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Differential Expression of the Two Distinct Replication Protein A Subunits from *Cryptosporidium parvum*

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Abstract Apicomplexan parasites differ from their host by possessing at least two distinct types (long and short) of replication protein A large subunits (RPA1). Different roles for the long and short types of RPA1 proteins have been implied in early biochemical studies, but certain details remained to be elucidated. In the present study, we have found that the *Cryptosporidium parvum* short-type RPA1 (CpRPA1A) was highly expressed at S-phase in parasites during the early stage of merogony (a cell multiplication process unique to this group of parasites), but otherwise present in the cytosol at a much lower level in other cell-cycle stages. This observation indicates that CpRPA1A is probably responsible for the general DNA replication of the parasite. On the other hand, the long-type CpRPA1B protein was present in a much lower level in the early life cycle stages, but elevated at later stages involved in sexual development, indicating that CpRPA1B may play a role in DNA recombination. Additionally, CpRPA1B could be up-regulated by UV exposure, indicating that this long-type RPA1 is probably involved in DNA repair. Collectively, our data implies that the two RPA1 proteins in *C. parvum* are performing different roles during DNA replication, repair and recombination in this parasite. J. Cell. Biochem. 104: 2207–2216, 2008.

Key words: replication protein A (RPA); *Cryptosporidium*; cryptosporidiosis; cell cycle; DNA repair; UV light

DNA replication is an essential process that progresses in a regulated fashion and requires the coordinated action of numerous multi-protein complexes for its completion. In eukaryotes, replication protein A (RPA) is a heterotrimeric complex that binds to single-stranded DNA (ssDNA) and has been demonstrated to be essential for DNA recombination, repair, and transcription [see Wold, 1997; Iftode et al., 1999 for review]. In humans and yeast, the proteins that comprise the RPA heterotrimer consist of RPA1 (~70 kDa), RPA2 (~32 kDa) and RPA3 (~14 kDa), in which the single RPA1 subunit is involved in the three major functions that include DNA replication, repair and recombination. The three RPA subunits and their sizes are, in general, conserved across many taxa, including yeast, plants, and animals [Wold, 1997; Iftode et al., 1999; Ishibashi et al., 2005]. However, significant deviations do exist, and this variation is exemplified in the protozoa [Brown et al., 1992, 1994; Pasion et al., 1994; Brown and Ray, 1997; Zhu et al., 1999; Millership and Zhu, 2002; Voss et al., 2002; Millership et al., 2004a; Rider et al., 2005].

Cryptosporidium parvum, *Toxoplasma gondii*, and *Plasmodium falciparum* represent members of the apicomplexan branch of protists and are causative agents of cryptosporidiosis, toxoplasmosis, and malaria, respectively. Each of these apicomplexan parasites contains loci predicted to encode long (~70–120 kDa, depending on species) and atypically short (~50 kDa) types of the RPA1 subunit, as well

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Abbreviations used: ERCC/XP, excision repair cross complementation/xeroderma pigmentosum; NER, nucleotide excision repair; RPA, replication protein A; SSB, single-strand binding; ssDNA, single-stranded DNA.

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as highly divergent RPA2 and RPA3 subunits [Zhu et al., 1999; Millership and Zhu, 2002; Voss et al., 2002; Millership et al., 2004a; Rider et al., 2005]. Another parasitic protist (*Crithidia fasciculata*) also possesses an unusually short-type RPA1 protein (~51 kDa) that is part of an RPA heterotrimer [Brown et al., 1992, 1994; Brown and Ray, 1997].

Among apicomplexan RPA proteins, a short-type RPA1 from *P. falciparum* has been studied, and the RPA proteins from *C. parvum* have been extensively characterized at the biochemical level [Zhu et al., 1999; Millership and Zhu, 2002; Voss et al., 2002; Rider et al., 2005]. *C. parvum* possesses loci encoding two RPA1 subunits—CpRPA1A (~50 kDa) and CpRPA1B (~70 kDa), a single RPA2 subunit (~34 kDa), and a single RPA3 subunit (~12 kDa) [Zhu et al., 1999; Millership and Zhu, 2002; Millership et al., 2004a; Rider et al., 2005]. Additionally, the *C. parvum RPA1B* locus is capable of producing a shorter ~50 kDa protein [Millership and Zhu, 2002]. Each of the *C. parvum* RPA1 subunits is capable of forming a heterotrimeric complex with the RPA2 and RPA3 subunits encoded by the genome, indicating conservation in the general composition of the RPA heterotrimer [Rider et al., 2005]. However, both CpRPA1A and CpRPA1B possess intrinsic ssDNA binding capabilities that differ significantly from one another and from their human counterpart [Millership and Zhu, 2002; Rider et al., 2005]. Although progress has been made in understanding the similarities and differences in biochemical terms, little is known about the biological functions of the long and short types of the RPA1 in protozoa. The presence of both types in members of the Apicomplexa offers a unique opportunity to expand our understanding of the RPA1 class of proteins.

