Functional characterization of replication protein A2 (RPA2) from Cryptosporidium parvum

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Replication protein A (RPA) is a heterotrimeric complex of single-stranded DNA-binding proteins that play multiple roles in eukaryotic DNA metabolism. The RPA complex is typically composed of heterologous proteins (termed RPA1, RPA2 and RPA3) in animals, plants and fungi, which possess different functions. Previously, two distinct, short-type RPA large subunits (CpRPA1 and CpRPA1B) from the apicomplexan parasite Cryptosporidium parvum were characterized. Here are reported the identification and characterization of a putative middle RPA subunit (CpRPA2) from this unicellular organism. Although the CpRPA2 gene encodes a predicted 40-1 kDa peptide, which is larger than other RPA2 subunits characterized to date, Western blot analysis of oocyst preparations detected a native CpRPA2 protein with a molecular mass of approximately 32 kDa, suggesting that CpRPA2 might undergo post-translational cleavage or the gene was translated at an alternative start codon. Immunofluorescence microscopy using a rabbit anti-CpRPA2 antibody revealed that CpRPA2 protein was mainly distributed in the cytosol (rather than the nuclei) of C. parvum sporozoites. Semi-quantitative RT-PCR data indicated that CpRPA2 was differentially expressed in a tissue culture model with highest expression in intracellular parasites infecting HCT-8 cells for 36 and 60 h. Sequence comparison suggests that RPA2 is a group of poorly conserved proteins. Nonetheless, functional analyses of recombinant proteins confirmed that CpRPA2 is a single-stranded DNA-binding protein and that it could serve as an in vitro phosphorylation target by a DNA-dependent protein kinase. The minimal length of poly(dT) required for CpRPA2 binding is 17 nucleotides, and the DNA-binding capability was inhibited by phosphorylation in vitro. These observations provide additional evidence on the divergence of RPA proteins between C. parvum and host, implying that the parasite DNA replication machinery could be explored as a chemotherapeutic target.

INTRODUCTION

Eukaryotic single-stranded DNA (ssDNA)-binding proteins, also known as replication protein A (RPA), participate in essential roles for DNA metabolism that include replication, repair and recombination. In all animals, plants and fungi, RPA is a heterotrimeric complex with subunits of approximately 70 kDa (RPA1), 32 kDa (RPA2) and 14 kDa (RPA3) (Iftode et al., 1999; Wold, 1997). In contrast, only three protists to date have been reported to possess subunits of the RPA complex: Cryptosporidium parvum, Plasmodium falciparum and Crithidia fasciculata (Brown et al., 1994; Brown & Ray, 1997; Millership & Zhu, 2002; Voss et al., 2002; Zhu et al., 1999). All three protists have a short-type RPA1 (large subunit), which has a molecular mass of only ~50 kDa, and this is due to the lack of the N-terminal protein-interacting domain present in plants, yeast and humans. Only Crithidia fasciculata has been observed to possess an RPA2 molecule and none of the protists has been shown to have an RPA3 molecule.

The biological and biochemical features of RPA proteins have been more intensively investigated in yeast and humans than any other organisms including protists, the most divergent taxonomic group of unicellular species. Although there are a limited number of reports investigating the function of the short-type RPA1 subunit in protists, virtually nothing is known about the function of RPA2. The only identification of a protist RPA2 is the 28 kDa protein from the cytosol of the trypanosomatid parasite Crithidia fasciculata (Brown et al., 1994). In contrast to RPA1 proteins that are relatively conserved among various species, the RPA2 subunit appears to be extremely poorly conserved among all organisms (see below for details).
which poses an extra burden on the identification and functional confirmation of protist RPA2 proteins. How this group of divergent proteins plays the same structural and functional roles in various organisms is an intriguing question.

The apicomplexan protist Cryptosporidium parvum is globally recognized as an intracellular parasite of humans and animals that can cause self-limiting diarrhea in immunocompetent individuals or life-threatening, prolonged opportunistic infections in immunocompromised patients. To date, there is still no effective treatment available for C. parvum infections (Griffin et al., 1998; Okhuysen & Chappell, 2002; Tzipori & Griffiths, 1998). We have previously identified and characterized two distinct, short-type RPA1 subunits from the parasite (humans have only one RPA1), indicating that protists may utilize a different mechanism in regulating DNA metabolism (Millership & Zhu, 2002; Zhu et al., 1999). In the present study, we have identified an RPA2 homologue (termed CpRPA2) from the C. parvum genome project. Despite its weak homology to other eukaryotic RPA2 proteins, the ssDNA-binding property and phosphorylation ability of CpRPA2 were verified and characterized.

