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Biochemistry

Conservation of structure and function of DNA replication protein A in the trypanosomatid *Crithidia fasciculata*

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ABSTRACT **Human replication protein A (RP-A) is a three-subunit protein that is required for simian virus 40 (SV40) replication *in vitro*. The trypanosome homologue of RP-A has been purified from *Crithidia fasciculata*. It is a 1:1:1 complex of three polypeptides of 51, 28, and 14 kDa, binds single-stranded DNA via the large subunit, and is localized within the nucleus. *C. fasciculata* RP-A substitutes for human RP-A in the large tumor antigen-dependent unwinding of the SV40 origin of replication and stimulates both DNA synthesis and DNA priming by human DNA polymerase α/primase, but it does not support efficient SV40 DNA replication *in vitro*. This extraordinary conservation of structure and function between human and trypanosome RP-A suggests that the mechanism of DNA replication, at both the initiation and the elongation level, is conserved in organisms that diverged from the main eukaryotic lineage very early in evolution.**

Trypanosomes are protozoan parasites of considerable medical and economic importance; they cause serious disease in humans and livestock. They are characterized by the presence of a kinetoplast, a massive network of catenated circular DNA molecules located at the base of the flagellum within the single mitochondrion of these cells. Trypanosomes diverged from the major eukaryotic lineage very early in the evolutionary history of eukaryotic cells relative to the separation of the "higher" evolutionary groups, the plants, animals, and fungi (1, 2). Studies of transcription in trypanosomes have revealed a number of unusual features, including trans-splicing, a process by which discontinuously synthesized precursor RNAs are spliced to yield mature messenger RNAs, and RNA editing, which involves the insertion and deletion of uridine residues in mitochondrial RNA transcripts. Studies of DNA replication in trypanosomes have focused mainly on the kinetoplast minicircle DNA, a molecule unique among mitochondrial DNAs for its high copy number and its cell cycle-regulated replication (for review, see refs. 3 and 4). The biochemical mechanism and the regulation of nuclear DNA replication in trypanosomes are poorly understood. Only one protein expected to be involved in nuclear DNA replication has been purified to homogeneity from a trypanosome, a type I topoisomerase from *Crithidia fasciculata* (5). Two RNase H activities in crude extracts of *C. fasciculata* have been described (6) and seem to be similar to the class I and class II RNase H enzymes of higher eukaryotes. Two DNA polymerases have been partially purified from *C. fasciculata* and *Trypanosoma brucei* (7, 8). These proteins are similar to DNA polymerases α and β of higher eukaryotes, but they were found to differ from their mammalian homologues in their response to several DNA polymerase inhibitors (7). Little else is known of other proteins involved in chromosomal DNA replication in trypanosomes and their biochemical properties—for example, whether they are homologous to known replication proteins from higher eukaryotes, whether they are regulated in a cell cycle-dependent fashion, and whether their properties can be exploited as targets for therapeutic drugs. Neither proteins involved in initiation of DNA replication nor proteins involved in the cell cycle regulation of replication have been identified.

Replication protein A [RP-A; also called RF-A or human single-stranded DNA-binding protein (SSB)] was first isolated from a protein fraction of human cells required for the *in vitro* replication of simian virus 40 (SV40) (9–11). It is a three-subunit, multifunctional SSB involved in both the initiation and elongation phases of SV40 replication. With topoisomerase I and the SV40 large tumor antigen (T-Ag), RP-A participates in the unwinding of the SV40 origin of replication during the initiation of DNA replication (12–14). RP-A stimulates DNA synthesis by DNA polymerases α and δ (15–17), suggesting that RP-A functions in the elongation phase of DNA replication *in vitro*. RP-A is present in the yeast *Saccharomyces cerevisiae* (18), indicating that its activity is evolutionarily conserved. In both yeast and human cells, RP-A is posttranslationally modified by phosphorylation of the middle subunit in a cell cycle-dependent manner (19), suggesting a role for RP-A in the cell cycle regulation of DNA replication. Extracts from cells in G_1 phase, the phase in which RP-A is unphosphorylated, have lower activity in unwinding the SV40 origin of replication and are 10 times less efficient at initiating SV40 replication, when compared to extracts from cells in S or G_2 phase, the phases during which RP-A is phosphorylated (20). A link between a regulatory role for RP-A and the cell cycle control machinery is suggested by the observation that G_1 extracts can be activated by a fraction that contains a kinase homologous to the $p34^{cdc2}$ kinase (21). The genes encoding the three subunits of RP-A in yeast are all essential for viability (22), indicating the essential nature of the functions of RP-A. In this report, the identification and characterization of the trypanosome homologue of RP-A are described.