Expression levels, phosphorylation status, and sub-cellular localization of RPA proteins have been correlated with the onset of DNA replication, responses to DNA damage, and initiation of recombination [Pasion et al., 1994; Wold, 1997; Perdigao et al., 1999; Voss et al., 2002; Marwedel et al., 2003; Binz et al., 2004]. Although different functions for the long and short types of RPA1 proteins have been suggested based on their differential expression patterns and ssDNA-binding properties [Millership and Zhu, 2002; Rider et al., 2005], their true biological roles remained to be elucidated. To facilitate a better understanding of the long- and short-type RPA1 proteins in *C. parvum*, we sought to examine their expression and sub-cellular distribution during the complex parasite life cycle (to gain insight into the roles of RPA proteins in replication and recombination) as well as in response to UV irradiation (to induce DNA repair). We discovered substantial differences in the timing of expression of these proteins during parasite development, as well as differences in abundance and sub-cellular distribution of these proteins in response to UV light. These differences suggest unique biological roles for the two *C. parvum* RPA1 proteins.

MATERIALS AND METHODS

Manipulation of Parasites

C. parvum oocysts (Iowa Strain) were obtained from Bunch Grass Farms (Deary, ID), purified by Percoll gradient centrifugation and stored at 4°C until use [Arrowood and Sterling, 1987]. In general, only oocysts that were less than 3 months old from the harvest date were used in experiments. Oocysts were cleaned by treating them with 10% Clorox on ice, followed by five to eight washes in water with oocysts being pelleted by centrifugation.

Detection of CpRPA1 Proteins During Development

Intracellular parasites at various developmental stages were prepared as described previously [Upton et al., 1995; Hijjawi et al., 2001; Millership et al., 2004b]. Briefly, HCT-8 cells in culture medium (RPMI-1640 medium containing 10% fetal bovine serum) were first seeded in 24-well plates containing poly-L-lysine treated glass coverslips. After incubation at 37°C overnight, Clorox-treated oocysts were added into the culture medium to allow for excystation and invasion into host cells for 2–3 h. Free sporozoites that failed to infect cells were removed by a medium exchange. At various time points after infection, monolayers on coverslips were rinsed with PBS and fixed in 10% formalin for immunofluorescence microscopy studies.

For very young parasites, aliquots of oocysts were seeded into cultures at 4-h intervals up to 20 h, and finally collected at 24 h to produce individual slides containing parasites ranging from 4 to 24 h in age. Additional cultures of parasites were generated that represented single time points every 12 h between 12 and

72 h. Parasites and host cells were examined by immunofluorescence microscopy as described above except that a dual labeling strategy was used where CpRPA1A (rabbit polyclonal) and CpRPA1B (affinity-purified chicken polyclonal) were differentiated using TRITC-labeled goat-anti-rabbit and FITC-labeled mouse-anti-chicken secondary antibodies. All samples were counterstained with DAPI to serve as a spatial reference and photographed as described above.

For treatments with bromodeoxyuridine (BrdU), parasites were cultivated up to 16–20 h, followed by an exchange of the medium with medium containing 10 μM BrdU as described [Striepen et al., 2004]. Parasite cultures were then allowed to continue development in BrdU-containing medium for an additional 5 h before being washed and fixed. Coverslips were incubated in 0.2 N HCl for 20 min and washed 3× with PBS before continuing with immunolabeling.

Optimization of UV Treatment of *C. parvum* Oocysts

Oocysts (10 million) were suspended in 0.5 ml of water at room temperature, placed in a Stratalinker UV crosslinker equipped with FG8T5 germicidal lamps (Stratagene), and exposed to various doses of UV-C light (0–300 mJ/cm^2). FG8T5 lamps emit UV-C with a peak emission at 254 nm. To ensure that this light was capable of penetrating water and/or PBS, absorbance spectra of these liquids were taken using a MultiScan Plate reader (Thermo) using UV-transparent plates. Absorbance across most of this range was found to be negligible. Following UV-C treatment, an equal volume (500 μl) of 2× excystation medium was added to the oocyst suspension and oocysts were incubated at 37°C for 60 min. An equal volume (1 ml) of PBS-buffered 20% formalin was added and the mixture was held at room temperature for at least 10 min. A 300 μl aliquot was taken and added to 300 μl of 10% BSA in 1× PBS. Samples were then examined using differential interference contrast on an Olympus BX51 microscope fitted with a digital camera. Digital images of each sample were then examined and the number of oocysts and empty oocyst shells were counted. The entire experiment was replicated and the data used to determine the inactivation rate under the tested conditions. Data was analyzed as a sigmoidal dose–response curve using Prism v4.0 software (GraphPad Software, Inc.).

