Differential expression and interaction of transcription co-activator MBF1 with TATA-binding protein (TBP) in the apicomplexan Cryptosporidium parvum

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All gene-specific transcriptional activators initiate gene transcriptions by binding to promoter sequences and recruiting general transcription factors including TATA-binding protein (TBP) to upstream of targeted genes. Some of them require multiprotein bridging factors (MBFs); for example, the type 1 MBF (MBF1) which interconnects the gene activator with TBP. In this study, the properties of a previously cloned type 1 multiprotein bridging factor (CpMBF1) and a newly identified TBP (CpTBP1) from the apicomplexan Cryptosporidium parvum were investigated. Genes encoding both proteins were differentially expressed as determined by semi-quantitative RT-PCRs during the parasite life cycle, but in different patterns. The highest level of expression of CpMBF1 was in the well-developed intracellular parasites, whereas that of CpTBP1 was found in intact oocysts and late intracellular stages, possibly correlated with the formation of oocysts. Both CpMBF1 and CpTBP1 were expressed as maltose-binding protein fusion proteins. The function of CpTBP1 was confirmed by its ability to bind a biotinylated DNA oligonucleotide containing TATA consensus sequence. The interaction between CpMBF1 and CpTBP1 was also observed by an electrophoretic mobility shift assay. Since little is known about the regulation and control of gene activity in Cryptosporidium parvum, this study may point to a new direction for the study of gene activation associated with the development of the complex life cycle of this parasite.

INTRODUCTION

Cryptosporidium parvum has been globally recognized as an important intracellular pathogen of humans and animals that can cause self-limiting diarrhoea in immunocompetent individuals, or prolonged, life-threatening opportunistic infections in immunocompromised patients with no effective treatment available to date (Okhuysen & Chappell, 2002; Tzipori & Widmer, 2000). This unicellular parasite belongs to the phylum Apicomplexa, which contains many pathogens of medical and veterinary importance (e.g. Plasmodium, Babesia, Toxoplasma, Eimeria and Cyclospora). Like other intestinal coccidia, the complex life cycle of C. parvum is constituted by a number of unique cell cycles. The first two asexual cycles are a type of cell multiplication (merogony) that typically produces eight and four merozoites, respectively. The second generation of merozoites may differentially develop into either microgametes or macrogametes (gametogony) that will be fused together to form a new generation of oocysts. Oocysts become infectious after four sporozoites are developed by a meiotic cell division (sporulation). Although unique, little is known about the regulation and control of gene activity associated with the cell cycles in C. parvum or other apicomplexans.

Multiprotein bridging factor type 1 (MBF1) was first identified in Drosophila as a mediator of the transcriptional activator FTZ-F1 and found to be evolutionarily conserved among a wide range of eukaryotic organisms including humans, animals, plants, insects, fungi and protists (Kabe et al., 1999; Liu et al., 2003; Takemaru et al., 1997, 1998; Zhu et al., 2000). MBF1 acts as a transcriptional co-activator that recruits DNA-binding regulators and TATA-binding protein (TBP) for the conditional or differential activation of gene expression. Under the condition of histidine starvation in Saccharomyces cerevisiae, MBF1 is responsible for the GCN4-dependent activation of transcription for the de novo synthesis of this amino acid (Takemaru et al., 1998). Although MBF1-deficient yeast cells can grow slowly without histidine, this growth relies solely upon the basal level expression of histidine synthetic genes and is sensitive to 3-amino-1,2,4-triazole (3-AT), an

Abbreviations: MBF1, multiprotein bridging factor type 1; MBP, maltose-binding protein; p.i., post-infection; TBP, TATA-binding protein.
inhibitor of imidazolglycerol-phosphate dehydratase encoded by the HIS3 gene. We have previously identified a *C. parvum* MBF1 gene (*CpMBF1*) and demonstrated that *CpMBF1* could functionally restore the 3-AT-resistant phenotype in an MBF1-deficient *S. cerevisiae* strain when introduced and expressed in this yeast (Zhu et al., 2000).