**METHODS**

**Preparation of C. parvum**. Oocysts of C. parvum (Iowa strain) were propagated, purified and provided by Ms Pat Mason at the Pleasant Hill Farm (Troy, ID, USA). The limited amount of bacterial contaminants and debris were removed with a Percoll gradient centrifugation technique and sterilized for 5 min in 10% Clorox on ice. After washing in sterile water five to eight times, the sterilized 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 sterile water and concentrated as described previously (Millership & Zhu, 2002; Zhu et al., 2000). Genomic DNA was isolated from the free sporozoites using the DNeasy isolation kit (Qiagen).

**Molecular cloning and heterogeneous expression of CpRPA2**. Using eukaryotic RPA2 protein sequences as a query, an RPA2 homologue was identified from the ongoing C. parvum genome project at the University of Minnesota (MN, USA) and designated CpRPA2. The entire putative open reading frame (ORF) of the CpRPA2 gene was cloned by PCR and expressed in Escherichia coli as a maltose-binding protein (MBP) fusion protein using the following primers: RPA2-F01 (5'-gcg aat tcA TGA ATT TTG GTG AAA ATA AT-3') and RPA2-R01 (5'-gcg aat tcT TAA TAT CCA GTA GCT CTC CAA GT-3') (lower-case letters represent artificially added EcoRI linkers). The ORF was amplified from C. parvum genomic DNA using a high-fidelity Pfu DNA polymerase (Stratagene) and cloned into a pMAL-p2x vector that contains a signal peptide to lead the MBP fusion protein into the bacterial periplasmic space (New England Biolabs). The sequence was confirmed in both directions by automated sequencing performed at Texas A&M University Gene Technologies Laboratory using an ABI PRISM 377 system (Applied Biosystems). The plasmids containing correctly oriented CpRPA2 sequence were transformed into the Rosetta strain of Escherichia coli (Novagen). Expression and purification of MBP fusion proteins was performed as described by the manufacturer, except that IPTG-induced expression was performed at a low temperature (16°C) overnight. The MBP fusion protein was observed to interfere with assays and was thus removed by the addition of factor Xz. Following cleavage, the free MBP-tag was removed by affinity chromatography with amylose resin, thus leaving the purified recombinant CpRPA2 protein (rCpRPA2).

**Antibody production and purification**. MBP-fused CpRPA2 was freshly emulsified with TiterMax Gold (TiterMax Gold USA) prior to each immunization. Polyclonal antibodies to MBP–CpRPA2 were raised in a specific pathogen-free rabbit that was initially immunized with 200 μg of antigen. Booster immunizations (100 μg each) were performed later at 30 and 60 days, respectively. Rabbit serum was collected prior to and after the immunization protocol. A negative and positive purification procedure was employed for the isolation of rCpRPA2-specific antibodies to ensure specificity using a previously reported protocol with slight modifications (Benet & van Cutsem, 2002). Our protocol was performed in three separate procedures: the first and second steps were used to remove the antiserum specific to the MBP-tag and non-specific binding with the aid of E. coli supernatants, respectively, while the third step was designed to purify the rCpRPA2-specific antibodies. Briefly, the MBP-tag (500 μg total) was immobilized onto a nitrocellulose membrane (5 cm × 5 cm) and, following drying, the membrane was blocked with 3% BSA in TBS containing 10 mM Tris/HCl and 500 mM NaCl for 2 h and washed twice with TBS for 10 min. Rabbit antiserum diluted (1:100) in 1% BSA/TBS was applied to the membrane and gently agitated for 3 h. The second phase of the purification utilized supernatants of E. coli prepared by sonication and applied to a second nitrocellulose membrane (5 cm × 5 cm). The membrane was air-dried and was washed three times in TBS and blocked as described above. The antiserum from the first phase of purification was applied to the second nitrocellulose membrane and incubated for 2 h with gentle agitation. The MBP–CpRPA2 (200 μg total protein) was then applied to a third nitrocellulose membrane (5 cm × 5 cm) as described above. The membrane was incubated in the antiserum preparation from the second step for 3 h and washed five times in TBS for 10 min with gentle agitation. The rCpRPA2-specific antibodies were eluted by incubating the membrane in 10 ml of 0.2 M glycine/HCl (pH 2.5) for 3 min. The eluted antibody solution was neutralized by the addition of 1:7 ml of 1 M Tris/HCl (pH 8.8) containing 7% BSA and used at 1/100 to 1/1000 final dilutions. All steps were performed at room temperature. Pre-serum was prepared in the same manner and failed to elicit a response both in Western blots and by indirect immunofluorescence.

