Nucleic Acids Research, 1997, Vol. 25, No. 19 **3881–3888**

A DNA helicase purified by replication protein A (RPA) affinity chromatography from mouse FM3A cells

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Received June 16, 1997; Revised and Accepted August 19, 1997

ABSTRACT

In an effort to identify cellular helicases that mimic the action of SV40 large T-antigen, we performed replication protein A (RPA) affinity chromatography on cell extracts from the mouse mammary carcinoma cell line FM3A. In this way, a novel DNA helicase was isolated and purified to near homogeneity. The most purified fractions showed the presence of two proteins of 28 and 21 kDa. Both proteins interacted with ^{32}P-labeled partially duplex DNA when bound to nitrocellulose membranes and were efficiently UV crosslinked to [α-^{32}P]dATP. Helicase activity was strongly stimulated by RPA on DNA substrates containing duplex regions longer than 18 bp. Only weak stimulation was observed in the presence of *Escherichia coli* single strand DNA binding protein (SSB). The enzyme unwinds DNA in the 5′–3′ direction in relation to the strand to which it binds. Only ATP and dATP were efficient as nucleoside triphosphate co-factors, and showed similar K_m values of ~0.6 mM. The properties of this enzyme suggest that it may take part in reactions mediated by RPA such as those predicted to occur at replication forks or alternatively may function during DNA repair or recombination.

INTRODUCTION

The unwinding of DNA is a prerequisite for a number of transactions such as DNA replication, transcription, recombination and repair. DNA helicases accomplish this task by coupling the hydrolysis of ribonucleoside or deoxyribonucleoside triphosphate to separate the two strands of the DNA double helix (1). DNA helicases have been isolated from a wide variety of sources ranging from bacteriophages to mammalian cells, and are now known to be implicated in several human diseases including Xeroderma pigmentosum, Cockayne's syndrome, Bloom's syndrome and Werner's syndrome (2). We have been particularly interested in the nature of DNA helicases required for DNA replication in mammalian cells. Currently, the precise nature of such enzymes is unknown despite extensive studies and their burgeoning number. No doubt, a central problem is the incertitude surrounding the existence of conserved sequences at mammalian replication origins and the absence of an *in vitro* replication assay to measure their activity (3).

The large T-antigen of SV40 (4) is probably the most extensively studied eucaryotic DNA helicase isolated so far, along with other DNA helicases of viral origin such as Herpes Simplex virus type 1 DNA helicase (5) and E1 DNA helicase of bovine papilloma virus (6). In the SV40 *in vitro* replication assay T-antigen binds to specific sequences within the SV40 origin of replication and initiates the structural changes that eventually lead to the priming of bidirectional DNA replication in the additional presence of the cellular proteins, replication protein A (RPA) and DNA polymerase α. This event is known to require highly specific interactions between RPA, DNA polymerase α and large T-antigen (7).

RPA is a eucaryotic single-stranded DNA binding protein required for DNA replication, DNA excision repair and homologous recombination (8). The protein is a stable complex of three subunits of 70, 32 and 14 kDa. All three subunits are essential for function and yeast cell viability (9). The requirement for RPA in the initiation of SV40 DNA replication cannot be replaced by other single strand DNA binding proteins such as SSB of *Escherichia coli* or the phage T4 gene 32 product, although these enzymes are good co-factors for T-antigen helicase activity. Rather, the interaction between T-antigen and RPA would appear to be necessary during the priming event carried out by DNA polymerase α (10).

As the unwinding of double-stranded DNA at replication origins is an essential and ubiquitous event in DNA replication, a counterpart of SV40 T-antigen has long been predicted to exist in mammalian cells. If this is the case, then such a DNA would also be expected to interact with RPA, and might be isolated on this basis. To this end, we chromatographed cell extracts from mouse FM3A cells on affinity columns containing recombinant human RPA (hRPA). A DNA helicase was isolated whose activity was specifically stimulated by hRPA.

MATERIALS AND METHODS

Nucleoside triphosphates, chromatography supports and chemicals

Radioactive [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]dATP (3000 Ci/mmol) were from ICN and [2,8-^{3}H]ATP (44 Ci/mmol) was from Amersham Corp. Nucleoside, deoxynucleoside triphosphates and various inhibitors were all obtained from Boehringer Mannheim. Protease inhibitors were from ICN. Phosphocellulose was from Whatman. Affi-gel 10 was from Bio-Rad S.A. and heparin–Sepharose was from Pharmacia Biochemicals. Bovine serum albumin (BSA) was from Boehringer Mannheim and mouse FM3A cell cultures were obtained from Computer Cell Culture, Belgium.

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Nucleic acids

The single-stranded DNA used for the standard helicase assays was a derivative of pTZ 19R (Pharmacia LKB) termed pTZAB8 (3164 nt) and contained as additional sequences the 5023–5297 bp region of the polyomavirus A2 genome (11). pTZAB8 was a kind gift of Dr Lacasa, France. Poly d(AT) was from Pharmacia Biotech and poly U was from Miles Laboratories Inc. Two oligonucleotides were purchased from Bioprobe Systems (France) and were repurified by polyacrylamide gel electrophoresis. The 18mer (5′-AGCGGATAA-CAATTTCAC-3′) and 56mer (5′-CATGCCTGCAGGTCGACTC-TAGAATTCCCCGGCCTCTGCTTAATACTAAAAAAAA-3′) were complementary to nucleotide sequences 6185–6203 and 6225–6280 of M13mp18, respectively, comprised within the pTZAB8 DNA sequence.