MATERIALS AND METHODS

Cell Growth. *C. fasciculata* Cf-C1 was grown, harvested, and stored frozen at −75°C as described (23).

Purification of RP-A. RP-A was purified from *C. fasciculata* as described for the purification of RP-A from yeast (18), with several modifications. Fifty grams of frozen cells (≈5 × 10^{11} cells) was thawed in 250 ml of 50 mM Hepes (pH 7.5), 10 mM EDTA, 0.5 M NaCl, 2.5 mM 2-mercaptoethanol, leupeptin (2 μg/ml), pepstatin A (1 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine. After homogenization with a motor-driven Teflon pestle, the lysate was stirred for 30 min at 0°C. The lysate was clarified by centrifugation in a Beckman Ti45 rotor (40,000 rpm for 30 min at 2°C). The supernatant was filtered through Miracloth and

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Abbreviations: RP-A, replication protein A; SV40, simian virus 40; ssDNA, single-stranded DNA; SSB, single-stranded DNA-binding protein; T-Ag, large tumor antigen.

centrifuged for an additional 90 min at 40,000 rpm at 2°C in a Ti45 rotor. The clear amber supernatant was loaded onto a 2.5 × 3 cm single-stranded DNA (ssDNA)-cellulose (Sigma) column equilibrated in 25 mM Hepes (pH 7.5), 1 mM EDTA, 10% (vol/vol) glycerol, 0.02% (wt/vol) Brij 58, 2.5 mM 2-mercaptoethanol, leupeptin (1 μg/ml), pepstatin A (0.5 μg/ml), and 0.25 mM phenylmethylsulfonyl fluoride (buffer A) containing 0.5 M NaCl. The column was washed with 3 vol of buffer A containing 0.75 M NaCl, and the protein was eluted with buffer A containing 1.5 M NaCl and 50% (vol/vol) ethylene glycol. Protein-containing fractions were pooled and dialyzed against 25 mM imidazole hydrochloride (pH 7.5), 0.1 mM EDTA, 10% glycerol, 0.02% Brij 58, 2.5 mM 2-mercaptoethanol, leupeptin (1 μg/ml), pepstatin A (0.5 μg/ml), and 0.25 mM phenylmethylsulfonyl fluoride (buffer B) containing 10 mM NaCl. The dialysate was clarified by passage through a 0.2-μm nitrocellulose filter and applied to a Mono Q column (HR 5/5; Pharmacia). The column was washed with 5 ml of buffer B containing 10 mM NaCl, and the protein was eluted with a 20-ml linear gradient from 10 mM to 400 mM NaCl in buffer B. This procedure yielded 300 μg of RP-A, as determined by a modified Bradford assay (24), using bovine serum albumin as a standard. The preparation was 94% homogeneous as judged by scanning densitometry of Coomassie brilliant blue-stained polyacrylamide gels. The molar ratio of the subunits was also determined by densitometry.

Reaction Conditions. SV40 origin unwinding assays (25 μl) were performed as described (13), with incubation at 37°C for 30 min. Reactions were terminated, and reaction mixtures were processed for electrophoresis as described (15) and electrophoresed for 12 hr at 25 V through a 1.5% agarose gel in 40 mM Tris acetate, pH 7.5/1 mM EDTA. DNA was detected by staining for 1 hr in ethidium bromide (0.5 μg/ml) followed by visualization with a UV transilluminator.

Binding of ssDNA by RP-A subunits was investigated by protein–DNA blotting of RP-A-containing fractions by using thermally denatured, ^{32}P-labeled pUC19 DNA as a probe, as described (25).

