived carcinogen, which is formed during cooking, frying and grilling of meat (Dietrich *et al.*, 2001).

The relationship of Cpnbd2 with the same group of transporters was less distinct. It was clustered in a clade which consisted of the NBDs of various ABC transporter proteins (Figure 4). The similarities of Cpnbd2 to these NBDs were generally moderate (Table 1). Interestingly,

Table 1. Quantitative analysis of amino acid similarities between Cpnbd1, Cpnbd2 and the NBD at the NH_2-terminal (_N) half and COOH-terminal (_C) half of the ABC proteins of several drug resistant parasitic protozoan and *H. sapiens*

ABC protein	Similarity (%)	
	Cpnbd1	Cpnbd2
CpABC_N	100	30
CpABC_C	46	43
CpABC1_N	100	30
CpABC1_C	46	46
CpABC2_N	58	28
CpABC2_C	40	49
CpABC3_N	37	36
CpABC3_C	41	35
EhPGP5_N	43	36
EhPGP5_C	37	36
EhPGP6_N	30	37
EhPGP6_C	40	32
HuABCA5_N	Nil	65
HuABCB1_N	35	39
HuABCB1_C	36	33
HuABCC1_N	68	32
HuABCC1_C	36	41
HuABCC2_N	68	32
HuABCC2_C	39	34
LaMDR1_N	37	36
LaMDR1_C	41	30
Ldmdr1_N	37	36
Ldmdr1_C	42	30
LeMDR1_N	36	35
LeMDR1_C	40	31
TbMRPA_N	59	39
TbMRPA_C	36	37
TbMRPE_N	60	38
TbMRPE_C	39	39
TcABCA1_N	Nil	54

(a) *Cpnbd1*/Cpnbd1

```
ggatgtggtaaatcaacattgattgaacttatattacaagaacttaaaccaagattaggaacaattcaatca
 G  C  G  K  S  T  L  I  E  L  I  L  Q  E  L  K  P  R  L  G  T  I  Q  S
aatggttcagttttttattgttcacagtcatcatggattattaatggtacagttagaagtaatattatactt
 N  G  S  V  F  Y  C  S  Q  S  S  W  I  I  N  G  T  V  R  S  N  I  I  L
gatttacctttgatcaagcctggtatgatattgttattaatgcttgttcattagtttatgatttaaaagct
 D  L  P  F  D  Q  A  W  Y  D  I  V  I  N  A  C  S  L  V  Y  D  L  K  A
atgccaaatggggatttaacagaaattggtgaaaatggtgttaaactttctggaggtcaa
 M  P  N  G  D  L  T  E  I  G  E  N  G  V  K  L  S  G  G  Q
```

(b) *Cpnbd2*/Cpnbd2

```
ggatgtggtaaatctacacttataaagcaaatgattggttttattaagcctgatcagggagatgttcactac
 G  C  G  K  S  T  L  I  K  Q  M  I  G  F  I  K  P  D  Q  G  D  V  H  Y
ggagatatttcttttatacataaccgtaaacaagctcgaacaatgatgagttatatgtcacaacagtatgca
 G  D  I  S  F  I  H  N  R  K  Q  A  R  T  M  M  S  Y  M  S  Q  Q  Y  A
cctcttgaaaagttaactgttgagcaaaatttagagatgattggaagaatgagagggttaagtacctcagaa
 P  L  E  K  L  T  V  E  Q  N  L  E  M  I  G  R  M  R  G  L  S  T  S  E
ctccaaaaagaaattgagcaccttctagcagatctggaaatagtagaataccgtgataaaaaaggaagtaaa
 L  Q  K  E  I  E  H  L  L  A  D  L  E  I  V  E  Y  R  D  K  K  G  S  K
ctttcaggaggacaa
 L  S  G  G  Q
```