**Western blot analysis**. This was performed using the affinity-purified, rCpRPA2-specific rabbit polyclonal antibody that was secondarily labelled with a monoclonal antibody against rabbit IgG conjugated with horseradish peroxidase (HRP) (Sigma Chemical). Approximately 1 × 10⁷ oocysts per well were lysed in loading buffer containing a protease inhibitor cocktail (Sigma Chemical) at 95°C for 5 min. Insoluble material was removed by centrifugation and soluble proteins were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose as described by mini trans-blot electrophoretic transfer (Bio-Rad). All immunological processing was performed as described by the Bio-Rad Western blot protocol. Briefly, following transfer of proteins, blots were incubated in 3% gelatin in TBS (20 mM Tris/HCl, pH 7.5; 500 mM NaCl) for 1 h. The primary antibody (either purified immune serum or pre-serum) was diluted with 1% gelatin in Tris/Tween 20-buffered saline (TTBS: 20 mM Tris/HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20) and incubated for 1 h at 37°C. Following washing with Tris/citrate-buffered saline (TCBS: 20 mM citrate, pH 5.5; 500 mM NaCl; 0.05% Tween 20), the blots were incubated in a secondary antibody conjugated to HRP (diluted in TCBS) for 1 h. Prior to analysis, blots were washed with TTBS and TBS, respectively, and colour development was performed with 3,3’-diaminobenzidine (Sigma Chemical) as substrate.
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* free sporozoites and intracellular stages developed in HCT-8 cells *in vitro* for various times (3–72 h) using RNase 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 parasites 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 the *CpRPA2* transcript by 23 thermal cycles using appropriate primer sets. The density of each amplicon was determined using GENETOOLS software v. 3.00.22 (Hitachi Software Engineering) and its relative level of transcripts at each time point was expressed as the ratio of signals between *CpRPA2* and 18S rRNA amplicons.

**ssDNA-binding assay.** The standard ssDNA binding reactions were performed in 15 μl Tris/Cl buffer (pH 7.4, 20 mM) containing 150 mM NaCl, 25% glycerol and 1 mM MgCl₂, which contained the indicated concentration of rCpRPA2 (Eckerich et al., 2001; Miller & Zhu, 2002). The 5’-biotinylated oligonucleotides ([dT]₁₀0, [dT]₂₀ ([dT]₁₂0, [dT]₁₂ ([dT]₁₂ ([dT]₂₅ ([dT]₃₀ and [dT]₅₀ (5 pmol each unless stated)) were combined with the protein and the reaction was incubated at 25 °C for 30 min. Control groups included MBP-tag (a non-specific protein) plus oligonucleotides and the oligonucleotides alone (no protein). The retardation of the oligonucleotide–protein complex was resolved on 6% native polyacrylamide gels, which were buffered with 50 mM Tris/borate (pH 7-5). 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 electrophoretic mobility shifting assay (EMSA) kit (Pierce).

**rCpRPA2 phosphorylation in vitro by DNA-dependent protein kinase (DNA-PK).** The phosphorylation of rCpRPA2 was investigated according to a previously described protocol with slight modifications (Brush & Kelly, 2000; Brush et al., 2001). Briefly, a reaction mixture (50 μl) in a buffer (50 mM HEPES; 100 mM KCl; 10 mM MgCl₂; 0.2 mM EGTA; 1 mM DTT) containing BSA (2-5 μg), calf thymus DNA (0-01 μg), γ³[P]ATP (100 μCi; 3-70 MBq), rCpRPA2 (0-02 μg) and DNA-PK (25 U) was first gently agitated at 30 °C for 30 min and halted by the addition of SDS-PAGE loading buffer. One of the DNA-PK-treated samples was heat-inactivated without adding loading buffer, followed by the addition of calf intestinal alkaline phosphatase (10 U) to test whether the newly phosphorylated rCpRPA2 could be dephosphorylated. The samples were heated at 95 °C for 5 min and subjected to SDS-PAGE (8%), and the separated proteins transferred to nitrocellulose membranes. The resulting blot was analysed by autoradiography. Reactions without dsDNA and/or DNA-PK were included as controls.