SV40 replication reactions were performed by using purified T-Ag, topoisomerases I and II, replication factor C, proliferating-cell nuclear antigen, and fraction IIA, as described (13).

DNA polymerase stimulation assays were performed with polymerase α/primase (333 ng/ml) with poly(dA)·oligo(dT) as template-primer DNA (17). Primase stimulation assays contained 20 mM Tris (pH 7.8), 8 mM $MgCl_2$, 4% glycerol, 4 mM dithiothreitol, bovine serum albumin (0.5 mg/ml), poly(dT) (0.1 mM nucleotide; average length of 300 nucleotides) as template DNA, 0.5 mM ATP, 50 μM [^{32}P]dATP (3000 cpm/pmol), polymerase α/primase (167 ng/ml), and *Escherichia coli* DNA polymerase I Klenow fragment (10 units/ml).

Immunization Procedure. Purified RP-A (150 μg) in 0.5 ml of phosphate-buffered saline (PBS; 20 mM KPO_4, pH 7.5/150 mM NaCl) was emulsified with 0.5 ml of 2× concentrated RIBI adjuvant (Ribi Immunochem) in PBS and injected subcutaneously into a female New Zealand White rabbit at five sites. The rabbit was boosted on day 28 following the same protocol and bled on day 38.

Immunoblots. Proteins were separated by SDS/PAGE (26) and transferred to 0.2-μm pore size nitrocellulose filters in 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). Filters were blocked in PBS plus 0.05% Tween 20 (PBST) containing 2% (wt/vol) bovine serum albumin and 5% (vol/vol) goat serum for 1 hr at 22°C. Filters were incubated in a 500-fold dilution of antiserum for 1.5 hr, washed three times with PBST, incubated with a 5000-fold dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody for 30 min, washed three times with PBST, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylyl phosphate (Promega).

Immunocytochemistry. Immunolocalizations were performed essentially as described (5), using a 200-fold dilution of antiserum raised against *C. fasciculata* RP-A.

RESULTS

Purification of RP-A From the Trypanosomatid *C. fasciculata*. The *C. fasciculata* homologue of RP-A was purified by using ssDNA-cellulose and Mono Q column chromatography. As is the case with RP-A from *S. cerevisiae* and human cells, the protein from *C. fasciculata* bound tightly to ssDNA, requiring greater than 0.75 M NaCl for elution. The Mono Q column removed trace contaminants present after the ssDNA column and concentrated the RP-A. Fig. 1*a* shows SDS/PAGE analysis of each step in the purification and Mono Q column fractions 1–10. The peak of *C. fasciculata* RP-A eluted from the Mono Q column at ≈125 mM NaCl, in fractions 5 and 6. These fractions contained three polypeptides with molecular masses of 51 kDa, 28 kDa, and 14 kDa