Enzymes

Escherichia coli SSB protein was purchased from Pharmacia Biotech and SV40 large T-antigen was purchased from Molecular Biology Resources Inc. Recombinant hRPA was purified from *E.coli* strain BL21 (DE3) containing the expression vector p11d-tRPA up to the hydroxyapatite step (12). Fractions containing 400–700 µg protein were dialyzed against a 400-fold excess of buffer containing 20 mM Tris–HCl, 200 mM NaCl, 2 mM DTT and 20% glycerol. The dialysate was passed through a 2 ml phosphocellulose column equilibrated with the same buffer and the non-adsorbed fractions were used as a source of pure hRPA after concentration through a Centricon 10 microconcentrator.

Preparation of DNA helicase substrates

Equimolar amounts of pTZAB8 single-stranded DNA and oligonucleotide were mixed in buffer containing 10 mM Tris–HCl, 1 M NaCl and 5 mM $MgCl_2$, heated at 100°C for 5 min and allowed to gradually cool to room temperature. The DNA was then precipitated with 2.5 vol absolute ethanol and the 3′-ends of the annealed oligonucleotides were labelled with [α-^{32}P]dCTP (3000 Ci/mmol) and the Klenow fragment under standard reaction conditions (13). In the case of the 18mer, labelling reactions with [α-^{32}P]dCTP contained an additional 10 µM dATP. When helicase polarity was to be measured, the 56mer annealed to pTZAB8 was cut by the restriction endonuclease *Msp*I within the partially duplex region before being labelled with [α-^{32}P]dCTP (3000 Ci/mmol) in the presence of 10 µM dGTP. This procedure gave rise to a linearized and blunt-ended pTZAB8 substrate with a duplex region of 32 bp at its 3′-end and a duplex region of 27 bp at its 5′-end. The release of the 32 bp fragment is indicative of helical movement in the 5′–3′ direction whereas displacement of the small fragment demonstrates movement in the 3′–5′ direction.

ATPase assay

Reactions were carried out in a final volume of 20 µl containing 100 µM ATP and 1 µCi [2,8-^{3}H]adenosine-5′-triphosphate in the same buffer as used for the strand displacement assays. After incubation for 60 min at 37°C, 1 µl of reaction mixture and 1 µl of a 0.1 M solution of ATP/ADP were spotted onto polyethyleneimine–cellulose thin plates pre-impregnated with fluorescent indicator 254 (Merck) and developed in 1.0 M formic acid and 0.5 M LiCl. The ADP and ATP spots were visualized under UV light, excised and counted.

Helicase assay

Reactions (20 µl) contained 50 mM HEPES–KOH, pH 7.7, 5 mM DTT, 7 mM $MgCl_2$, 100 µg/ml BSA, 2 mM ATP and 54 fmol 3′-^{32}P labelled partial duplex DNA substrate and hRPA where indicated. After incubation at 37°C for 60 min, the reactions were stopped by the addition of 5 µl 5× loading dye containing 50% glycerol, 1% SDS, 0.25% bromophenol blue and 0.25% xylene cyanol. The samples were electrophoresed for 2 h through a 10% polyacrylamide gel in 1× Tris–glycine buffer (13) containing 0.1% SDS at 250 V. Bands were visualized by autoradiography and the level of radioactivity in individual bands was calculated using phosphoimager (Storm: Molecular Dynamics) analysis.

Preparation of the hRPA affinity columns

Recombinant hRPA (500–800 µg) from the phosphocellulose step was dialyzed overnight against a 500-fold volume of coupling buffer (30 mM HEPES–KOH, pH 7.6, 0.2 M NaCl, 20% glycerol, 0.25 M EDTA) at 4°C. The dialyzate was then mixed with 0.25–0.5 ml Affi-gel 10 after removal of the iso-propanol from the latter by successive washes in water and coupling buffer, and the two were incubated at 4°C for 2 h on a rotary shaker. After a brief impulse in a an Eppendorf benchtop centrifuge, the protein concentration in the supernatant was measured in order to estimate coupling efficiency. This was always in the range of 95%. The Affi-gel 10-hRPA affinity matrix was introduced into a Bio-Rad column (dimensions 0.5 × 5 cm) and washed copiously with interaction buffer (30 mM HEPES–KOH, pH 7.6, 2 mM DTT, 1 mM EDTA, 20% glycerol, 0.01% NP-40) containing 0.25 M NaCl followed by equilibration in interaction buffer containing 30 mM NaCl. The columns were kept for 24–48 h before use and always discarded after each purification.