**Immunofluorescence microscopy.** *C. parvum* sporozoites were prepared by excystation of fresh oocysts in PBS containing 0.5% trypsin and 0.25% taurodeoxycholic acid for 1 h at 37 °C. Excysted free sporozoites were washed three times in PBS by centrifugation, fixed in a PBS-buffered formalin solution for at least 15 min and washed again in PBS and water (twice). Sporozoites suspended in water were then adhered to microscope slides pre-coated with 0.1% poly-L-lysine, air-dried and extracted with cold methanol/acetic acid (1:1, v/v) for 5 min at −20 °C. Following blocking in PBS containing 5% BSA and 0.05% Tween 20 for 1 h at room temperature, samples were incubated in primary antibodies diluted in PBS containing 1% BSA and 0.05% Tween 20 for 1 h. Each sample was then incubated with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody (monoclonal anti-rabbit-TRITC, Sigma Chemical). Wash steps between each stage were accomplished with PBS containing 0.05% Tween 20. Slides were mounted using anti-quenching medium (Molecular Probes) and viewed with a BX51 Olympus microscope equipped with a FITC/TRITC filter set and photographed with an Olympus PM10SP camera.

**RESULTS**

**RPA2 proteins are a group of poorly conserved molecules in eukaryotes**

The presence of two distinct, short-type RPA1 subunits in apicomplexans prompted us to search for their corresponding smaller subunits. However, using human and yeast RPA2 proteins or their homologues from other species as queries, only a limited number of hits with extremely low identity scores were retrieved from the ongoing *C. parvum* or other apicomplexan genome-sequencing projects (e.g. *Plasmodium falciparum*, *Toxoplasma gondii* and *Eimeria tenella*). Among them, we have identified one ORF from the *C. parvum* genome that appears to be a good candidate gene for the RPA2 subunit, since it contains RPA2 consensus sequences based on the National Center for Biotechnology Information (NCBI) Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/). However, only a limited number of conserved amino acids is present in various RPA2 proteins (Fig. 1a). The *RP2* subunit is a group of poorly conserved proteins since pair-wise sequence identities between and among a wide range of organisms were mostly around 15–36% (Fig. 1b). It is noticeable that RPA2 proteins within the same major taxonomic group can also be divergent (i.e. RPA2 proteins of the plants *Oryza sativa* and *Arabidopsis thaliana* share 37% identity, while those from yeast/fungi share only 23–32% identity) (Fig. 1b).

**Native CrpRPA2 is a 32 kDa protein that is mainly present in the cytosol of free sporozoites**

The putative *CpRPA2* ORF was predicted to start from the first uninterrupted initiation codon (ATG) based on a generally accepted practice. However, the deduced amino acid sequences predicted a 40-1 kDa polypeptide, which was significantly larger than the 27–34 kDa proteins in other eukaryotic organisms (Fig. 2a) (Iftode et al., 1999; Wold, 1997). On the other hand, our Western blot analysis of oocyst soluble proteins using affinity-purified anti-rCpRPA2 antibodies detected a single band at approximately 32 kDa (Fig. 2b), which was within the range of other known eukaryotic RPA2 proteins. The 8 kDa mass difference between the calculated and observed CpRPA2 proteins suggest that this gene might be translated at an alternative initiation codon since five methionine codons

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The difference between calculated and observed proteins, we
sion. However, regardless of the discrepancy in the mass
molecular and proteomic analyses to reach a firm conclu-
when aligned (Fig. 1a). Both possibilities require further
of conserved regions at the C terminus of CpRPA2
gone post-translational cleavage based on the presence
could be found within the first 165 nt of the putative ORF. It
Alignment and identities (%) of RPA2 subunits among various eukaryotic organisms.