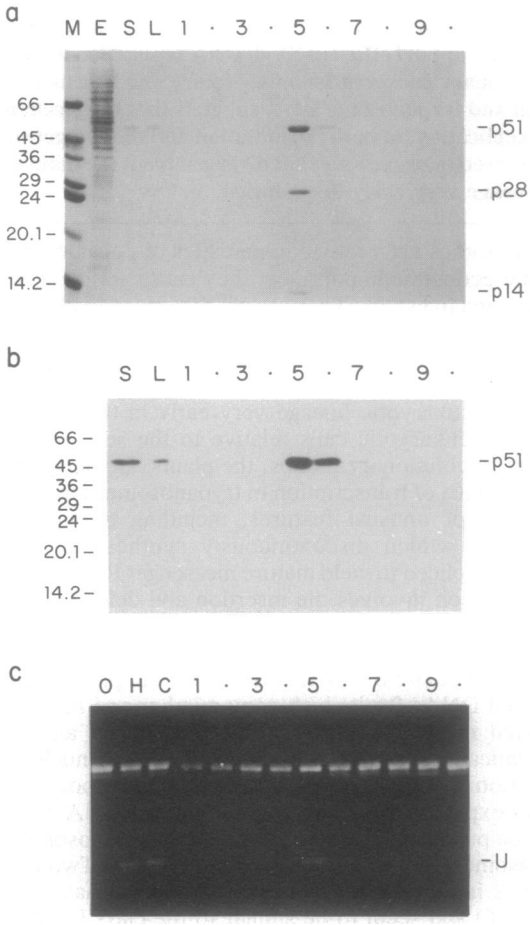

FIG. 1. Cofractionation of *C. fasciculata* RP-A protein, ssDNA binding activity, and SV40 origin unwinding activity. (*a*) SDS/PAGE and Coomassie brilliant blue stain analysis of molecular mass markers (lane M; in kDa), the clarified extract (lane E), ssDNA-cellulose eluate (lane S), Mono Q load (lane L), and Mono Q fractions 1–10 (lanes 1–10). The positions of the three RP-A subunits are indicated (p51, p28, and p14). (*b*) ssDNA binding activity of Mono Q fractions 1–10 analyzed by protein–DNA blotting of these fractions, using ^{32}P-labeled ssDNA as a probe. The position of the large subunit of RP-A is indicated (p51). (*c*) SV40 origin unwinding assays containing T-Ag, topoisomerase I, SV40 origin-containing plasmid, and no RP-A (lane O), 500 ng of human RP-A (lane H), 500 ng of *E. coli* ssDNA binding protein (lane C), or Mono Q fractions 1–10 (lanes 1–10). The position of the underwound form is indicated (U).

(p51, p28, and p14), in a molar ratio of 0.87:1.0:0.98. The three-subunit structure of RP-A in yeast and human cells is conserved in *C. fasciculata*, although the molecular masses of the subunits differ [69 kDa, 36 kDa, and 13 kDa for yeast (18); 70 kDa, 34 kDa, and 11 kDa for human (10)].

Identification of the Subunit That Binds to ssDNA. For yeast and human RP-A, the large subunit is responsible for binding ssDNA (18, 25). The subunit of *C. fasciculata* RP-A that binds ssDNA was identified by blotting SDS/PAGE-separated samples of the ssDNA-cellulose pool, Mono Q load, and Mono Q fractions 1–10 onto a nitrocellulose filter and probing with ^{32}P-labeled ssDNA (Fig. 1*b*). This analysis demonstrated that only the large subunit of *C. fasciculata* RP-A (p51) binds ssDNA and that the ssDNA binding activity cofractionates with the three RP-A polypeptides on the Mono Q column.

***C. fasciculata* RP-A Substitutes for Human RP-A in Unwinding the SV40 Origin of Replication.** In the presence of T-Ag, topoisomerase I, and RP-A, SV40 DNA is converted to a topologically underwound form, which is an early intermediate in the initiation of SV40 DNA replication, prior to primer formation and origin-specific DNA synthesis (12, 13, 27). Although human RP-A is required for SV40 DNA replication, yeast RP-A (18), *E. coli* SSB (12), adenovirus DNA-binding protein, and herpes simplex virus infected-cell polypeptide 8 (15) can each substitute for human RP-A in the formation of the underwound form, in the absence of DNA synthesis. To determine whether *C. fasciculata* RP-A could function in this reaction, unwinding assays were performed using Mono Q column fractions instead of human RP-A (Fig. 1*c*). The same Mono Q fractions that contain *C. fasciculata* RP-A and ssDNA binding activity (fractions 5 and 6) also substituted for human RP-A in unwinding the SV40 origin of replication to produce the underwound form of DNA.

Identification of RP-A Subunits in Fresh *C. fasciculata* Lysates. The molecular masses of the subunits of *C. fasciculata* RP-A differ from those found for yeast and human RP-A subunits. To ensure that the purified RP-A represented the intact form of each of the subunits, as found *in vivo*, samples of purified *C. fasciculata* RP-A and fresh *C. fasciculata* lysates were subjected to immunoblot analysis with an antiserum raised against purified *C. fasciculata* RP-A (Fig. 2). The antiserum identifies polypeptides of 51 kDa and 28 kDa in both the purified RP-A (lanes 1 and 2) and the cell lysate (lane 3). Several polypeptides of >66 kDa are identified faintly in the cell lysate, but are not seen when similar blots are probed with antiserum that has been affinity purified using p51 (data not shown). The antiserum does not identify p14 in any of the samples. Since the major forms of p51 and p28 have the same molecular masses in freshly lysed cells as they do in the purified samples, these likely represent the intact subunits and not proteolytic degradation products, despite their being smaller than the corresponding subunits of human and yeast RP-A.