In this study, we expressed the *CpMBF1* protein in *Escherichia coli* as a maltose-binding protein (MBP) fusion protein and demonstrated the interaction of *CpMBF1* with a recombinant *C. parvum* TBP (*CpTBP1*) that was recently identified from the parasite’s genome. Using semi-quantitative RT-PCR and Western blotting analysis, we found that the *CpMBF1* gene was differentially expressed in association with the parasite cell cycle. We also characterized the DNA-binding property of the *CpTBP1* protein and found that the *CpTBP1* gene was also differentially expressed during the complex parasite life cycle, but in a different pattern in comparison to that of *CpMBF1*.

**METHODS**

**Parasite preparation.** *C. parvum* oocysts (Iowa strain) purified from the feces of infected calves by sucrose gradient centrifugation were purchased from Pleasant Hill Farm (Troy, ID, USA). The limited amount of bacterial contaminants and debris was removed with a Percoll gradient centrifugation technique and sterilized for 5 min in 10% Clorex on ice. After washing in sterile water five to eight times, the oocysts were excysted for 1–1.5 h at 37°C in PBS (pH 7.5) containing 0-25% trypsin and 0-75% taurodeoxycholic acid. Sporozoites were washed three to five times with water and concentrated as described previously (Millership & Zhu, 2002). Genomic DNA and total RNA were isolated from the free sporozoites using DNaseasy and RNeasy isolation kits (Qiagen), respectively. Free sporozoites were also used to infect human HCT-8 cells in *vitro* to prepare various parasite intracellular stages for the isolation of total RNA and proteins, or for the detection of *CpMBF1* protein by indirect immunofluorescence microscopy (see below for more details).

**Semi-quantitative RT-PCR.** This was performed similar to a previously described protocol (Abrahamsen & Schroeder, 1999). However, the quantity of products was densitometrically (rather than radioactively) measured in agarose gels. Total RNA was isolated from *C. parvum* oocysts, free sporozoites and intracellular stages developed in HCT-8 cells in *vitro* for various times (3–72 h) using RNeasy kit (Qiagen). All RNA samples were subject to intensive RNase-free DNase digestion until no products could be amplified by PCR. Since RNA samples isolated from intracellular parasite were mixed with host and parasite RNA, all samples were first normalized using *C. parvum* 18S rRNA by a semi-quantitative RT-PCR with a pair of previously described primers (i.e. 995F and 1206R) (Abrahamsen & Schroeder, 1999). Twenty thermal cycles were employed so that the densities of RT-PCR amplicons could be measured within linear ranges in agarose gels. The RNA concentrations from various samples were adjusted to produce comparable amounts of *C. parvum* 18S rRNA amplicons by RT-PCR. The adjusted amounts of total RNA were then used for RT-PCR amplification of *CpMBF1* and *CpTBP1* transcripts by 23 thermal cycles using appropriate primer sets, i.e. *CpMBF1-004F* (5′-AGT CAG GAT TGG AAT CAA GTG C-3′) and *CpMBF1-380R* (5′-CAT CTG GAC ATC TTT TGA ACC-3′) for *CpMBF1* and *CpTBP1-001* (5′-ATG AGT GAT GAC ATA CTA AGT TC-3′) and *CpTBP1-750R* (5′-TCA TTC TCT GCT GTA TGG ACA AAG AA-3′) for *CpTBP1*, respectively. The density of each amplicon was determined using GENETOOLS software v. 3.00.22 (Hitachi Software Engineering) and its relative level was normalized by comparison with that of 18S rRNA control. All experiments were repeated in triplicate.

**Production of polyclonal antibody and immunolabelling of *CpMBF1*.** A short peptide (CpMBF1-11–18, 9KGGSSRPKGIKSE-QDLNQ9) was synthesized by the peptide synthesis facility at the Department of Veterinary Pathobiology, Texas A&M University. The short peptide was freshly cross-linked to keyhole limpet haemocyanin (Sigma) prior to each immunization. Polyclonal antibodies to *CpMBF1* were raised in a specific pathogen-free rabbit that was initially immunized with 200 μg of antigen. Booster immunizations (100 μg) were performed later at 30 and 60 days, respectively. Rabbit serum was collected prior to and after the immunization protocol.