Fig. 1. Alignment and identities (%) of RPA2 subunits among various eukaryotic organisms. (a) Alignment of RPA2 amino acids (aa) from Cryptosporidium (Cry. parvum), Crithidia (Cri. fasciculata), Saccharomyces (Sac. cerevisiae), Arabidopsis thaliana and Homo sapiens. Positions containing >50% and 100% identical aa among all five species are shaded and underlined, respectively. A solid arrow indicates stretches of unalignable 67 aa omitted from CpRPA2. (b) A table of pair-wise aa identity between RPA2 proteins from various eukaryotic organisms (pair-wise comparison settings: open gap penalties, 5; extended gap penalties, 0-1), including Cry. parvum (GenBank accession no. AY219916), H. sapiens (NP_002937), Cri. fasciculata (Q23697), Encephalitozoon cuniculi (NP_585792), Sac. cerevisiae (NP_014087), A. thaliana (AAD18120), O. sativa (BAB40535) and Schizosaccharomyces (Sch.) pombe (Q92373).

were able to confirm that the 40 kDa rCpRPA2 translated
from the entire putative ORF possesses ssDNA-binding capability and that it could be phosphorylated by a DNA-PK as described below. Semi-quantitative RT-PCR analysis was performed to measure the relative levels of CpRPA2 transcripts during the C. parvum life cycle in vitro (Fig. 2c).

After the density of each cDNA amplicon was measured in
agarose gels and normalized with respect to the 18S rRNA
transcripts during the
was performed to measure the relative levels of CpRPA2
capability and that it could be phosphorylated by a DNA-PK

standard, apparent differential expressions were observed throughout the in vitro life cycle. Although mRNA was detected in all samples when 40 thermal cycles were used (from sporozoites to 72 h post-infection) (data not shown), the transcription of CpRPA2 was most apparent at 36 and 60 h post-infection. These time points probably represent the typical life stages of Type II meronts and gamonts, suggesting that the CpRPA2 gene is more actively transcribed during late developmental stages.

Using an affinity-purified anti-CpRPA2 antibody in indirect immunofluorescence microscopy, we were able to clearly detect CpRPA2 protein in excysted free sporozoites (Fig. 3). It was interesting that, although some fluorescent signals were detectable in the nuclei, CpRPA2 was intensively labelled in the anterior half of the sporozoite cytoplasm and clearly separated from nuclei (counterstained with YOYO-1 dye). This observation suggests that the majority of CpRPA2 protein is not present in the nuclei in excysted free sporozoites. Similar observations have been reported in non-replicating organisms, in which the majority of purified RPA complex is in the cytoplasm fraction of gently lysed cells (Fairman & Stillman, 1988; Wold & Kelly, 1988; Brush et al., 1995). It is unclear why the protein is found mostly in the anterior region. However, one possibility is that the detected CpRPA2 may represent newly synthesized protein that is still associated with endoplasmic reticulum and/or Golgi bodies at the anterior region of sporozoites. The limited amount of CpRPA2 protein co-localized with nuclear DNA agreed with the lack of DNA replication
and minimum repair) activity in the sporozoite stage as observed previously in other organisms (Kim et al., 1992; Wold et al., 1989). We are currently studying the expression pattern of CpRPA2 during the C. parvum intracellular development in comparison with that of CpRPA1 and CpRPA1B. Although our preliminary data indicate that all three RPA proteins are differentially expressed during the parasite’s complex life cycle (data not shown), we will report and discuss these findings separately upon the completion of this analysis.

rCpRPA2 binds to ≥17 nt oligonucleotides

To confirm that CpRPA2 possessed ssDNA-binding properties, we cloned its entire ORF into an MBP fusion vector and expressed the recombinant protein in bacteria. The rCpRPA2 was purified to apparent homogeneity with an amylose-based affinity chromatography (Fig. 2a). It must be noted that even with the use of a periplasmic exported fusion system (i.e. pMAL-p2x vector), the protein appeared to be toxic to the E. coli, since bacterial growth was halted after prolonged passage of the transformants. Therefore, bacterial hosts needed to be freshly transformed with pMal-p2x-CpRPA2 plasmids for each fusion protein production. Such an apparent cytotoxicity was observed when expressing the two C. parvum large subunits (CpRPA1 and CpRPA1B) in bacteria, which was hypothesized to be caused by their non-specific ssDNA-binding action that interferes with DNA metabolism during the bacterial cell cycle (Millership & Zhu, 2002).