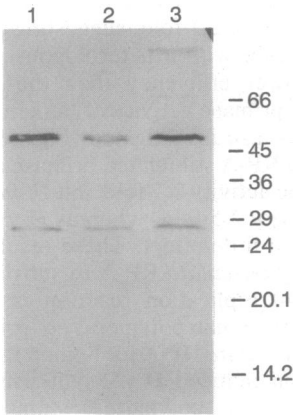

FIG. 2. Immunoblot of purified RP-A and freshly lysed *C. fasciculata*. Purified RP-A (20 ng, lane 1; 10 ng, lane 2) and *C. fasciculata* lysed by boiling in SDS reducing buffer (3.8×10^6 cells; lane 3) were electrophoresed in an SDS/15% polyacrylamide gel and transferred to nitrocellulose. RP-A was detected by using antiserum raised against purified *C. fasciculata* RP-A. The positions of molecular mass markers (in kDa) are indicated.

***C. fasciculata* RP-A is a Nuclear Protein.** Human RP-A is located almost exclusively in the nucleus (28). The intracellular localization of *C. fasciculata* RP-A was determined by immunofluorescence microscopy. Fixed *C. fasciculata* were incubated with polyclonal rabbit antiserum raised against purified RP-A. Bound antibody was then detected with a fluorescein isothiocyanate-labeled secondary antibody. The positions of the nuclear and kinetoplast (mitochondrial) DNA were detected with the DNA-binding dye 4′,6-diamidino-2-phenylindole. Fig. 3*a* shows the staining of the nuclei and kinetoplasts with 4′,6-diamidino-2-phenylindole. Fig. 3*b* shows fluorescein isothiocyanate staining of the same field of cells. Anti-RP-A antibody is found exclusively in the nucleus of each cell, indicating that *C. fasciculata* RP-A is a nuclear protein, consistent with it functioning in chromosomal replication. Use of preimmune serum in place of anti-RP-A antiserum produced no visible staining (not shown).

***C. fasciculata* RP-A Cannot Replace Human RP-A in the SV40 DNA Replication Reaction.** *C. fasciculata* RP-A was tested for its ability to substitute for human RP-A in the *in vitro* SV40 replication system reconstituted with purified proteins and fraction IIA (Fig. 4*a*). In this reaction, saturating concentrations of human RP-A (37.5 μg/ml) yielded an incorporation of 25.4 pmol of dAMP. Substituting *C. fasciculata* RP-A at as much as 50 μg/ml yielded an incorporation of only 1.9 pmol of dAMP, which is <10% of the incorporation seen with human RP-A. This level of DNA synthesis was similar to that seen when yeast RP-A or *E. coli* SSB was substituted for human RP-A (3.5 and 2.5 pmol of dAMP incorporated, respectively), but greater than the peak level of synthesis seen with T4 gene *32* protein (0.3 pmol of dAMP with T4 gene *32* protein at 50 μg/ml).

The products of these SV40 replication reactions were analyzed on a denaturing agarose gel (Fig. 4*b*). Ten-fold less of the human RP-A-containing reaction mixtures was analyzed to compensate for the ≈10-fold greater synthesis in these reactions. The products of the reactions containing human RP-A (lanes 2–5) migrated as unit-length linear single-stranded molecules or as form I molecules, with a smear of DNA strands that are smaller than unit length. The products of the reactions containing yeast RP-A (lanes 10–13) were similar, but included strands of greater than unit length, probably resulting from background repair synthesis. The