Real-time PCR Analysis of Repair Gene Transcripts

We first tested whether the expression of *CpRPA1A* and *CpRPA1B* genes were altered in response to UV light. In order to gain greater insight into gene regulation in the parasite in response to UV-induced DNA damage, we have also identified and included an additional 30 loci from the *C. parvum* genome that may be involved in DNA repair and recombination, particularly those involved in nucleotide excision repair (NER) in this study. These include ssDNA-binding proteins (RPA, Rad51, RecA homologs), homologs of ERCC/XP group protein coding genes, homologs of DNA synthesis proteins (DNA polymerases, Ligase, PCNA, RFC) and loci involved in transcriptional initiation (TBF, SSL). Several of these loci were previously identified [Millership and Zhu, 2002; Millership et al., 2004a; Rider et al., 2005; Rochelle et al., 2005]. Primers for these loci were designed using BeaconDesigner Software (Bio-Rad) (see Table S1 for a list of loci and primer sequences used). The quality of all primer pairs were first evaluated by PCR using *C. parvum* genomic DNA (gDNA) as template (data not shown).

Oocysts were brought to and maintained at ambient temperature for at least 2 h in a biological safety cabinet to reduce potential effects of temperature changes on gene expression in all oocysts. Aliquots (10 million oocysts in 100 μl of PBS) were exposed to UV-C treatment (21 mJ/cm^2) or mock treated (placed in the crosslinker, but not irradiated) and then maintained at ambient temperature for an additional 30 min to allow time for more apparent gene expression profile changes. Three hundred microliters (300 μl) of RLT buffer (the lysis buffer in Qiagen's RNeasy RNA isolation kit) were added to each sample and samples were immediately flash frozen in liquid nitrogen. Samples were subjected to several freeze-thaw cycles to disrupt the oocyst wall, and stored overnight at −80°C. RNA was isolated as described previously [Rider et al., 2005]. A SYBR-green-based real-time quantitative RT-PCR (qRT-PCR) was performed on the samples using a Quantitect One-Step RT-PCR Kit (Qiagen) supplemented with the passive dye FITC (0.01 mM) for use in an iCycler (Bio-Rad)

as described previously [Rider et al., 2005]. The 18S ribosomal RNA transcript was used as a standardization control for all loci to calculate the ΔC_T [Rider et al., 2005] and differences between treated and untreated samples were calculated as the $\Delta\Delta C_T$. Fold changes were estimated by calculating $2^{\Delta\Delta CT}$ that assumes a $2\times$ increase in the number of amplicons after each thermal cycle. The experiment was replicated and the data was examined to identify any significant changes in transcript abundance using Student's *t*-test.

Detection of CpRPA1 Changes Due to UV Treatment

The production and affinity purification of polyclonal antibodies to CpRPA1 proteins have been reported elsewhere [Millership and Zhu, 2002; Rider et al., 2005]. Oocysts were treated as described above for transcript analysis, but subjected to one of two different treatment options: Room temperature formalin-fixed and PBS-washed at various time points following UV-C treatment (i.e., 0, 30, 60, 90, and 120 min) or excysted at 37°C for 90 min followed by washing and fixation. Aliquots (10 μl) containing UV treated or mock treated samples were spotted onto poly-L-lysine coated coverslips and air-dried overnight at ambient temperature. Unexcysted samples were heated at 80°C for 10 min to permeabilize oocysts [Smith et al., 2004]. Oocyst or sporozoite preparations were extracted with cold methanol, blocked in 10% BSA–PBS for 1 h, incubated individually with CpRPA1A and CpRPA1B polyclonal antibodies and TRITC-conjugated secondary antibodies as described previously [Millership and Zhu, 2002]. Digital images were taken using an Olympus BX51 microscope fitted with a digital camera and appropriate filters. All images were examined using the same exposure parameters to allow comparison among samples.

UV-C Treatment of Cultured Parasites

Parasites were cultured as described above, but at 24 h post-inoculation, the culture medium was replaced with 500 μl of $1\times$ PBS. Samples were then exposed to 21 mJ/cm^2 UV (ID_{20}) or mock treated. The PBS was replaced again with fresh culture medium. Samples were incubated for 0, 45 or 90 min in a cell culture incubator. After the culture medium was removed, samples were fixed for 20 min at room temperature in $1\times$ PBS containing 10% formalin. Fixed samples were stored in 70% ethanol until treated for immunofluorescence microscopy.