*C. parvum* was grown in *vitro* as described previously (Hijjawi et al., 2001), except that, after inoculation into human HCT-8 cells (ATCC CCL-244), cultures were kept in a 5% CO2 incubator and the maintenance medium contained 5% fetal calf serum. HCT-8 cells were grown on poly-l-lysine-treated glass coverslips for 24 h in maintenance medium prior to infection with parasites. Freshly excysted sporozoites (at a ratio of one sporozoite per 10 cells) in medium were added to host cell monolayers and incubated at 37°C for 4 h, at which time point the medium was replaced. Subsequent medium changes were carried out every 24 h. Infected and uninfected samples were fixed in a 10% formalin solution balanced with PBS for 10 min at 2, 4, 8, 12, 16, 24, 48 and 72 h post-infection (p.i.). In addition to the parasites in cultured cells, intact oocysts and free sporozoites were prepared, fixed with formalin, applied onto the poly-l-lysine-treated coverslips and air-dried for immunofluorescence microscopy analysis. Immunolabelling of the *CpMBF1* protein in *C. parvum* extra- and intra-cellular life cycle stages was performed as described previously, with slight modifications (Millership & Zhu, 2002; Zhu & Keithly, 1997). Fixed cells were extracted with 50% methanol/50% acetone (v/v) for 5 min at −20°C. Samples were rehydrated with water and then PBS. Following blocking in 10% fat-free dried milk in PBS for 1 h at room temperature, the samples were incubated with affinity-purified primary antibodies in PBS containing 3% milk for 1 h at room temperature and treated with an FITC-conjugated monoclonal antibody against rabbit IgG (Sigma Chemical). Wash steps between stages were accomplished with PBS containing 0-05% Tween 20. Slides were mounted using a SlowFade Light Antifade mounting medium (Molecular Probes) and examined using an Olympus BX51 Epi-Fluorescence microscope system equipped with FITC/TRITC filters.

**Expression of recombinant *CpMBF1* and *CpTBP1* proteins.** The sequence of the *CpMBF1* gene, which encodes 147 aa with an estimated molecular mass of 16-4 kDa, has been described previously (Zhu et al., 2000). The *CpTBP1* gene was newly identified from the *C. parvum* genome project (University of Minnesota, MN, USA) using various eukaryotic TBPs as queries (Fig. 1). The entire open reading frames (ORFs) of *CpMBF1* and *CpTBP1* were amplified from *C. parvum* genomic DNA using a high-fidelity *Pfu* DNA polymerase (Stratagene) with primer sets *CpMBF1-F01* (5′-tcg aat tca GTA TGC AGG ATT GGA ACA AA-3′) and *CpMBF1-R01* (5′-tcc gaa ttc TTA ATC ATT ATT ATT ATC AGG-3′), and *CpTBP1-F01* (5′-ggg aat tca GTA TGG ATG ATC TAC AA-3′) and *CpTBP1-R01* (5′-ggg aat tca TAT TCT CTT CGT CAA AGA AA-3′) using appropriate primer sets, i.e. *CpMBF1-004F* (5′-AGT CAG GAT TGG AAT CAA GTG C-3′) and *CpMBF1-380R* (5′-CAT CTG GAC ATC TTT TGA ACC-3′) for *CpMBF1* and *CpTBP1-001* (5′-ATG AGT GAT GAC ATA CTA AGT TC-3′) and *CpTBP1-750R* (5′-TCA TTC TCT GCT GTA TGG ACA AAG AA-3′) for *CpTBP1*, respectively. The density of each amplicon was determined using GENETOOLS software v. 3.00.22 (Hitachi Software Engineering) and its relative level was normalized by comparison with that of 18S rRNA control. All experiments were repeated in triplicate.
Electrophoretic mobility shift assays (EMSAs). The interaction between CpTBP1 and DNA was determined by a standard single-stranded-DNA-binding assay (Bellorini et al., 1995; Cang et al., 1999; Poon et al., 1993; Rechkoblit et al., 2001; Srinivasan & Gopinathan, 2002), in which indicated concentrations of MBP–CpTBP1 and biotin-labelled TATA-box consensus oligonucleotide (5’-GCA GAG CAT ATA AGG TGA GGT AGG A-3’) were mixed in 15 μl Tris/HCl buffer (20 mM pH 7.4, 150 mM NaCl, 10% glycerol, 10 mM MgCl2, 1 mM DTT, 1 mM ZnCl2) and incubated at 25°C for 30 min. Control groups included MBP tag (as a non-specific protein) plus oligonucleotide and the oligonucleotide alone (no protein). The retardation of the oligonucleotide–protein complex was resolved on 6% native polyacrylamide gels, which were run at 37°C for 3.5 h. Following electrophoresis, reactions were transferred onto Zeta-Probe GT nylon membranes (Bio-Rad) and the free and protein-bound oligonucleotides were detected using a Pierce LightShift chemiluminescent EMSA kit (Pierce).