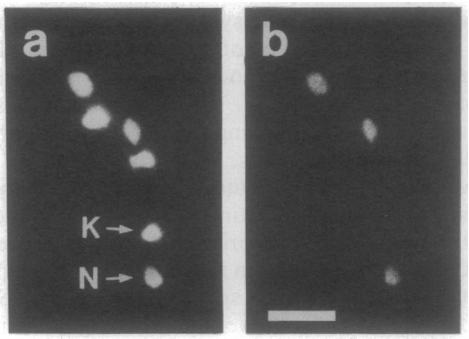

FIG. 3. Intracellular localization of RP-A using indirect immunofluorescence. (*a*) 4′,6-Diamidino-2-phenylindole-stained DNA structures. (*b*) Cells were treated with antiserum raised against purified *C. fasciculata* RP-A, followed by a fluorescein isothiocyanate-labeled second antibody. The positions of the nuclear DNA (N) and the kinetoplast (mitochondrial) DNA (K) are indicated. (Bar = 5 μm.)

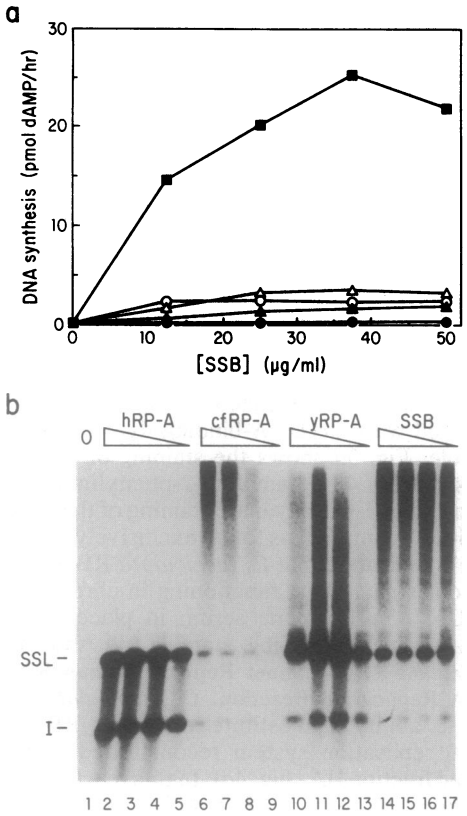

FIG. 4. *C. fasciculata* RP-A substitution in SV40 replication reactions. (*a*) Human RP-A (■), yeast RP-A (△), *C. fasciculata* RP-A (▲), *E. coli* SSB (○), and T4 gene *32* protein (●) at 0–50 μg/ml were incubated with optimized amounts of T-Ag, topoisomerase I, topoisomerase II, proliferating-cell nuclear antigen, replication factor C, fraction IIA (which contains DNA polymerases α and δ and maturation factors), and SV40 origin-containing plasmid, and DNA synthesis was measured. (*b*) Samples of the replication products were analyzed on denaturing agarose gels. Reactions contained the following concentrations of the indicated proteins. Lanes: 1, 0 μg/ml; lanes 2, 6, 10, and 14, 50 μg/ml; lanes 3, 7, 11, and 15, 37.5 μg/ml; lanes 4, 8, 12, and 16, 25 μg/ml; lanes 5, 9, 13, and 17, 12.5 μg/ml. For human RP-A (lanes 2–5), 10-fold less of the replication products was analyzed than in all other lanes. The positions of unit-length single-stranded linear molecules (SSL) and denatured form I molecules (I) are indicated. hRP-A, human RP-A; cfRP-A, *C. fasciculata* RP-A; yRP-A, yeast RP-A.

reactions with *E. coli* SSB (lanes 14–17) showed a larger proportion of ssDNA molecules of greater than unit length. In the *C. fasciculata* RP-A reactions (lanes 6–9), most of the synthesis resulted in the production of these abnormal, long single strands, with very little synthesis of unit-length single-stranded linear molecules or of form I molecules. Thus *C. fasciculata* RP-A substitutes poorly for human RP-A in SV40 DNA replication, yielding <10% of the DNA synthesis seen with human RP-A and resulting largely in the synthesis of abnormally long DNA strands.