Purification of DNA helicase

All steps were carried out on ice. Cytosol extracts were prepared from a batch of 5×10^{10} FM3A cells according to the methods described in reference 14. Essentially, frozen cells were resuspended in a 2-fold volume of hypotonic buffer (20 mM HEPES–KOH, pH 7.5, 5 mM NaCl, 1.5 mM $MgCl_2$, 10 mM DTT) containing protease inhibitors at the following concentrations (2 µg/ml aprotinin, 25 µg/ml antipain, 2 µg/ml leupeptin, 5 µg/ml pepstatin, 0.25 mM PMSF and 1 mM EGTA). After 25–35 strokes with a Teflon coated homogenizer, the cell suspensions were adjusted to 0.2 M NaCl and centrifuged at 30 000 r.p.m. in a 50.2Ti Beckman rotor at 4°C for 1 h. Subsequent steps were carried out at 4°C. The supernatants (~250–300 ml) were then dialyzed twice against 2 l of buffer H (20 mM HEPES–KOH, pH 7.0, 50 mM NaCl, 10% glycerol, 1 mM DTT, 0.25 mM PMSF) for 2 h each time. The protein concentrations of the dialyzed extracts ranged from 8 to 14 mg/ml. After dialysis, a precipitate formed and was removed by centrifugation in a Sorval SS34 rotor at 18 000 r.p.m. for 30 min at 4°C. The NaCl concentration of the dialyzed fractions was then adjusted to 0.2 M NaCl and applied to a 100–150 ml column of phosphocellulose pre-equilibrated in buffer H containing 0.2 M NaCl. The column was then washed with 2 vol buffer H containing 0.2 M NaCl and proteins were successively eluted with 2 vol buffer H containing 0.4 and 1 M NaCl, and 15 ml fractions were collected. The 0.2 and 0.4 M NaCl fractions were collected, frozen in liquid nitrogen and stored at –70°C. The 1 M

NaCl fraction containing ~100 mg protein was dialyzed overnight on ice against buffer H containing 30 mM NaCl and 0.01% NP-40 after the addition of protease inhibitors as described above. At this stage another precipitate formed and this was again removed by centrifugation in the same way as described above. The supernatant was then allowed to charge slowly onto the Affi-gel 10-hRPA column after passing through an 'in line' column containing 5 ml Affi-gel 10 that had only been equilibrated overnight in interaction buffer containing 30 mM NaCl. Both columns were washed with interaction buffer containing 100 mM NaCl and after deconnection of the pre-Affi-gel 10 column, the hRPA affinity column was eluted successively with interaction buffer containing 0.25 and 1 M NaCl and 0.5 ml fractions were collected. After the addition of protease inhibitors as described above, the active fractions were pooled and dialyzed against interaction buffer containing 0.2 M NaCl. The dialysate was then charged onto a 1 ml column of heparin–Sepharose pre-equilibrated in the same buffer and the column was successively eluted stepwise with 5 ml vol of interaction buffer containing 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 M NaCl. Fractions of 1 ml were collected. Active fractions were frozen in liquid nitrogen and stored at –70 °C after the addition of protease inhibitors as described already.

Identification of dATP and DNA interacting peptides

Unless otherwise indicated, all operations were carried out at 4°C. For the identification of peptides interacting with the DNA helicase substrate, ~20 ng of the 0.5 M NaCl eluate from the heparin–Sepharose column was precipitated with 3 vol of acetone on ice and centrifuged for 10 min in an Eppendorf benchtop centrifuge. The pellet was resuspended in 10 µl PAGE-loading buffer (13) containing 100 mM DTT and subjected to electrophoresis on a 12% polyacrylamide gel along with relevant molecular weight standards according to standard procedures (13). Proteins were transferred overnight to a nitrocellulose filter in carbonate buffer (3 mM Na_2CO_3 and 10 mM $NaHCO_3$, pH 9.8) at 4°C and at 100 mA. The filter was blocked with blocking buffer (50 mM $NaPO_4$ pH 7.3, 1 mM $MgCl_2$, 2 mM DTT, 0.3% Tween 20 and 1% BSA) for 30 min and then incubated for another 30 min in 2 ml blocking buffer containing 2 µM dATP and 8.4 pmol of partially duplex 18mer helicase substrate previously labelled with 40 µCi [α-^{32}P]dATP. The filter was washed to remove non-specifically bound DNA, allowed to drip dry and subjected to autoradiography.

The dATP interacting peptides were identified by UV cross-linking of [α-^{32}P]dATP followed by SDS–PAGE and autoradiography. An aliquot (20 ng) of the purified 0.5 M NaCl fraction from heparin–Sepharose was adjusted to 7 mM $MgCl_2$, 10 µM dGTP, 0.16 µM [α-^{32}P]dATP (50 µCi) and 147 µg/ml pTZAB8 single-stranded DNA. The sample was incubated at 37°C for 5 min and then divided into 25 µl drops that were spotted onto an ice cold aluminium block covered with Parafilm. The block was introduced into a UV Stratalinker 1800 and illuminated for 5 min. The samples were recovered into one tube and precipitated with acetone as described above. After SDS–PAGE the gel was dried and autoradiographed at –70°C.