Figure 1. Nucleotide and deduced amino acid sequences of (a) *Cpnbd1*/Cpnbd1, and (b) *Cpnbd2*/Cpnbd2. Nucleotide sequences are indicated by small letters, whereas the deduced amino acid sequences are indicated by capital letters and are represented by single-letter codes.

```
                  *   10    *   20    *   30    *   40    *   50
Cpnbd1     : GCGKSTLIELILQELKPRLGTIQS-------------NGSVFYCSQSSWIING  :  40
CpABC_N    : GSGKTTLIELILQELKPRLGTVQS-------------NGSVFYCSQSSWIING  :  40
CpABC1_N   : GSGKTTLIELILQELKPRLGTIQS-------------NGSVFYCSQSSWIING  :  40
CpABC2_N   : GCGKSSLIKAILNEIRPSASNIYVR---------PRETNSILSYSPQLPWIPSG  :  45
CpABC3_N   : GCGKSSILKLILRLYDPDKGKILLDGLNIKKYDLAFLREQITIVDQESKLFND  :  53
HuABCC1_N  : GCGKSSLLSALLAEMDKVEGHVAI-------------KGSVAYVPQQAWIQND  :  40
HuABCC2_N  : GSGKSSLISAMLGEMENVHGHITI-------------KGTTAYVPQQSWIQNG  :  40

                *   60    *   70    *   80    *   90    *  100    *
Cpnbd1     : TVRSNIILDLP-FDQAWYDIVINACSLVYDLKAMPNGDLTEIGENGVKLSGGQ  :  92
CpABC_N    : TIRSNIILDLP-FDQAWYDIVINACSLVYDLKAMPNGDLTEIGENGINLSGGQ  :  92
CpABC1_N   : TVRSNIILDLP-FDQAWYDIVINACSLVYDLKAMPNGDLTEIGENGINLSGGQ  :  92
CpABC2_N   : SVRSAILLGRE-WNREKYELIVDCCQLKEDFSSWQRGDLRVVDEGGCSLSGGQ  :  97
CpABC3_N   : TIRQNILYGNENATEKEIENALRLSQAWDFVNSFRDGLETLVGNEGGLLSGGQ  : 106
HuABCC1_N  : SLRENILFGCQ-LEEPYYRSVIQACALLPDLEILPSGDRTEIGEKGVNLSGGQ  :  92
HuABCC2_N  : TIKDNILFGTE-FNEKRYQQVLEACALLPDLEMLPGGDLAEIGEKGINLSGGQ  :  92
```

Figure 2. Comparison of the amino acid sequences of Cpnbd1 and the NBDs of ABC proteins from *C. parvum* and *H. sapiens*. Similar amino acids shared by all, 70% and 50% of the aligned sequences, are boxed by black, dark grey and light grey, respectively.

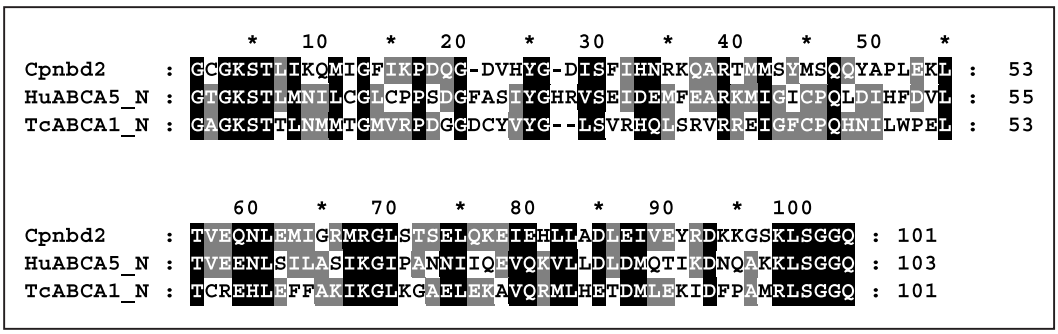

Figure 3. Comparison of the amino acid sequences of Cpnbd2 and the NBDs of ABC proteins from *C. parvum*, *H. sapiens* and *T. cruzi*. Similar amino acids shared by all and two of the aligned sequences, are boxed by black and dark grey, respectively.

