# *In vitro* analysis of the zinc-finger motif in human replication protein A

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Human replication protein A (RPA) is composed of 70, 34 and 11 kDa subunits (p70, p34 and p11 respectively) and functions in all three major DNA metabolic processes: replication, repair and recombination. Recent deletion analysis demonstrated that the large subunit of RPA, p70, has multiple functional domains, including a DNA polymerase $\alpha$-stimulation domain and a single-stranded DNA-binding domain. It also contains a putative metal-binding domain of the 4-cysteine type (Cys-Xaa$_4$-Cys-Xaa$_{13}$-Cys-Xaa$_2$-Cys) that is highly conserved among eukaryotes. To study the role of this domain in DNA metabolism, we created various p70 mutants that lack the zinc-finger motif (by Cys → Ala substitutions). Mutation at the zinc-finger domain (ZFM) abolished RPA's function in nucleotide excision repair (NER), but had very little impact on DNA replication. The failure of zinc-finger mutant RPA in NER may be explained by the observation that wild-type RPA significantly stimulated DNA polymerase $\delta$ activity, whereas only marginal stimulation was observed with zinc-finger mutant RPA. We also observed that ZFM reduced RPA's single-stranded DNA-binding activity by 2–3-fold in the presence of low amounts of RPA. Interestingly, the ZFM abolished phosphorylation of the p34 subunit by DNA-dependent protein kinase, but not that by cyclin-dependent kinase. Taker together, our results strongly suggest a positive role for RPA's zinc finger domain in its function.

Key words: DNA replication, DNA repair, *Xeroderma pigmentosum* group A complementing protein, single-stranded DNA binding

## INTRODUCTION

Human replication protein A [RPA; also known as human single-stranded DNA (ssDNA)-binding protein], is a three-subunit complex that was initially discovered as an essential factor for the replication of simian virus 40 (SV40) DNA *in vitro* [1–3]. Eukaryotic RPA consists of a 70, a 34 and an 11 kDa subunit (p70, p34 and p11 respectively) [2,3], all of which are tightly associated with each other during interphase [4], but are separately localized during mitosis, which suggests that the functional activity of RPA may be regulated throughout the cell cycle. The p34 subunit is phosphorylated at the G1/S boundary and dephosphorylated during G2/mitosis [5,6]. Phosphorylation of p34 is also induced by DNA damage [7,8], suggesting a role for RPA phosphorylation in DNA metabolism.

In replication, RPA interacts with the SV40-encoded large tumour antigen (SV40 Tag) at the origin of SV40 DNA replication, which initiates unwinding [9–11]. It also interacts with the DNA polymerase $\alpha$–primase complex (pol $\alpha$–primase), which is necessary for the initiation of SV40 DNA replication [9,10,12,13]. During elongation, RPA stabilizes the ssDNA and stimulates pol $\alpha$ and pol $\delta$ [14–16], even though the latter may not be essential for DNA replication [17].

RPA also functions in damaged DNA repair [18–20] and homologous recombination [21]. In nucleotide excision repair (NER), RPA forms a complex with *Xeroderma pigmentosum* group A complementing protein (XPA) [22–25], a protein that specifically recognizes UV damage [26]. RPA stimulates the interaction of XPA with DNA through an RPA–XPA complex at damaged DNA sites [23,24,27]. It has been shown [27] that RPA–XPA interaction at damaged DNA is necessary for DNA repair because mutant RPA has been found not to interact with XPA and also to support NER only poorly [27]. RPA is also involved in a later stage of NER, possibly by promoting pol $\delta/\epsilon$ action in the filling-in reaction [28].

Previous studies on RPA–ssDNA interaction and limited proteolysis strongly suggest that RPA undergoes a structural change when it interacts with DNA [29,30]. p70 has multiple functional domains, including a pol $\alpha$ stimulation domain, an ssDNA-binding domain, and a conserved zinc-finger domain of the 4-cysteine type [17,31,32]. The zinc-finger domain in several DNA-binding proteins, such as transcription factors and adenovirus DNA-binding protein, plays a key role in the interaction with DNA [33–35]; however, deletion of RPA's zinc-finger domain seems to have no significant effect on its ssDNA-binding activity [17,30,32].

In order to understand the structure–function relationship, we investigated the role of RPA's zinc-finger domain in its function. We found that the zinc-finger domain is necessary for DNA repair and is involved in the structural change that occurs during RPA–DNA interaction. This structural change allows RPA to form a stable interaction with DNA and the phosphorylation of p34 by DNA-dependent protein kinase (DNA-PK).

## EXPERIMENTAL

### Preparation of plasmids, cell extracts, proteins and antibodies

The SV40 replication origin-containing plasmid, pSV01ΔEP, has been described previously [25]. UV-irradiated pBS (3 kbp;

Abbreviations used: CDK, cyclin-dependent protein kinase; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; GST, glutathione S-transferase; NER, nucleotide excision repair; NP40, Nonidet P40; pol, DNA polymerase; PCNA, proliferating-cell nuclear antigen; RF-C, replication factor C; RPA, human replication protein A; SV40, simian virus 40; SV40 Tag, SV40-encoded large tumour antigen; ssDNA, single-stranded DNA; XPA, *Xeroderma pigmentosum* group A complementing protein; ZFM, zinc-finger mutant.