RESULTS

Purification of RPA-dependent DNA helicase activity

In order to isolate and identify cellular DNA helicases that interact with RPA, we realized affinity chromatography of mouse FM3A cell extracts on Affi-gel 10-RPA affinity columns (Table 1). Extracts were first passed through a phosphocellulose column in order to remove intrinsic mouse RPA, which elutes in the 0.2 M NaCl non-adsorbed fraction (15), and to reduce the number of proteins binding to the hRPA-affinity column. The 1 M NaCl eluate from the phosphocellulose column was used as starting material after dialysis against buffer containing 30 mM NaCl. At this stage, the detection of DNA helicase was made difficult by the presence of contaminating nuclease activity and low activities (data not shown). However, after passage through the Affi-gel 10-RPA affinity matrix, DNA helicase activity could be detected in the 0.25 M NaCl eluted fractions but only if hRPA was added to the helicase assays containing the 56mer DNA helicase substrate (Fig. 1A). No activity could be detected in the absence of hRPA. As elution of the DNA helicase activity was incomplete even after passage of 5 ml 0.25 M NaCl buffer, a volume equivalent to 10–20-fold that of the column, we continued elution with 1 M NaCl buffer. However, this did not improve the elution profile and DNA helicase activity continued to leave the column as a broad peak (Fig. 1A). Also, no activity was detected in the 0.1 M NaCl wash of the Affi-gel 10-RPA column or in a 0.8 M NaCl eluate from a heparin–Sepharose column previously charged with the non-adsorbed fraction from the Affi-gel 10-RPA column, indicating that all the activity present in the 1 M NaCl phosphocellulose fraction had probably been adsorbed to the column (data not shown). Fractions containing comparable activity were pooled and further purified by heparin–Sepharose chromatography. The RPA-dependent DNA helicase activity was eluted over two salt concentrations, 0.4 and 0.5 M NaCl (Fig. 1B), again reflecting the tendency of this protein to stick to the column.

Table 1. Purification table of DNA helicase

Helicase fraction	M	Protein (mg)	DNA helicase activity		
			Total (units)[a]	Specific (units/mg)	Yield (%)
Crude extract		2700	(–)[b]	(–)	(–)
Phosphocellulose 1 M		104	(–)[b]	(–)	(–)
hRPA-Affigel 10		0.040	1320	32 200	100
Heparin–Sepharose	0.4	(–)[c]	200	(–)	15
	0.5	0.001[d]	500	300 000	22

[a]One unit was defined as the activity required to displace 50% of 54 fmol of 3′-^{32}P-labelled 56mer DNA substrate under standard conditions for 1 h at 37°C.
[b]Activity in the crude lysate and phosphocellulose fraction could not be reliably measured.
[c]The protein concentration in the heparin–Sepharose 0.4 M NaCl fraction was below the sensitivity range of the Bradford assay.
[d]Protein concentration was based on silver staining using BSA as a protein standard and includes both the amounts of the 28 and 21 kDa bands (Fig. 2).

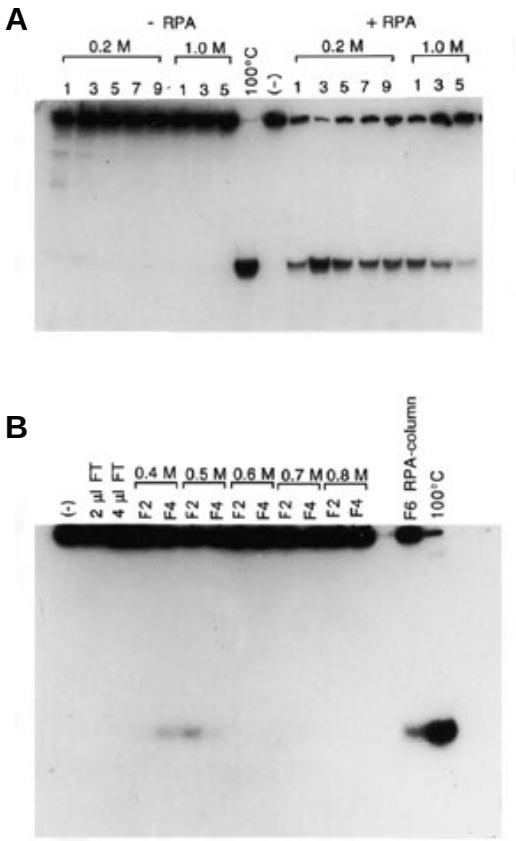

Figure 1. DNA helicase activity of fractions from the Affi-gel 10-RPA affinity and heparin–Sepharose columns. Assays were carried out under standard conditions as described in Materials and Methods using the 56mer DNA helicase substrate and 2 µl of each fraction. (**A**) DNA helicase activity of fractions from the Affi-gel 10-RPA affinity column in the presence or absence of RPA. (**B**) DNA helicase activity of fractions from the heparin–Sepharose column. When present, RPA was at 0.9 µg per reaction, and 2 µl of the indicated fractions from the column were employed. A buffer solution containing NaCl at the molarities 0.4, 0.5, 0.6, 0.7 and 0.8 was used to elute the heparin–Sepharose column. F2 and F4, fractions two and four of each salt elution step respectively. –, no fraction added; 100°C, the DNA helicase substrate was denatured at 100°C; FT, flow through fraction; F6 RPA-column, F6 of the 0.2 M eluate from the RPA-affinity column in (A).