RESULTS

C. parvum RPA1 Expression is Developmentally Regulated

We used immunofluorescence microscopy to examine the expression of CpRPA1A during parasite development. Parasites were cultured in vitro on HCT8 host cells for up to 48 h prior to examination. An attempt was made to identify parasites of all developmental stages during the first round of merogony, although parasites with two or four nuclei were much less abundant. The signal for CpRPA1A was relatively strong in some cells, and weak in others, where the strongest staining cells are early stage meronts that typically displayed weaker or non-specific DAPI nuclear stain (Fig. 1 and Supplemental Fig. I). The CpRPA1A staining in many cells of this type displayed a punctate pattern similar to replication and repair-associated nuclear proteins from other organisms [Lisby and Rothstein, 2005].

The weak DAPI staining in a cell has been known as an indicator that the cell is in its S-phase and active in replicating its DNA [Honore et al., 2002; Yamada et al., 2005]. This observation strongly suggests that CpRPA1A is associated with DNA replication. To further test this hypothesis, parasites were grown in the presence of 5-bromodeoxyuridine (BrdU) and examined for both CpRPA1A and BrdU using immunofluorescence. Parasites and host cells where DNA had recently been replicated could be identified by the presence of incorporated BrdU. Using this approach, young parasites expressing RPA1A were identified, as well as parasites with both BrdU and RPA1A. This indicates that RPA1A expression is present during DNA replication, but also slightly precedes DNA replication (Fig. 1).

We also examined the expression of CpRAP1B during parasite development over the 48-h period following infection of HCT8 host cells. CpRPA1B only produced weak signal in younger parasite cultures. However, in older cultures (24–48 h), parasites displaying increased CpRPA1B signal became apparent (Fig. 1). Developing oocysts and stages believed to be microgametes were particularly intense. The expression of CpRPA1B protein is consistent

with previously reported transcript levels from parasites grown under similar conditions [Rider et al., 2005]. This suggests that CpRPA1B may be utilized when type two merogony, and/or gamete and zygote formation is taking place. Combined, the data suggest that the two RPA1 proteins from *C. parvum* play important roles at very different stages of development.

UV-C Reduced the Excystation of *C. parvum* Oocysts in a Dose-Dependent Manner

RPA proteins have been found to play a role during DNA repair. To determine if this may be the case for either of the *C. parvum* RPA1 proteins, we wished to examine expression after DNA damage. The inactivation of *C. parvum* oocysts by UV treatment has been previously reported [see Rochelle et al., 2005 for review]. These early studies were mainly designed to investigate the disinfecting effect of UV treatment on the parasite. Our data first confirmed the observations of others that UV-C light from germicidal lamps is capable of reducing the excystation of *C. parvum* oocysts. We found that UV-C at relatively low levels was sufficient to inhibit excystation, but that complete (100%) inhibition of excystation under the conditions used was not achieved. The calculated UV irradiation dose required for 50% reduction in excystation (ID_{50}) was 66.13 ± 1.38 mJ/cm^2. We utilized a much lower dose in our experiments to prevent killing parasites, since the goal of our experiments was to identify an irradiation dose that could cause DNA damage (rather than to completely inactivate all oocysts) and be applied to the subsequent experiments. The levels of UV irradiation used for our experimental treatments (21 mJ/cm^2) have independently been reported to cause DNA damage as evidenced by an accumulation of cyclobutyl-thymine dimers in individual oocysts [Al-Adhami et al., 2006].