The MBP-tag was found to interfere with the ssDNA-binding property of CpRPA2 and thus was removed from the fusion protein. MBP-free rCpRPA2 was purified and utilized to study the ssDNA-binding properties by EMSA. First, we demonstrated that rCpRPA2 was able to bind to biotinylated (dT)_{40} oligonucleotides (Fig. 4a). We also observed a concentration-dependent binding activity of rCpRPA2 to the oligonucleotide (data not shown). In a second study, the ability of rCpRPA2 to bind to various lengths of ssDNA [(dT)_{15..40}] was investigated, and this revealed that rCpRPA2 was able to bind oligo(dT) of ≥17 nt in length (Fig. 4b). These observations taken together verified that the CpRPA2-encoded protein belongs to the family of eukaryotic ssDNA-binding proteins.

rCpRPA2 could be phosphorylated in vitro

Previous studies in eukaryotic organisms have shown that the phosphorylation of RPA2 proteins might play an important role in regulating the biological functions of these organisms (Henricksen et al., 1996; Treuner et al., 1999a, b). In those studies, the phosphorylation of recombinant and native RPA2 proteins was achieved by DNA-PKs and/or cyclin-dependent protein kinases. In this study, we tested whether rCpRPA2 could be phosphorylated by DNA-PK. In the presence of dsDNA to activate the kinase, we observed that DNA-PK was able to transfer the radioactive phosphate group to rCpRPA2 (Fig. 5a, lane ‘Pi’). The detected radioactivity in DNA-PK-treated rCPRPA2 resulted neither from self-phosphorylation by rCpRPA2 nor from the potential ATP-binding property of this protein, since no radioactivity was detectable in the control containing ATP and rCpRPA2 (but without DNA-PK) (Fig. 5a, lane ‘Ctl’), and the phosphorylated rCPRA2 could be dephosphorylated using calf intestinal alkaline phosphatase (Fig. 5a, lane ‘dePi’). After confirming that rCpRPA2 could be phosphorylated by DNA-PK, we further investigated the effects of phosphorylation on the ssDNA-binding properties of this protein with EMSA. Again, our data demonstrated that the rCpRPA2 could bind to ssDNA (Fig. 5b, lane 3). However, following phosphorylation by DNA-PK, rCpRPA2 apparently lost its ability to bind to ssDNA (Fig. 5b, lane 4).

DISCUSSION

C. parvum belongs to the phylum Apicomplexa, many members of which are pathogens of medical and veterinary importance (e.g. malarial parasites – Plasmodium spp.; opportunistic pathogens in AIDS patients – C. parvum and T. gondii; as well as veterinary important coccidial parasites – Eimeria spp.). This group of protists has at least three distinct life stage replication processes: sporogony,
merogony and gamogony. Both sporogony and merogony are cell multiplication processes that produce more than two daughter cells in each cell cycle and differ from the host cell somatic duplication, which implies that a unique mechanism may be involved in DNA replication in apicomplexan parasites. To date, little work has been published on how C. parvum (or members of the phylum Apicomplexa) perform the processes associated with DNA metabolism.

RPA participates in essential roles for DNA metabolism, which include replication, repair and recombination. All animals, plant and fungi studied to date possess only one type of RPA trimeric complex, with subunits of approximately 70 kDa (RPA1), 32 kDa (RPA2) and 14 kDa (RPA3). The DNA binding activity of RPA has been isolated to RPA1 and RPA2 (Iftode et al., 1999; Wold, 1997). In addition to ssDNA-binding, RPA1 has been shown to interact with a number of cellular proteins that regulate the cell cycle, DNA repair and recombination. Whereas RPA2 is thought to have a regulatory function, thought to be controlled by phosphorylation (Brill & Bastin-Shanower, 1998), the role of RPA3 still remains ambiguous, but studies indicate that it is probably associated with assembly and stabilization of the trimeric subunit (Iftode et al., 1999; Wold, 1997).