The interaction between CpMBF1 and CpTBP1 was assayed by incubating 100 ng MBP–CpMBF1 or MBP tag with various concentrations of MBP–CpTBP1 in 15 mM Tris/HCl buffer at 37°C for 30 min. The reactions were separated on 6% native polyacrylamide gels and transferred onto nitrocellulose membranes. Free CpMBF1 and CpTBP1-bound CpMBF1 proteins were labelled using rabbit antiserum against CpMBF1 as primary antibody and horseradish peroxidase-conjugated secondary antibody, followed by a colour development with 3,3’-diaminobenzidine.

**RESULTS**

Characterization of the newly identified CpTBP1 gene

Using TBP sequences from other eukaryotic organisms including humans and yeast as queries in BLASTN searches, only one TBP gene homologue (CpTBP1) was retrieved from the nearly complete C. parvum genome-sequencing project. The 750 bp CpTBP1 ORF encodes a 27.8 kDa protein that contains two TBP-unique motifs (RXXXYXTALIF and RXXXXXVLLXF) based on the National Center for Biotechnology Information (NCBI) Conserved Domain Search database (http://www.ncbi.nlm.nih.gov/Structure) (Fig. 1). While these two regions are well conserved in terms of sequence and position, there is a 4 aa insertion within region 2. This raises the interesting possibility that the DNA-binding site recognized by C. parvum protein and/or the DNA-binding property may slightly differ from that recognized by its hosts, which might be associated with the general AT-rich feature of the C. parvum genome (~72%).

sequence alignments of apicomplexan TBPs since amino acid identity between CpTBP1 and its counterparts in Plasmodium spp. is only 33–36% (in comparison to 45–89% among animals, plants and fungi).

**CpMBF1** and **CpTBP1** are differentially expressed in different manners

Semi-quantitative RT-PCR analysis was performed to measure the relative levels of transcripts during C. parvum life cycle in vitro for both the CpMBF1 and the CpTBP1 gene. After the density of each cDNA amplicon was measured in agarose gels and normalized with respect to the 18S rRNA standard, apparent differential expression patterns were observed for both CpMBF1 and CpTBP1 transcripts. Although CpMBF1 mRNA transcripts were detectable in all parasite life cycle stages, relatively small amounts of transcripts were detected in free sporozoites, early (3–12 h p.i.) and late (48–72 h p.i.) life cycle stages (Fig. 2). However, the transcription of CpMBF1 was much higher during the 24 and 36 h p.i. in comparison to other life cycle stages (Fig. 2), which typically represented the life cycle stages between first and second generations of merogony. Such an observation is further supported by

**Fig. 1.** Sequence alignment of TBPs at the two conserved regions. Shaded amino acids represent residues conserved among all species. Amino acids conserved among apicomplexans are boxed.
indirect immunofluorescence microscopy. Using rabbit anti-CpMBF1 antibody to probe various parasite intracellular life cycle stages, fluorescence was detectable only in well-developed meronts or gamonts (Fig. 3). Little or no CpMBF1 protein was detectable among oocysts, sporozoites and merozoites during their early invasion stages (data not shown). Furthermore, Western blot analysis only detected CpMBF1 protein among intracellular parasite-infected host cells, but not in the protein extracts isolated from intact oocysts and excysted sporozoites (data not shown). The antibody, also, did not cross-react with the host-cell proteins in Western blot analysis (data not shown) or immunofluorescence microscopy (Fig. 3).