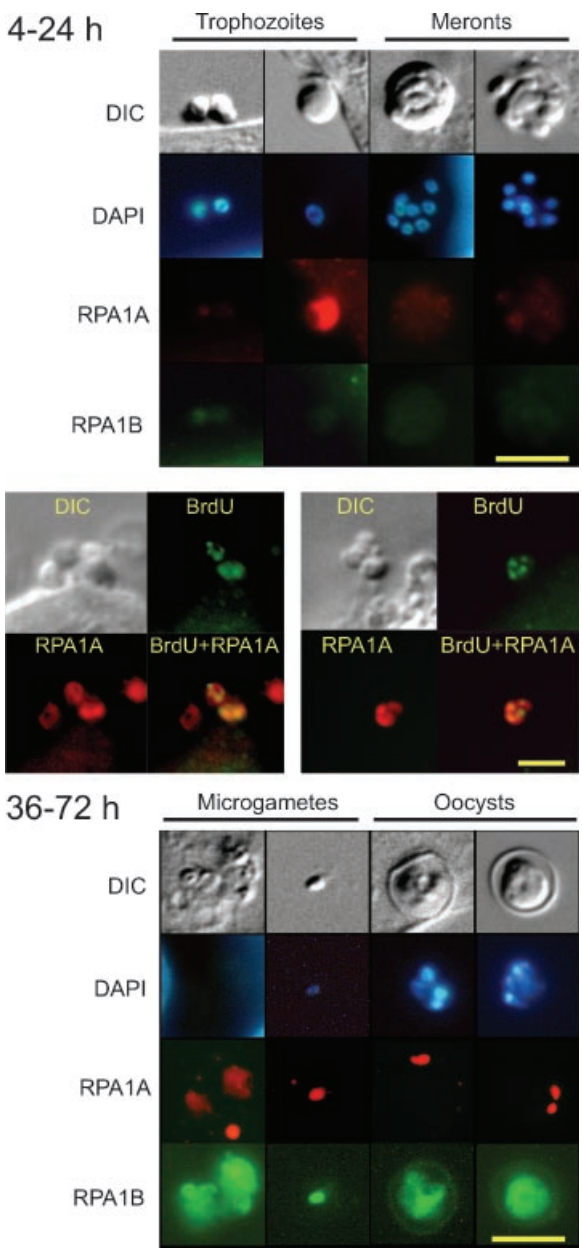

Fig. 1. Top: Expression of CpRPA1A (red) and CpRPA1B (green) during early cultivation on HCT8 host cells *in vitro* (4–24 h). Gray-scale panels are DIC images taken of the parasites and DAPI staining is presented in blue. The early stages include growing parasites of increasing age (columns 1–3) and merozoites that are about to be released (column 4). CpRPA1A signal appears to fluctuate periodically during these replicative phases, while CpRPA1B remains near the limits of detection (greenish background equivalent to the host cells). DAPI stain for the parasite in panel 2 was relatively low. Middle: Recent incorporation of 5-bromodeoxyuridine (BrdU, green) into parasite DNA correlates with maximal RPA1A expression (red), although it appears that RPA1A expression precedes DNA replication slightly since some parasites express RPA1A but have not yet incorporated any detectable BrdU. Bottom: Parasites from late growth cultures (36–72 h). Colors are the same as indicated for the top panel. CpRPA1B displays substantial increases in certain stages—particularly developing oocysts (columns 3–4) and microgametes (columns 1–2). The yellow scale bars represent 5 μm.

The Expression of *CpRPA1B* was Up-Regulated in Response to UV-C Treatment

The expression levels of RPA1 and NER genes in oocysts in response to the UV-C treatment (21 mJ/cm^2) as determined using real-time qRT-PCR analysis are summarized in Table I. Our data show that *CpRPA1B* and RAD3 (likely involved in DNA repair) gene expression

TABLE I. Transcript Levels for *C. parvum* Nucleotide Excision Repair (NER) and DNA Replication Gene Homologues Following Treatment with UV-C

		Delta CT			
Group	Locus (homolog)	UV treated average	Mock treated average	*t*-test (*P*)	Fold change
RPA	cgd3_2210 (RPA1A)	16.7 ± 0.57	17.55 ± 1.20	0.231	1.803
RPA	cgd7_4620 (RPA1B)	12.5 ± 0.71	14.2 ± 0.00	0.038	3.249
RPA	cgd2_4080 (RPA2)	15.25 ± 0.07	15.15 ± 0.35	0.366	0.933
ERCC/XP	cgd4_440 (RAD2)	11.3 ± 0.42	11.3 ± 0.99	0.500	1.000
ERCC/XP	cgd7_820 (RAD3)	12 ± 0.14	13.4 ± 0.00	0.003	2.639
ERCC/XP	cgd6_1530 (RAD4)	13.55 ± 0.21	13.35 ± 0.21	0.223	0.871
ERCC/XP	cgd6_2610 (RAD10)	14.65 ± 0.64	14.55 ± 0.07	0.423	0.933
ERCC/XP	cgd4_2360 (RAD23 related)	13.6 ± 0.57	14.5 ± 0.14	0.080	1.866
ERCC/XP	cgd1_3420 (XPF/MUS81)	18.3 ± 0.57	17.8 ± 1.41	0.344	0.707
Synthesis	cgd3-4290 (DNA pol alpha)	16.4 ± 2.12	8.95 ± 0.49	0.020	0.006
Synthesis	cgd6-4410 (DNA pol delta)	14.15 ± 0.78	13 ± 0.57	0.116	0.451
Synthesis	cgd8_1240 (DNA pol epsilon)	4.05 ± 0.78	5.55 ± 1.77	0.193	2.828
Synthesis	cgd3_3470 (PCNA)	15.8 ± 1.56	15.45 ± 0.78	0.401	0.785
Synthesis	cgd8_2150 (PCNA)	13.75 ± 0.78	14.8 ± 0.42	0.118	2.071
Synthesis	cgd3_1450 (RFC-like)	6.6 ± 1.84	6.95 ± 0.35	0.408	1.275
Synthesis	cgd3_3170 (RFC-like)	15.65 ± 2.47	15.85 ± 3.75	0.478	1.149
Synthesis	cgd7_4690 (RFC-like)	17.35 ± 0.92	18.5 ± 0.57	0.135	2.219
Ligation	cgd3_3820 (Ligase I)	17 ± 0.71	18.35 ± 0.49	0.079	2.549
ERCC/XP	cgd8_1940 (RAD25)	13.4 ± 0.85	14.95 ± 0.35	0.070	2.928
SSB	cgd4_2060 (RecA-like)	13.1 ± 1.83	13.7 ± 1.27	0.370	1.516
SSB	cgd6_4800 (RecA-like)	20.8 ± 1.97	21.85 ± 0.21	0.267	2.071
SSB	cgd7_1690 (DMC)	11.15 ± 0.35	10.75 ± 1.48	0.373	0.758
SSB	cgd5_410 (RAD51)	17.9 ± 2.96	19.25 ± 1.34	0.309	2.549