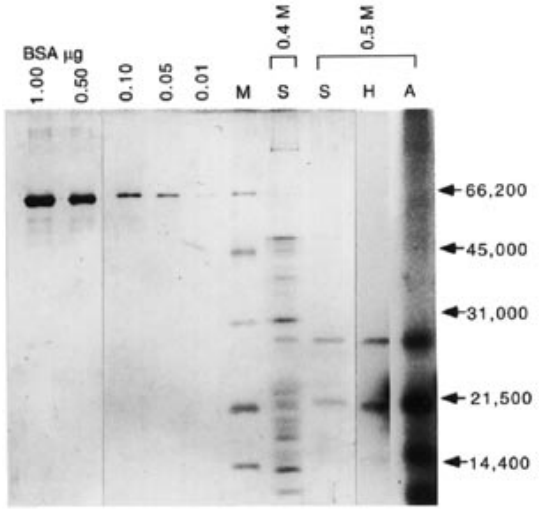

Figure 2. Protein constituents of the DNA helicase containing heparin–Sepharose fractions and their interaction with the ^{32}P-labelled DNA helicase substrate and [α-^{32}P]dATP. On the left, the various amounts of silver-stained BSA used to estimate the protein concentration in the different fractions. Lanes 0.4 M and 0.5 M, indicate 0.4 M and 0.5 M NaCl eluted fractions from heparin–Sepharose; lanes S, silver stained fractions; lane H, bands interacting with the ^{32}P-labelled DNA helicase substrate after transfer to a nitrocellulose membrane; lane A, proteins that were cross-linked to [α-^{32}P]dATP. On the left, molecular weights of the protein standards of lane M in kDa.

Biochemical analysis of the protein constituents present in the heparin–Sepharose fractions

When the purity of the heparin–Sepharose 0.4 and 0.5 M NaCl fractions was examined by SDS–PAGE followed by silver staining, the 0.5 M NaCl fraction was found to be relatively pure compared to the 0.4 M NaCl eluted fraction, as it displayed the presence of only two major bands of 21 and 28 kDa (Fig. 2, compare 0.4 M lane S with 0.5 M lane S). To determine if these proteins were associated with the DNA helicase activity, we transferred the proteins to a nitrocellulose filter and probed them with the 3′-^{32}P-labelled (18mer) DNA helicase substrate in the presence of 2 µM cold dATP. Also, we attempted to UV crosslink them to 0.16 µM [α-^{32}P]dATP in the presence of pTZAB8 single-stranded DNA and an ~60-fold excess of cold dGTP. Autoradiography revealed only two radioactive bands with the same mobility as the silver stained bands when the filters were incubated with the ^{32}P-labelled DNA helicase substrate (Fig. 2, lane H). When the proteins in the same fraction were crosslinked to [α-^{32}P]dATP under UV light, the same two bands were visible along with two additional, smaller bands (Fig. 2, lane A). Therefore, we concluded that both the 21 and 28 kDa proteins are implicated in the DNA helicase activity of this fraction.

Dependence on hRPA for DNA helicase activity

As shown above, we were unable to demonstrate the presence of DNA helicase activity in the fractions from the hRPA-affinity column unless recombinant hRPA had been added to the reactions containing the standard, partially duplex 56mer substrate. In Figure 3, we demonstrate that this stimulation by hRPA is specific, as *E.coli* SSB protein could not efficiently replace hRPA in these reactions. When increasing concentrations of SSB protein were added to the reactions containing a constant amount of purified DNA helicase, only a mild effect of SSB concentration was observed and stimulation was at most 10%. T4 phage gene 32 protein was similarly ineffective (data not shown). This was in contrast to the concentration-dependent increase in DNA helicase activity seen on addition of hRPA. At a concentration of 0.9 µg hRPA per reaction, nearly 60% of the 56mer was displaced from the partially duplex DNA substrate. The hRPA dependent increase in DNA helicase activity occurred within the range of hRPA concentrations required to cover between 20 and 120% of the single-stranded DNA if one assumes one binding site for hRPA every 30 nt (15). This suggests that maximum activity required at least complete saturation of the single-stranded DNA.

The observed stimulation of DNA helicase activity by hRPA was in contrast to the slight inhibitory effect of hRPA on DNA-dependent ATPase activity (discussed later and Table 3).