***C. fasciculata* RP-A Stimulates Human DNA Polymerase α/Primase.** Human RP-A stimulates DNA synthesis by human DNA polymerase α/primase ≈5-fold, suggesting a role for RP-A in the elongation phase of replication (15, 16). This stimulation appears to be specific, since *E. coli* SSB, T4 gene *32* protein, adenovirus DNA-binding protein, and herpes simplex virus infected-cell polypeptide 8, all of which are SSBs that stimulate their cognate DNA polymerases and are required for DNA replication (29–32), fail to stimulate human polymerase α (15). The effect of *C. fasciculata* RP-A on DNA synthesis by human polymerase α was examined (Fig. 5*a*). Under the conditions used, human RP-A at a

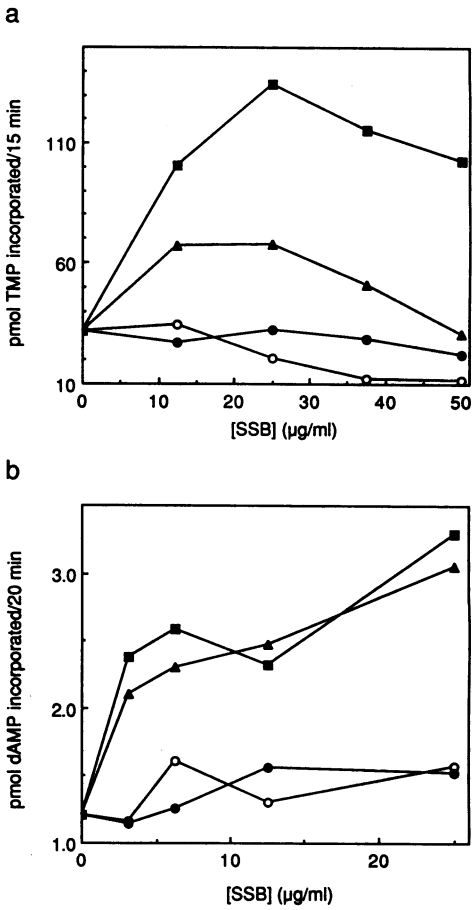

FIG. 5. Stimulation of polymerase α/primase activity by *C. fasciculata* RP-A. (*a*) DNA synthesis by human DNA polymerase α/primase using poly(dA)·oligo(dT) as primer-template DNA in the presence of increasing concentrations of human RP-A (■), *C. fasciculata* RP-A (▲), SSB (○), or T4 gene *32* protein (●). (*b*) DNA primase activity of human DNA polymerase α/primase measured using poly(dT) as template DNA in the presence of increasing concentrations of human RP-A (■), *C. fasciculata* RP-A (▲), SSB (○), or T4 gene *32* protein (●).

concentration of 25 μg/ml stimulated DNA synthesis 4.2-fold, and *E. coli* SSB and T4 gene *32* protein both inhibited DNA synthesis slightly, all consistent with previous findings. At 25 μg/ml, *C. fasciculata* RP-A stimulated DNA synthesis by human polymerase α 2.1-fold, suggesting that it is capable of specific interaction with a heterologous polymerase, although with a lower efficiency than the cognate RP-A. Stimulation of the primase activity of human DNA polymerase α/primase was also investigated (Fig. 5*b*). Both human and *C. fasciculata* RP-A stimulated primase activity; human RP-A increased the activity 2.7-fold and *C. fasciculata* RP-A increased the activity 2.5-fold, whereas *E. coli* SSB and T4 gene *32* protein had little effect. These results indicate that the inability of *C. fasciculata* RP-A to substitute for human RP-A in the SV40 replication reaction is not due to an inability to stimulate human polymerase α/primase. Whereas substituting *C. fasciculata* RP-A for human RP-A in the SV40 replication reaction reduced DNA synthesis 13.6-fold, the same substitution in the polymerase stimulation assay reduced synthesis only 2-fold and had a negligible effect in the primase stimulation assay. Further interactions between RP-A and the other replication proteins must account for the specific requirement for human RP-A in the SV40 replication reaction, perhaps in a complex involved in initiation of DNA synthesis, between the origin-unwinding and elongation phases of replication.