Only loci that were detected in all samples are presented here. Significant changes in expression (based on Student's *t*-test of the ΔC_T, $P < 0.05$) were observed for only a few genes, including the up-regulation of *CpRPA1B* and *RAD3*, and the down-regulation of DNA polymerase alpha. Other loci of note include: *DNA ligase I*, *RAD25*, and a *RAD23-related* gene that were near the threshold of significance. The *18s rRNA* transcript was used as the normalization control.

was up-regulated in oocysts within 30 min after UV-treatment (seen as a decrease in the ΔC_T). Moderate increases in the expression of the *CpRPA1A* and RAD51 genes were also observed. On the other hand, the DNA polymerase alpha (replication-associated) gene expression was down-regulated. Substantial changes in the expression of most of the other loci under investigation were not observed (Table I). These observations in general agree with our working hypothesis that transcripts for DNA repair proteins are being favored at the expense of transcripts encoding DNA replication proteins and that CpRPA1B is associated with DNA repair.

To determine if the up-regulation of CpRPA1 transcript leads to increased CpRPA1 protein levels, we examined the CpRPA1 proteins present in UV-C treated oocysts at different times following UV exposure using indirect immunofluorescence microscopy (Fig. 2). All samples displayed similar DAPI staining indicating that penetration of small molecules into the oocysts occurred similarly in all treatments. Very weak signals were observed for both RPA1 proteins in untreated oocysts. No obvious changes in CpRPA1A protein levels were observed in UV-treated oocysts (data not shown). On the other hand, short-term exposures under epi-fluorescence revealed a mixed population of oocysts with regard to CpRPA1B signals in UV-C treated samples at 90 min post-exposure. CpRPA1B signals in the 90-min samples varied from one oocyst to another with some oocysts displaying increased fluorescent signals. Other samples produced homogeneous populations of oocysts bearing relatively weak signals for CpRPA1B. Long epi-fluorescence exposures of the samples displaying negligible CpRPA1B revealed a relatively uniform signal among oocysts that was near the levels of background (see Fig. 2, inset of 90 min with no UV-C treatment). Western blots of sporozoite proteins obtained from UV treated and mock treated oocysts did not show substantial differences in protein abundance between samples, probably due to the lower sensitivity of this method (data not shown). We also examined the sub-cellular distribution of the CpRPA1 proteins in sporozoites derived from UV-C treated oocysts by using indirect immunofluorescence microscopy. Cytosolic GAPDH was examined as control. Gross changes in expression or nuclear localization for these proteins were not as obvious as in the oocyst