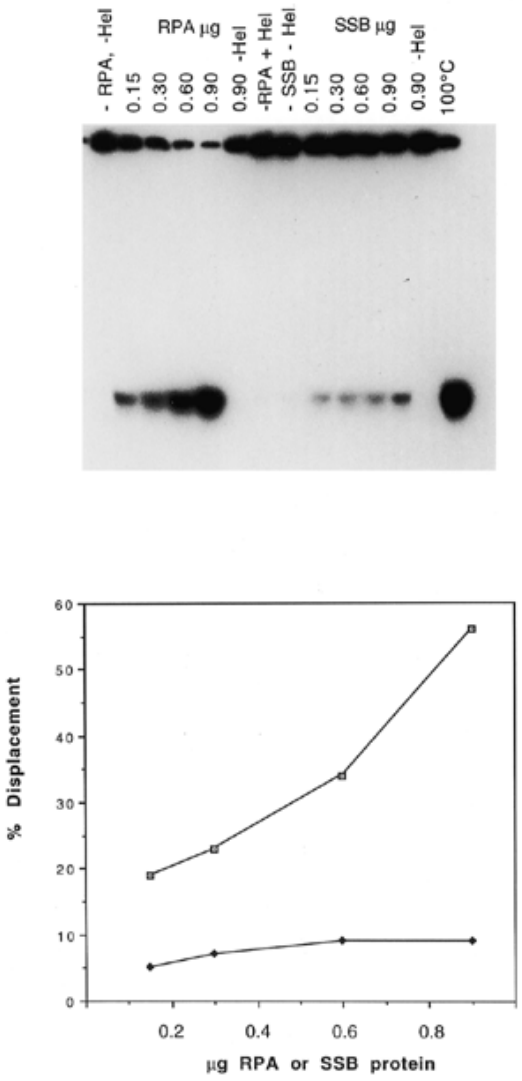

Figure 3. Specific requirement of RPA for DNA helicase activity. DNA helicase activity was measured under standard conditions using 0.6 ng of the purified heparin–Sepharose fraction. The amounts of RPA and SSB protein are indicated above each lane. –RPA, no RPA added; –SSB, no SSB added; –Hel, no DNA helicase added. Other symbols are as in Figure 1. Below, a line graph of the data presented in the upper figure. The amount of radioactivity in the displaced bands was estimated by Imagequant analysis of a corresponding phosphoimage, and is expressed as a percentage of the radioactivity in the 100°C lane which was taken to be 100% after correction for background levels in the –RPA –Hel lane. Dot within a square, RPA added; closed diamond, SSB added.

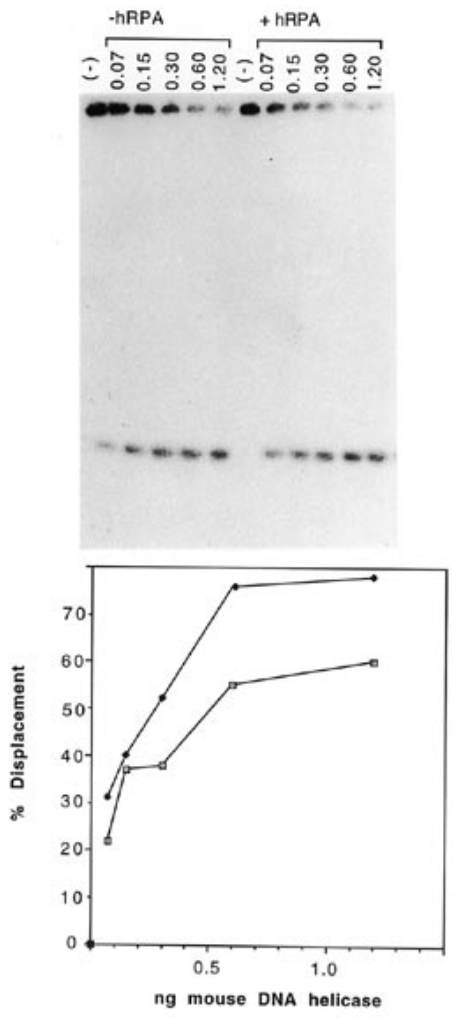

Figure 4. DNA helicase activity on a substrate molecule containing an 18 bp double-stranded region. The amounts of DNA helicase added to the assays are indicated above each lane (ng). Other symbols are as in Figures 1 and 3. Below, line graph of the data shown in the figure estimated as described in the legend to Figure 3. Dot within a square, with RPA; closed diamond, without RPA.

Dependence on hRPA requires DNA substrates with partially duplex regions longer than 18 bp

In order to determine if dependence on hRPA was a function of the length of the partially duplex region of the helicase substrate, we examined the dependence on hRPA using an 18mer partially duplex DNA helicase substrate. Unlike the 56mer partially duplex substrate, DNA helicase activity was considerable on this substrate, even in the absence of hRPA demonstrating that the length of the partially duplex region is critical for dependence on hRPA (Fig. 4). Also, the amount of DNA helicase required to displace the 18mer was two to three times less than with the 56mer partially duplex substrate (data not shown).

Polarity of the DNA helicase

DNA helicases can translocate along DNA in either the 3′–5′ or 5′–3′ direction. In order to determine the direction of translocation of the purified DNA helicase, we constructed a linear pTZAB8 DNA substrate containing partially duplex regions of 27 bp at the 5′-end and 32 bp at the 3′-end, both of which were labelled with ^{32}P and blunt-ended at their opposing ends by the Klenow enzyme (see Materials and Methods). The release of the small fragment would indicate a DNA helicase with 3′–5′ polarity, whereas, release of the large fragment would indicate an enzyme with 5′–3′ polarity. When this substrate was added to the helicase assays containing SV40 T-antigen, only the 27 bp fragment was displaced, confirming the 3′–5′ polarity of the SV40 T-antigen (3). The mouse DNA helicase displaced preferentially the large fragment indicating a polarity of 5′–3′ for this enzyme (Fig. 5).

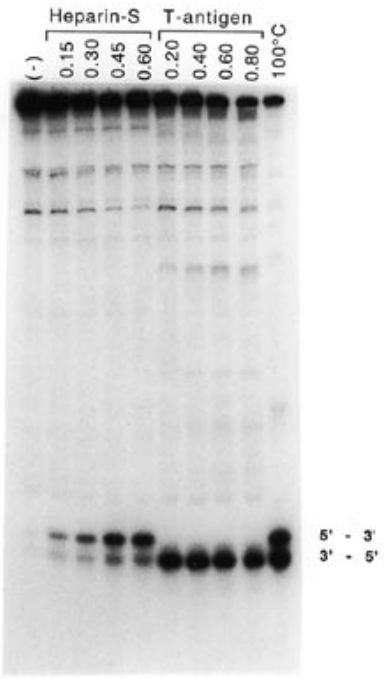

Figure 5. DNA helicase strand polarity. The amounts of DNA helicase added to the assays are indicated above each lane for the heparin–Sepharose fraction (ng) and for T-antigen (μg). Other symbols are as in Figures 1 and 3; 5′–3′, 32mer; 3′–5′, 27mer.