On the other hand, although semi-quantitative RT-PCR analysis revealed that the CpTBP1 gene was differentially expressed, the pattern of expression was completely different from that of CpMBF1. In fact, CpTBP1 was highly expressed in intact oocysts, free sporozoites and intracellular stages at 12–36 h and 60–72 h p.i., but lowly transcribed in parasites at their early stages of invasion (i.e. 3–6 h and 48 h p.i.) (Fig. 2), suggesting strong promoter-regulated gene activation among those early and late intracellular parasite developmental stages.

**Interactions of CpTBP1 with CpMBF1 and DNA oligonucleotide**

Both CpMBF1 and CpTBP1 were successfully cloned into pMAL-c2x vector and expressed as MBP fusion proteins. Using an amylose resin-based affinity chromatography, both recombinant proteins were purified to homogeneity (Fig. 4). Although full-length recombinant proteins were obtained at their expected sizes, some minor smaller protein fragments were observed among affinity-purified proteins, which might represent partial translation of foreign proteins in bacteria or limited degradation of these fusion proteins during the purification.

TBP is known in other eukaryotes to bind to TATA-box consensus sequences. We tried to use several different double- and single-stranded sequences under varying conditions to get the protein to bind without success. However, using the bacterial TATA-box consensus sequence, we observed binding. To confirm the binding function of the newly identified CpTBP1 protein, we used a single-stranded DNA oligonucleotide with a 5’-biotin tag to investigate CpTBP1-binding properties. Gel shift assays showed that the protein was able to bind a specific biotinylated oligonucleotide under specified conditions (Fig. 5a). The control protein for the MBP tag only (Fig. 4b, lane 2) did not bind to the oligonucleotide, indicating that activity was due to the TBP portion of the recombinant protein. Activity of CpTBP1 was observed to be magnesium-dependent (data not shown).

The interaction between CpMBF1 and CpTBP1 was also demonstrated by an electrophoretic mobility shift assay (Fig. 5b). In this assay, the CpMBF1-specific antibody recognized a single band when CpMBF1 was present with
MBF1 or alone (Fig. 5b, lanes 1 and 3) and no cross-reaction was observed between the antibody and CpTBP1 (Fig. 5b, lanes 2 and 4). When CpMBF1 was reacted with different amounts of CpTBP1 and separated, two bands were recognized by the antibody: a lower band corresponding to the free CpMBF1 and a higher band representing the complex formed between CpMBF1 and CpTBP1 (Fig. 5b, lanes 5 and 6). These observations confirmed that CpMBF1 is indeed a transcriptional co-activator that could interact with CpTBP1. However, it must be noted that reactions were labile in nature. Attempts using both GST pull-down and immunoprecipitation assays could not yield very conclusive results. We are currently investigating alternative approaches, including the use of various cross-linkers and fluorescence resonance energy transfer technology, to further characterize the interactions between CpMBF1 and CpTBP1.