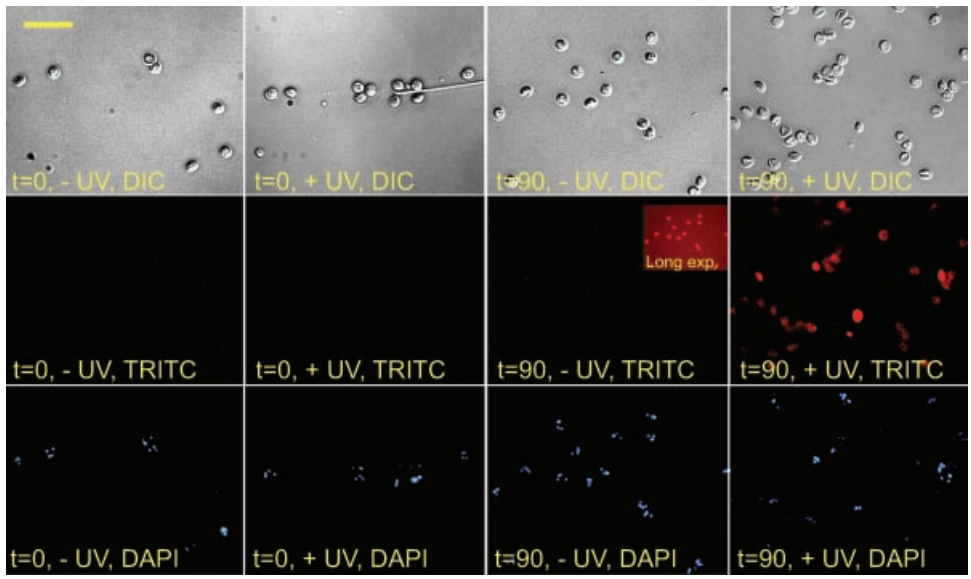

Fig. 2. CpRPA1B protein expression in oocysts after UV treatment. Each column of images represents a different set of conditions (labeled at top). Images from different treatments were taken with the same exposure settings for comparison among treatments. Images at the top are DIC, middle represents indirect immunofluorescence for CpRPA1B (red), and bottom is DAPI nuclear stain (blue). Short-term exposures, reveal that CpRPA1B was discernibly more intense in UV treated oocysts by 90 min after UV exposure. Limited CpRPA1B staining could be observed in all samples if long exposure times were used, but with concomitant increases in background signal (an example is presented as the inset for the 90 min time point without UV exposure). All samples displayed similar staining with DAPI, which served as a control. The scale bar represents 20 μm.

samples examined for CpRPA1B and the bulk of the signal for all three proteins was cytosolic. However, analyses of digital images taken of the sporozoites revealed very small but significant decreases in nuclear localization for CpRPA1A and small but significant increases in nuclear localization of CpRPA1B (data not presented).

The above data suggested that CpRPA1B levels were increased by UV treatment/DNA damage. However, both oocysts and sporozoites had previously been shown to express low levels of CpRPA1B [Millership and Zhu, 2002; Rider et al., 2005] and the small changes identified by immunofluorescence were not able to be confirmed using an alternative method (e.g., Western blotting). To further test whether CpRPA1B was truly inducible by DNA damage, we examined intracellular growing parasites exposed to UV-C light. As observed previously with the developmental study, CpRPA1B was undetectable in parasites that were not exposed to UV light. However, CpRPA1B was induced in some intracellular parasites that were exposed to UV light and fixed at either 45 or 90 min after UV exposure (Fig. 3). Three interesting phenomena were revealed with this experiment: (1) UV-induced RPA1B was clearly observed only in mature Type I meronts that

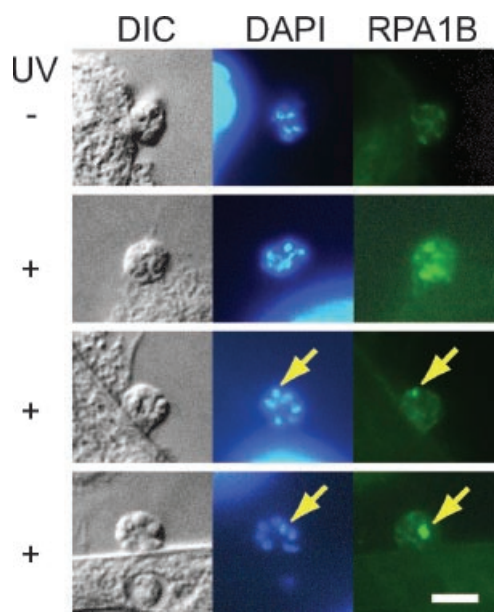

Fig. 3. UV-induced expression of RPA1B in meronts. Top row indicates typical RPA1B expression in mature meronts as controls. Bottom three rows are images taken of mature meronts that were fixed 90 min after exposure to 21 mJ/cm^2 UV-C irradiation. In some meronts, individual merozoites may display RPA1B signal that corresponds well with individual nuclei (arrows). The white scale bars represent 5 μm.

had undergone merogony (contained merozoites); (2) individual merozoites within the meront responded in an autonomous manner (e.g., some expressed CpRPA1B and others did not); and (3) CpRPA1B was often localized to the same regions as the DAPI signals from individual merozoite nuclei. Approximately 1 meront in every 15–20 mature meronts (depending on replicate) from the UV-treated group displayed a clear CpRPA1B immunofluorescent signal, while the mock treated group failed to display increased CpRPA1B immunofluorescent signals in any of the approximately 300 mature meronts that were examined. This data clearly indicates a change in CpRPA1B expression that is associated with DNA damaging levels of UV exposure.