Characterization of other requirements for DNA helicase activity

In Table 2 we list the various characteristics of the purified mouse DNA helicase in the standard DNA helicase assay containing the 56mer partially duplex substrate. Displacement of the 56mer was entirely dependent on the presence of ATP and Mg^{2+}. No other nucleoside triphosphates could replace ATP efficiently with the notable exception of dATP which was as effective as ATP. Neither ATPγS, p[NH]ppA or ADP could replace ATP. At a concentration of 2 mM ATP, DNA helicase activity was inhibited 38 and 73% by 0.2 and 2 mM ATPγS, respectively. This was in contrast to p[NH]ppA which was not inhibitory even at 2 mM and ADP which only inhibited by 30% at a concentration of 2 mM. The enzyme was sensitive to *N*-ethylmaleimide (NEM) demonstrating the necessity for (a) sulhydryl group(s) for DNA helicase activity. The enzyme was only mildly inhibited by increased salt concentrations of 50 and 100 mM NaCl.

Characterization of DNA-dependent ATPase activity

In Table 3 we summarize the characteristics of the ATPase activity present in the purified fractions. The DNA helicase displayed an identical K_m value of 0.6 mM for the hydrolysis of both ATP and dATP which is, to our knowledge, among one of the highest K_m values reported for a DNA helicase (16). As was the case for the DNA helicase activity, there was an absolute requirement for Mg^{2+} and activity was inhibited by *N*-ethylmaleimide. Only poly (dA).oligo (dT) was more effective than pTZAB8 for ATP hydrolysis, and only low levels of activity were observed with the double-stranded DNA molecules, pUC-19 and λ phage, demonstrating the absolute necessity for single-stranded regions of DNA for the ATPase activity of this enzyme. Poly U was also ineffective, suggesting that this enzyme is unlikely to function on RNA substrates. When hRPA was added to the assays, a slight inhibition of ATPase activity was evident suggesting that hRPA might hinder widespread access of the DNA helicase to single-stranded DNA. *Escherichia coli* SSB protein was inhibitory at all concentrations, indicating that SSB protein cannot be displaced from single-stranded DNA coated by the mouse DNA helicase. Low concentrations of NaCl (50 mM) stimulated ATPase activity, in contrast to the mild inhibition by salt of the DNA helicase activity (Table 2). hRPA by itself did not display any ATPase activity showing that this preparation was not contaminated with DNA helicases from *E.coli*.

Table 2. Requirements for DNA helicase activity

Additions or omissions	Concentration (mM)	DNA fragment displaced (%)
Complete[a] + ATP	0.2 or 2	26.2 or 44.4
Omit $MgCl_2$ or ATP or hRPA		<0.8
Omit DTT + NEM	5	<3.5
Complete + ATPγS	0.2 or 2	32 or 12
Complete + p[NH]ppA	0.2 or 2	49.9 or 49.9
Complete + ADP	0.2 or 2	45.5 or 30.7
Omit ATP, add GTP	0.2 or 2	<0.1 or 8.7
Omit ATP, add CTP	0.2 or 2	<0.1 or 12
Omit ATP, add UTP	0.2 or 2	<0.1 or <0.1
Omit ATP, add ATPγS, p[NH]ppA or ADP	2	<0.1
Omit ATP, add dATP	0.2 or 2	31.7 or 37.4
Omit ATP, add dTTP or dGTP or dCTP	2	3.0 or 2.7 or 4.5
Complete + NaCl	50 or 100	33 or 29

[a]Complete reactions contained 7 mM $MgCl_2$, 0.9 μg hRPA, 54 fmol of 3′-^{32}P-labelled partial duplex DNA (56mer), 2 mM ATP and 0.4 ng helicase (heparin–Sepharose 0.5 M fraction). Other conditions are described in Materials and Methods.

Table 3. Influence of various additions and omissions on the DNA-dependent ATPase activity

Additions or omissions	Amount added	ADP formed (pmol)
Complete[a]		300
Omit $MgCl_2$		<5
Omit DTT add NEM	5 mM	<30
Complete + hRPA	0.9 µg	228
Complete + *E.coli* SSB	0.3 or 1 µg	<8 or <4
Omit helicase add hRPA	0.9 µg	<1
Omit pTZAB8 DNA		<4
Omit pTZAB8 add poly (dA).oligo(dT)	10 µM	760
Omit pTZAB8 add pUC-19	10 µM	<32
Omit pTZAB8 add λ phage DNA	10 µM	<10
Omit pTZAB8 add poly U	10 µM	<11
Omit pTZAB8 + hRPA	0.9 µg	<12
Complete + NaCl	50 or 100 mM	740 or 360
K_m for ATP or dATP[b]		0.6 mM

[a]The complete reaction contained 100 µM [2,8-^{3}H]adenosine 5′ triphosphate, 50 ng pTZAB8RF DNA (10 µM as nucleotide) and 0.4 ng enzyme (heparin–Sepharose 0.5 M fraction). Other conditions and the experimental procedures are described in Materials and Methods.