**DISCUSSION**

MBF1 is a co-activator that mediates transcriptional activation by interconnecting the general transcription factors with the TATA box-containing promoter.
factor TBP and gene-specific activators (e.g. Drosophila nuclear receptor FTZ-F1 or yeast leucine zipper protein GCN4) (Dragoni et al., 1998; Kabe et al., 1999; Li et al., 1994; Liu et al., 2003; Takemaru et al., 1997). We have previously identified its C. parvum counterpart and demonstrated that the CpMBF1 gene is able to functionally complement MBF1 deficiency in yeast (Zhu et al., 2000). However, the general biological features including the level of expression, localization and TBP-binding property were not fully characterized before. Here, we found that CpMBF1 is differentially expressed with most transcripts and proteins localized in the well-developed intracellular parasite (Figs 2 and 3). The CpMBF1 transcription level is low in free sporozoites and intracellular parasites during their early invasion stages, and CpMBF1 protein is barely detectable in intact oocysts and free sporozoites, suggesting that CpMBF1 may play a role in activating certain genes during the intracellular development of C. parvum. In addition to the activation of histidine synthetic genes in yeast under histidine starvation, MBF1-mediated pathways are known to cause cell differentiation in insects, the formation of stalks in the slime mould Dictyostelium discoideum and the differentiation of endothelial cells in mammals (Dragoni et al., 1998; Kabe et al., 1999; Singleton et al., 1991; Takemaru et al., 1997). These observations, together with the parasite life cycle stage-dependent expression pattern, imply that CpMBF1 may be involved in the cell differentiation of C. parvum during its complex life cycle. However, it is yet unclear what genes in the parasite are regulated by CpMBF1-dependent pathway(s), which certainly warrant further investigations.

We have also identified, cloned and expressed the CpTBP1 gene which is essential for all TATA-box-regulated gene transcriptions. This permits the study of the interaction between CpMBF1 and CpTBP1 proteins, as well as that between CpTBP1 and the TATA-box consensus sequence in vitro. Indeed, under appropriate conditions, we observed the formation of CpMBF1–CpTBP1 and CpTBP1–oligonucleotide complexes using MBP fusion proteins (Fig. 5a), which validated the functions of both proteins in C. parvum. The establishment of the CpTBP1–DNA binding assay also has great potential for testing the inhibitory effects of other compounds in vitro. However, the TATA-box consensus sequence used in this study was adopted from other studies and may not represent the true consensus sequence in C. parvum. We are currently scanning the potential TATA-box candidate sequences within the C. parvum intergenic regions for investigating their TBP-binding properties.

CpTBP1 appears to be the only TBP-encoding gene in the parasite, since intensive BLAST searches using TBP homologues failed to identify any other homologues from the nearly complete C. parvum genome sequence. The binding of TBP to the TATA-box that is located upstream of the 5' untranslated region (5'-UTR) initiates the general downstream gene transcription machinery by recruiting a variety of transcription factors including TFIIA, B, E, F, H and J (Kraemer et al., 2001; Ouyang et al., 2000; Pugh, 2000; Vasanthi et al., 2003; Wu & Chiang, 2001). Most of these factors can be identified from the C. parvum genome (http://cryptodb.org/) (data not shown), suggesting that the general gene transcriptional machinery is conserved between this parasite and other eukaryotes. Although the CpTBP1 gene is differentially expressed during the C. parvum complex life cycle, its expression pattern differs significantly from that of CpMBF1, indicating that other transcriptional activators and/or co-activators in addition to CpMBF1 are present in regulating specific gene transcriptions in this parasite. Based on the transcription level of CpTBP1 (Fig. 2), it appears that more genes are regulated by transcriptional factors in oocysts than other stages. Cryptosporidium causes one of the opportunistic infections in AIDS patients for which no effective treatment is yet available (Okhuysen & Chappell, 2002; Tzipori & Widmer, 2000). The pathogenesis of cryptosporidiosis is associated with the complex life cycle of this pathogen. However, little is known about the gene activation and DNA replication of this parasite. Analysis of TATA-box consensus sequences using model bioinformatics tools and the C. parvum genome sequence may be undertaken to identify potential
genes whose activation may be regulated by transcriptional factors. The identification of *C. parvum* genes regulated by CpMBF1 and other transcriptional factors or co-activators will clearly provide a new direction to study the gene regulation associated with the complex parasite life cycle. If CpMBF1-dependent gene transcriptional activation is proved to be essential for the parasite's intracellular development, this pathway may be pursued as a novel molecular target for the development of drugs against *C. parvum* or other apicomplexan-based diseases.

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