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450 J/m$^2$) and p5A (4.5 kbp) were prepared as described previously [36]. SV40 Tag was prepared as described by Lee et al. [37]. The ammonium sulphate fraction (35–65 %) and the phosphocellulose/1.0 M NaCl fraction of HeLa cell cytosolic extracts were prepared as described [25,37]. Purified human DNA pol $\delta$ and replication factor C (RF-C) were gifts from Dr. J. Hurwitz (Sloan-Kettering Cancer Institute, New York, NY, U.S.A.). DNA-PK was partially purified from M059K cell extracts to provide the double-stranded DNA–cellulose fraction as described [38] and cyclin-dependent protein kinase (CDK) was obtained from Oncogene Science (Cambridge, MA, U.S.A.). Rabbit polyclonal antibodies against p34 and p70 of RPA were as described previously [39]. A plasmid expressing the glutathione S-transferase (GST)–XPA fusion gene was obtained from Dr. K. Tanaka (Osaka University, Osaka, Japan) and glutathione–Sepharose 4B and Protein A–Sepharose CL 4B were purchased from Pharmacia Biotech.

### Plasmid constructs and site-directed mutagenesis

cDNA encoding the p70 subunit of human RPA was cloned into baculovirus transfer vector pVL941-SW as described previously [39]. The site-directed mutagenesis kit was purchased from Stratagene. For the change Cys-486 → Ala, the plasmid pVL941SW-70 [carrying the wild-type (wt) p70 gene] and the following primers were used in the PCR reaction, with the underlined nucleotides changes from the wt sequence: 5′-CCG ACT CAG GAC GCC AAT AAG AAA GTG-3′ and 5′-CAC TTT CTT ATT GGC GTC CTG AGT CGG-3′. The resulting mutant was named zinc-finger mutant 1 (ZFM1). For the creation of ZFM2, the double mutations at sites 481 and 486 (Cys-481 → Ala and Cys-486 → Ala), ZFM1 was used as the template and the following primers were used in the PCR reaction: 5′-C ATG TAC CAA GCC GCC CCG ACT CAG GAC-3′ and 5′-GTC CTG AGT CGG GGC GGC TTG GTA CAT G-3′. For ZFM4, the mutation with the change of all four cysteines (the sites 481, 486, 500 and 503) into alanines, ZFM2 served as the template and the following primers were used in the PCR reaction: 5′-GA TTG TAC CGC GCT GAG AAG GCC GAC ACC GAA TTT C-3′ and 5′-G AAA TTC GGT GTC GGC CTT CTC AGC GCG GTA CAA TC-3′. The mutation procedure was performed according to the Stratagene protocol. Briefly, the PCR reactions (50 $\mu$l each) were carried out using a Perkin-Elmer DNA Thermal Cycler 480 with the following temperature regimes: the first cycle, 95 °C for 30 s; the following 16 cycles, 95 °C for 30 s, 55 °C for 1 min and 68 °C for 24 min. The high fidelity of Pfu DNA polymerase plus the low number of PCR cycles greatly reduces the number of undesirable mutations occurring during the PCR process. *Dpn*I (15 units) was used to digest the parental plasmid at 37 °C for 1–2 h. *Dpn*I-digested PCR products were transformed into 50 $\mu$l of supercompetent Epicurean coli XL-1 Blue cells (supplied with the Stratagene mutagenesis kit). The plasmid DNAs were purified using the QIAGEN Midi Plasmid Kit and were used for DNA sequencing or baculovirus transfection. All mutations mentioned above were verified by DNA sequencing prior to use.

### Transfection of baculovirus, preparation of recombinant baculovirus and co-infection and overexpression of wt and mutant RPA

The vectors pVL941-SW containing the gene encoding p34 or p11 of RPA have been described previously [39]. The transfer vectors, carrying p34, p11 and wt or mutant p70 of RPA (2.0 $\mu$g) together with 0.5 $\mu$g of baculovirus (PharMingen), were respectively transfected into Sf9 insect cells according to the published procedure [39]. The recombinant baculovirus was harvested after 5 days of transfection at 27 °C and further amplified through three rounds of infection into Sf9 insect cells until the population of the recombinant baculovirus reached $(1–2) \times 10^8$ plaque forming unit/ml. An equal amount of each of the recombinant baculoviruses, wt p11, wt p34, and either wt p70 or mutant p70, was co-infected into Sf9 insect cells to overexpress wt or mutant RPA for 48 h at 27 °C [39]. For the preparation of 1 litre of lysate, $3 \times 10^7$ Sf9 cells were seeded into each of 33 dishes ($150 \times 25$ mm in size) with 30 ml of Grace's medium per dish, and co-infected with each recombinant baculovirus at a multiplicity of infection of 15. The co-infected Sf9 cells were harvested from the dishes, washed with PBS and lysed for 1 h on ice with 50 ml of lysis buffer [50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 0.5 % (v/v) Nonidet P40 (NP40), 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.1 mM NaF, 10 mM $\beta$-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 0.1 $\mu$g/ml leupeptin and 0.2 $\mu$g/ml antipain]. The cell and lysis buffer mixtures were centrifuged at 35000 rev./min (80000 ***g***) at 4 °C for 30 min using a Beckman 45 Ti rotor. The lysate (supernatant) was used for further purification.