DISCUSSION

Apicomplexan parasites are unique in that they possess both long and short versions of the RPA1 loci that probably arose from a common ancestor [Rider et al., 2005]. In other eukaryotic organisms, RPA1 proteins of either the long type or the short type have been reported, with the short-type apparently being present only among protozoa [Brown et al., 1994; Zhu et al., 1999; Voss et al., 2002; Rider et al., 2005]. The systems that have been more extensively studied possess long type RPA1 proteins that have been implicated in essential roles in DNA metabolism. Early studies have shown that transcripts for the two RPA1 genes in *C. parvum* were differentially expressed, and their encoded proteins have different ssDNA-binding properties, suggesting that they might play unique roles in DNA metabolism [Millership and Zhu, 2002; Rider et al., 2005]. Examination of the expression patterns of *CpRPA1A* and *CpRPA1B* genes and their encoded proteins in *C. parvum* has revealed that these two proteins can be implicated in DNA replication, DNA repair, and DNA recombination. However, it appears that *C. parvum* prefers to utilize the two RPA proteins for specialized roles—being either replication-oriented (CpRPA1A) or recombination/repair-oriented (CpRPA1B). This does not preclude the possibility that these proteins have partially redundant or overlapping biological functions. A division of RPA1 functions is not unprecedented. Similar observations have been identified in the reference plant *Arabidopsis thaliana*, where one essential RPA1 is suspected of being utilized for DNA replication, and a second, non-essential RPA1, has been demonstrated to help protect against DNA damage [Ishibashi et al., 2005]. Additionally, three distinct RPA heterotrimers have been found in rice, and their sub-cellular distributions suggests unique roles for each heterotrimer [Ishibashi et al., 2001, 2005, 2006].

Cell-cycle regulation of RPA has been demonstrated in all of the systems where this has been examined. In several systems, it appears that the phosphorylation of RPA (RPA2, in particular) is intimately involved in this regulation during the cell cycle and in response to DNA damage [Pasion et al., 1994; Brown and Ray, 1997; Wold, 1997; Iftode et al., 1999; Perdigao et al., 1999; Voss et al., 2002; Marwedel et al., 2003]. In protozoa, however, RPA abundance has been demonstrated to fluctuate in a cell-cycle-dependent manner [Brown and Ray, 1997; Voss et al., 2002]. This lends itself to speculation that the CpRPA1 proteins are also being regulated in a cell-cycle-dependent manner. CpRPA1A is the short type RPA1 in *C. parvum* and is primarily expressed in early intracellular stages of the parasite. Mertonts of *C. parvum* can be observed that have a smooth appearance and no obvious merozoites within the meront, while other meronts may display signs of merozoites within the meront. The distinction between parasites that have undergone this "cellularization" to complete endopolygeny is important for differentiating between immature growing meronts and mature meronts. Maximal CpRPA1A expression is associated with immature parasites (meronts) that frequently display reduced DAPI staining and no obvious internal cellularization (merozoites). However, in meronts with distinct merozoites and obvious nuclei, CpRPA1A protein is low. The reduced DAPI staining observed here in *C. parvum* parasites appeared to correlate with the onset of the S-phase because independent experiments showed that BrdU incorporation during DNA replication occurs in parasites with increased RPA1A expression. Interestingly, similar experiments in mouse and human cells indicate that early S-phase cells present reduced DAPI nuclear staining [Honore et al., 2002; Yamada et al., 2005]. Although markers for cell-cycle stages other than S phase are not readily available for *C. parvum*, CpRPA1B was probably UV induced at the M-phase/G1 transition since division to

produce the individual merozoites had clearly occurred but the merozoites had not yet been released to initiate a second round of parasitic growth. It is not clear if RPA1B expression is cell-cycle regulated in later stage cultures since cells normally expressing CpRPA1B at high levels were much less abundant.

CpRPA1B is up-regulated at the level of transcript and at the level of protein and is mobilized to the nucleus following exposure to UV-induced damage. DNA repair has been detected in oocysts following similar UV treatment [Oguma et al., 2001; Morita et al., 2002; Rochelle et al., 2005]. This suggests a role for CpRPA1B in DNA repair-related processes. Transcript levels for RAD51 and RAD3 (known to be involved in DNA repair in other organisms) were also up-regulated moderately or significantly, respectively, following UV exposure (Table I), lending additional support to the idea that changes in *CpRPA1B* expression coincide with DNA repair. The presence of an RPA1 protein more dedicated to DNA repair may beneficial to this parasite, since the parasite oocysts (an environmental stage) are always at risk for DNA damage caused by UV irradiation from the sun. Similarly, extracellular motile stages (merozoites) may need to be prepared for environmental variables that could induce DNA damage.

In the absence of DNA damage, *CpRPA1B* is poorly expressed in early parasite cultures, but does become apparent in later parasite cultures. These late-stage parasites may represent type two meronts and/or young reproductive stages. Thus, CpRPA1B may play a role in recombination as well as repair.

The observations presented here, combined with previous biochemical analyses, strongly suggest divergent roles for the two RPA1 proteins in *C. parvum*. However, many new questions arise about the specific roles the long and short RPA1 proteins play in *C. parvum* and whether or not these observations will apply directly to other apicomplexans. Additional work on *C. parvum*, as well as other more genetically tractable apicomplexans may help to clarify these issues.

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