[b]The K_m value for ATP or dATP was measured under standard reaction conditions containing 1 µCi [2,8-^{3}H] adenosine 5′ triphosphate or 1 µCi [α-^{32}P]dATP and various concentrations of cold ATP or dATP. The K_m value was taken from the value $-1/K_m$ at the 1/[S] intercept on a Lineweaver–Burk plot.

DISCUSSION

The advent of high expression vectors for mammalian RPA has provided an opportunity to purify by affinity chromatography proteins implicated in the DNA transactions mediated by RPA. Here, we deliberately searched among the proteins binding to Affi-gel 10-RPA columns for DNA helicase activity, as RPA was shown to interact with, and enhance the DNA helicase activity of large T-antigen at the SV40 origin of replication. In this way, a novel DNA helicase was isolated and purified to near homogeneity. The most purified preparations consisted of two bands of 28 and 21 kDa, indicating that either one or both of these two proteins compose the DNA helicase activity. In order to pursue this issue further, we examined their interaction with the ^{32}P-labelled DNA helicase substrate and their crosslinking to [α-^{32}P]dATP and found that both were equally competent for these two reactions. Thus, both proteins probably contribute to the DNA helicase activity of this fraction. The two proteins may represent two distinct DNA helicases, although their co-purification and the restricted characteristics of the enzyme activities suggest the presence of only one enzymatic entity. Alternatively, the helicase could be a heterodimer made up of 21 and 28 kDa subunits. However, it would be extremely unusual for a DNA helicase to consist of two different subunits, each comprising of SSDNA and [α-^{32}P]dATP binding activities. Rather, the two proteins may represent the catalytic remnants of a larger protein digested by proteolysis during the course of purification. This interpretation would be consistent with their relatively small size compared to other DNA helicases, the presence of [α-^{32}P]dATP and SSDNA binding sites on both proteins and the presence of smaller bands capable of interacting with [α-^{32}P]dATP but not with SSDNA (Fig. 2, lane A), probably indicating more extensive proteolytic removal of the SSDNA binding region. Confirmation of this hypothesis, however, must await N-terminal amino acid sequencing, and identification of the genes coding for these proteins, both of which are presently underway.

The DNA helicase activity of the preparation was dependent on the presence of hRPA in the assays when the substrate molecule contained a duplex region larger than 18 bp. The requirement for RPA could not be replaced by *E.coli* SSB protein (Fig. 2) or the product of phage T4 gene 32 (data not shown). Thus, retention of the DNA helicase on the hRPA affinity column has functional significance, in that only RPA served as a co-factor for the DNA helicase activity on DNA helicase substrates with duplex regions of 56 bp.

There exists a previous report of DNA helicase activity co-purifying with RPA (17), and a number of DNA helicases specifically stimulated by RPA have been isolated from HeLa cells and calf thymus (18–20). However, to our knowledge, this is the first DNA helicase to be purified by RPA affinity chromatography whose activity depends on RPA. Certain of the chromatographic properties of our DNA helicase, for instance, elution at 1 M NaCl on phosphocellulose and at 0.5 M KCl on heparin–Sepharose, resemble those of another DNA helicase purified from human HeLa cells (18) that also showed strong dependence on RPA. However, unlike our enzyme, the human DNA helicase possessed 3′–5′ strand polarity for helicase translocation, making it unlikely that they are the same enzyme. Also, our enzyme showed an elevated K_m value of 0.6 mM for ATP or dATP hydrolysis which is very different from the K_m values of 40 and 15 µM found respectively for ATP and dATP for the human enzyme (18).

The ATPase activity of the DNA helicase was strongly inhibited by *E.coli* SSB protein but only weakly inhibited by RPA at concentrations of SSB protein and RPA sufficient to cover all the single-stranded DNA in the assays. This suggests that either the molecular structure of RPA bound to single-stranded DNA facilitates the access of this DNA helicase to the DNA, or else this DNA helicase is able to destabilize hRPA bound to single-stranded DNA, a feature reminiscent of T-antigen alleviation of RPA inhibition of DNA polymerase α primase activity on single-stranded DNA (21). Clearly, further experiments are needed to clarify this issue and, indeed, the details of the mechanism through which RPA stimulates DNA helicase activity. Likewise, it will be interesting to identify the subunit of RPA responsible for the interaction with this DNA helicase.

Although it is tempting to suggest that the DNA helicase described in this study could function like large T-antigen in initiation of DNA replication at mammalian origins of replication, two characteristics of this DNA helicase, i.e. 5′–3′ polarity and strong dependence on RPA for unwinding of substrates with double stranded regions larger than 18 bp, are unlike those of T-antigen which possesses 3′–5′ polarity and functions efficiently, like most other DNA helicases, in the presence of SSB protein. Thus, there is nothing to exclude for the moment that this DNA helicase might be involved in other reactions mediated by RPA such as DNA recombination and repair.

ACKNOWLEDGEMENTS

We would like to thank Dr Wold for kindly providing us with the expression vector p11d-tRPA used for purifying recombinant human RPA. We would also like to thank Miss Isabelle le Pipe for her excellent technical assistance during the period February–May 1996. This work was supported in part by a grant from 'Comit de Val de Marne de la ligue contre le cancer' to G.B.

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