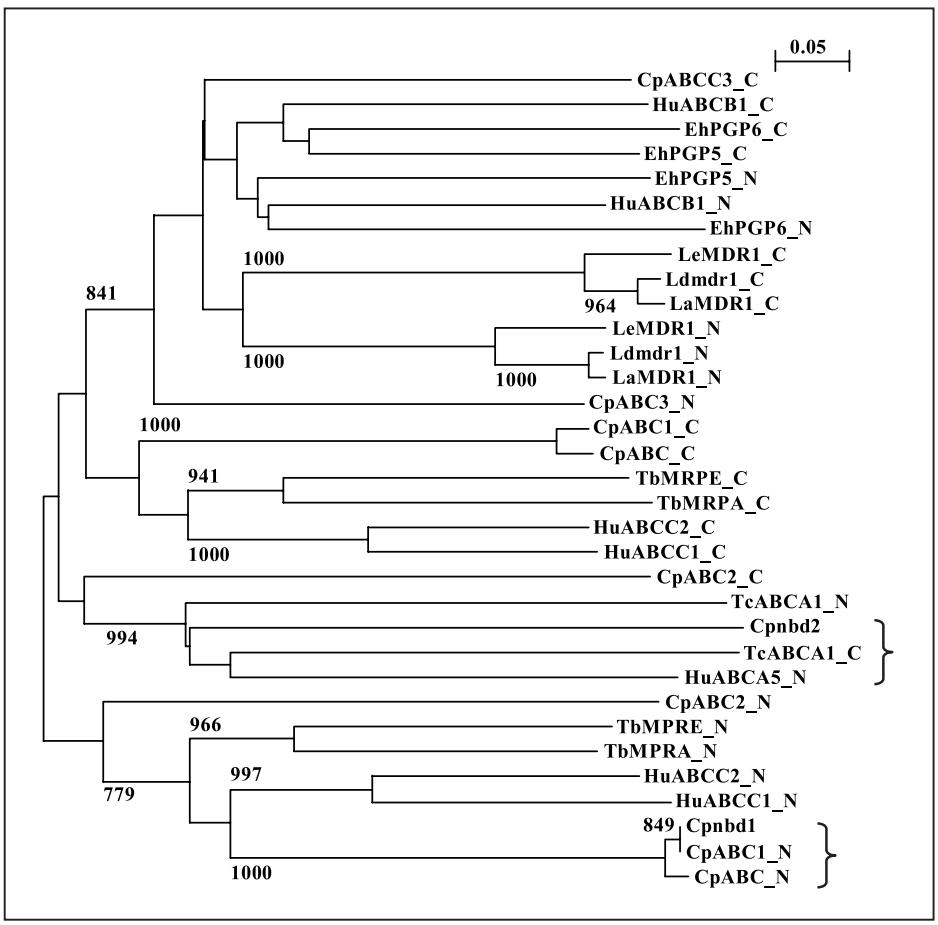

Figure 4. Phylogenetic relationship of NBDs among various ABC superfamily of proteins. Cpnbd1 is closely related to CpABC_N and CpABC1_N, the ABC proteins of *C. parvum* KSU-1 isolate and *C. parvum* SFGH1 isolate, respectively. Cpnbd2, however, is moderately related to the *T. cruzi* (TcABC1_C) and human (HuABCA5_N) ABC proteins.

Cpnbd2 did show significant high similarity to the NBDs at the NH_2-terminal halves of two ABC proteins [the *H. sapien* ATP-binding cassette A5 (HuABCA5) and *T. cruzi* ABCA1 transporter (TcABCA1)]. It was 65% similar to HuABCA5_N and 59% similar to TcABCA1_N. However, neither the substrate nor the function of HuABCA5 and TcABCA1 is known (Dean *et al.*, 2001). The possible function of Cpnbd2, therefore, cannot be inferred from the phylogenetic analysis.

The study of ABC protein family in *C. parvum* is considered at its infancy as no functional study has thus far been conducted on any of the characterised ABC proteins of the parasite. In intracellular stages, CpABC1 is located at the host-parasite boundary of mature meronts. The location of CpABC1 suggests that it could be a component of one or more of the membranes (the meront plasma membrane, the parasitophorous vacuole membrane, or the feeder organelle) at the host-parasite boundary (Zapata *et al.*, 2002). Similar observation was reported by Perkin *et al.* (1999) on CpABC. The peculiar location of CpABC and CpABC1, suggests that these proteins may play a role in metabolic interaction between the parasite and the infected host. The localisation of both proteins also correlates well and supports a role as drug efflux pumps that transport endogenous and xenobiotics away from the parasite.

Since some genes of the P-gp and MRP subfamilies of transporters have been associated with MDR phenomenon in cancer patients and certain drug resistance pathogens, the presence of such homologous protein in *C. parvum* may also attribute to the intrinsic MDR phenotype of the parasite.

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