DISCUSSION

A trypanosome homologue of the essential replication protein RP-A has been identified by several criteria. The protein, as purified from *C. fasciculata*, binds tightly to ssDNA-cellulose and is a 1:1:1 complex of three subunits. It binds ssDNA via the largest subunit (p51), is located in the nucleus, functions in a SV40 origin unwinding assay, and stimulates human DNA polymerase α/primase. Proteins that bind tightly to ssDNA but do not function in eukaryotic nuclear DNA replication either fail to substitute in the SV40 origin unwinding assay [e.g., T4 gene *32* protein and calf thymus protein UP1 (15)] or fail to stimulate human DNA polymerase α/primase [e.g., *E. coli* SSB, adenovirus DNA-binding protein, and herpes simplex virus infected-cell polypeptide 8 (15, 16)]. One property of human RP-A that the *C. fasciculata* protein does not share is the ability to support SV40 replication *in vitro*, a property that the yeast RP-A homologue also lacks (18). This conservation of subunit structure and of numerous activities indicates that the protein described here represents the trypanosome homologue of RP-A.

The large subunit of RP-A from *C. fasciculata* is unusually small, measuring 51 kDa, compared to 69 kDa for the yeast subunit and 70 kDa for the human subunit. The molecular mass of 51 kDa represents the intact large subunit, as indicated by Western blot analysis of freshly lysed cells. One function of the large subunit of yeast and human RP-A is the binding of ssDNA. Protein–DNA blotting analysis of *C. fasciculata* RP-A indicates that p51 retains this activity, despite being significantly smaller than the yeast or human large subunits. It will be of interest to determine whether a particular domain is missing in the large subunit of *C. fasciculata* RP-A and if so what the function of that domain is in yeast and human cells.

C. fasciculata RP-A fails to substitute for human RP-A in the SV40 replication reaction, despite functioning in the SV40 origin unwinding reaction, stimulating the primase activity of human DNA polymerase α/primase to the same level as human RP-A, and stimulating the polymerase activity of polymerase α/primase to 50% of the level seen with human RP-A. The reduction in polymerase α stimulation could be more pronounced in the complete replication reaction, due to interactions with other replication proteins and due to polymerase α's function in the initiation of leading-strand synthesis (17, 33–35). Human RP-A interacts specifically with T-Ag (36). The functional significance of this interaction is suggested by the observation that human RP-A, the 70-kDa subunit of human RP-A, and *E. coli* SSB all inhibit DNA polymerase α/primase activity on an artificial replication fork template, but only the inhibition by human RP-A can be overcome by the addition of T-Ag (37). Absence of an important interaction between *C. fasciculata* RP-A and T-Ag might result in the inability of *C. fasciculata* RP-A to substitute for human RP-A in SV40 replication. Failure of *C. fasciculata* RP-A to stimulate polymerase δ may also contribute to its inactivity in SV40 replication, although this is unlikely since stimulation of polymerase δ by SSBs appears to be nonspecific (15).

Nuclear DNA replication in trypanosomes is poorly understood. The striking conservation of the subunit structure and the multiple activities of RP-A in *C. fasciculata* suggest that the basic DNA replication machinery found in higher eukaryotes is conserved in organisms that diverged from the main eukaryotic lineage very early in evolution. Proteins involved in replication, particularly the DNA polymerases, represent good targets for therapeutic drugs, and this conservation of structure and function will facilitate the identification and purification of the other proteins involved in DNA replication in trypanosomes. In yeast and human cells, the middle subunit of RP-A is subject to cell cycle-dependent phosphorylation (19). Determination of the phosphorylation state of *C. fasciculata* RP-A during the cell cycle may indicate if replication proteins in trypanosomes are subject to the same cell cycle regulation as their homologues in higher eukaryotes. Finally, since RP-A functions in the initiation of SV40 replication and interacts specifically with the viral initiator protein T-Ag (36), *C. fasciculata* RP-A will be valuable in studying initiation of DNA replication in trypanosomes.

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