While C. parvum is a eukaryote, our studies have indicated that its DNA-binding proteins differ from other known eukaryotic RPA complexes. Indeed, in contrast to its human and animal hosts, C. parvum possesses two distinct RPA1 subunits (CpRPA1 and CpRPA1B) (Millership & Zhu, 2002; Zhu et al., 1999). These two subunits are also significantly smaller than other eukaryotic RPA1 subunits and possess different DNA binding properties in vitro. Our preliminary localization analyses indicate that CpRPA1 and CpRPA1B probably act independently through the parasite’s life cycle (data not shown). The identification of CpRPA2 provides us with the opportunity to further understand the structure and action of the RPA complex during the life cycle of C. parvum. However, it is unclear whether CpRPA2 interacts with one or both of CpRPA1 or CpRPA1B subunits. Indeed, we have been unsuccessful in identifying a second possible RPA2-encoding gene from the complete C. parvum genome. The search of RPA2 homologues from the complete P. falciparum genome was not successful, which was probably due to the high divergence of this protein or possible intron interruption(s) present in the P. falciparum homologue(s). However, one can not rule out the possibility that, although P. falciparum possesses a RPA1 homologue (i.e. PfrPA1), it probably lacks a second ssDNA-binding protein, such as RPA2. This notion is in part supported by a previous study which showed only a single species of protein (PfrPA1) from crude nuclear extract was able to form an ssDNA–protein complex (Voss et al., 2002).

Unlike RPA1 which has defined regions of homology, no apparent regions have been identified to date within RPA2,
except for two short motifs at the N and C termini (Fig. 1). However, in general, RPA2 has three domains: the N terminus contains the phosphorylation site, the central domain is the DNA-binding region and the C-terminal is the binding domain for other proteins (Iftode et al., 1999). Comparison of the characterized RPA2 proteins and annotated homologues from the genomes of various organisms supports the notion that RPA2 is a group of highly divergent molecules (Fig. 1). The lack of an in vitro culture system for C. parvum complicates our ability to investigate the replication pathways of this unique protist and thus the use of recombinant proteins is the only feasible way to perform functional protein studies on this parasite. In this study, we have confirmed that rCpRPA2 is an active ssDNA-binding protein and that it can be phosphorylated by DNA-PK (Fig. 4a). In addition, the phosphorylation appears to inhibit binding of rCpRPA2 to ssDNA in vitro (Fig. 4b). The functional significance of the N-terminal RPA2 phosphorylation is not fully understood (Iftode et al., 1999). In some cases when RPA2 is phosphorylated it becomes disassociated from RPA1, but this is not always the case (Treuner et al., 1999a, b). Indeed, deletion of the human RPA2 (HsRPA) N-terminal region (containing the phosphorylation sites) has no effect on replication in SV40 DNA replication (Iftode et al., 1999; Wold, 1997). However, recent data indicated that RPA2 phosphorylation might play an important role in DNA repair (Iftode et al., 1999; Wold, 1997). HsRPA2 becomes hyper-phosphorylated when human cells are damaged by UV radiation, and this coincides with the inability to support SV40 DNA replication in vitro (Boubnov & Weaver, 1995; Carty et al., 1994; Fried et al., 1996). Although our data clearly indicated that rCpRPA2 could be phosphorylated in vitro by DNA-PK, it was unclear whether or how the phosphorylation of CpRPA2 might occur in vivo. Nonetheless, the ability of rCpRPA2 to serve as a substrate for phosphorylation clearly pointed out a possible new direction to further investigate the function and regulation of this important parasite protein.

Interest in the HsRPA complex stems from the importance of this trimer in DNA metabolism, and thus cancer biology. To date, several anti-cancer drugs have been identified that interact with the RPA complex (Liu et al., 2000; Peters et al., 2001). One of these, tirapazamine, is a hypoxia-activated cytotoxic agent which is currently in phase III clinical studies and has been localized to RPA2 (Peters et al., 2001). Indeed, monoclonal antibodies against HsRPA2 inhibit DNA replication, by impeding RPA stimulation of DNA polymerase alpha, but do not stop the RPA complex from binding to DNA (Basilion et al., 1999). Alternative methods have also been utilized in the form of anti-sense technologies to inhibit the RPA complex (Basilion et al., 1999; Gomes & Wold, 1996; Kenny et al., 1990). Together with the evidence that C. parvum possesses a novel CpRPA2 and two distinct, short-type large subunits (Millership & Zhu, 2002), all of which differ significantly from its host, one could speculate that the parasite’s DNA replication proteins may serve as a novel target for drugs against cryptosporidiosis and possibly other apicomplexan-based diseases.

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