### Purification of wt or mutant RPA

The RPA purification procedure was as outlined previously [17,39] with slight modifications. Briefly, 1 litre lysates from Sf9 cells, co-infected with recombinant baculoviruses encoding wt p11, wt p34, and either wt p70 or mutant p70, were adjusted to 0.5 M NaCl and loaded onto a ssDNA–cellulose column which was equilibrated with buffer A [25 mM Tris/HCl (pH 7.5), 10 % (v/v) glycerol, 0.02 % (v/v) NP40, 1 mM DTT, 0.5 mM EDTA, 0.1 mM PMSF, 0.1 $\mu$g/ml leupeptin and 0.2 $\mu$g/ml antipain] containing 0.5 M NaCl. The column was successively washed with 20 column vols. of buffer A containing 0.5 M NaCl and 0.8 M NaCl. The proteins were eluted with buffer A containing 2.0 M NaCl/40 % ethylene glycol. The eluted fractions were diluted five times with the buffer A, and loaded onto an Affi-Gel Blue (Bio-Rad) column which was equilibrated with the buffer A containing 0.5 M NaCl. After washing the column with buffer A containing 0.5 M NaCl and 0.8 M NaCl, proteins were eluted with the same buffer containing 2.5 M NaCl/40 % ethylene glycol. The RPA-containing fractions were dialysed against buffer A containing 50 mM NaCl and further purified using a Q-Sepharose column with a linear salt gradient (50 mM–0.4 M NaCl). The wt or mutant RPA was analysed using Western blotting as well as 12 % SDS/PAGE. Fractions with at least 90 % purity were collected and stored at −80 °C until use.

### Western blotting

Western blots were performed as described previously [17]. Briefly, wt or mutant RPA was run on a 12 % SDS/PAGE gel and transferred to a nitrocellulose membrane (BA83, Bio-Rad). After the transfer, either anti-p70 or anti-p34 polyclonal antibody was used to detect RPA subunits, which were then revealed by $^{125}$I-labelled Protein A (Amersham Corp.) treatment and visualized by autoradiography.

### RPA–ssDNA-binding assay

Oligo(dT)$_{50}$ was 5′-end labelled with [$\gamma$-$^{32}$P]ATP (Du Pont) and T4 polynucleotide kinase (USB, Cleveland, OH, U.S.A.) based on the manufacturer's instruction. The indicated amount of wt or mutant RPA was incubated with 40 fmol of 5′-end $^{32}$P-labelled Oligo(dT)$_{50}$ at room temperature for 15 min in a reaction mixture consisting of 60 mM Hepes/KOH (pH 7.8), 14 mM

$MgCl_2$, 1 mM DTT, 0.2 $\mu$g/$\mu$l BSA and either 100 mM or 500 mM NaCl. Protein–DNA complexes were analysed using 5% polyacrylamide gels in 1× TBE buffer (acrylamide/bisacrylamide = 79:1). The gels were dried and exposed to X-ray films (Kodak). The bands of interest were excised from the gels and their radioactivities were detected using a Beckman scintillation counter LS 6500.

### *In vitro* SV40 DNA replication

The assay was performed as described by Wobbe et al. [40] with minor modifications. The reaction mixture (40 $\mu$l) was composed of 40 mM creatine phosphate-di-Tris salt (pH 7.7), 0.05 $\mu$g/$\mu$l creatine kinase, 7 mM $MgCl_2$, 0.125 $\mu$g/$\mu$l BSA, 0.5 mM DTT, 4 mM ATP, 335 $\mu$M UTP, GTP and CTP, 100 $\mu$M dTTP, dCTP and dGTP, 25 $\mu$M [$\alpha$-$^{32}$P]dATP (30000 c.p.m./pmol), 60 ng/$\mu$l SV40 Tag, 10 ng/$\mu$l pSV0IΔEP and the indicated amounts of wt or mutant RPA. The reaction was incubated at 37 °C for 1 h. The reaction was stopped by adding 80 $\mu$l of the stop solution (0.1 M $NaHPO_4$ and 1.0 mg/ml carrier DNA), subsequently mixed with 5 ml of pre-chilled 5% (w/v) trichloroacetic acid (TCA), and incubated on ice for 5 min. The reaction mixture was then filtered through a glass fibre filter (2.5 cm circles, retention > 2.3 $\mu$m; Sigma). The filter was washed with pre-chilled 5% trichloroacetic acid, 1% trichloroacetic acid and 95% ethanol in that order, and dried through strong illumination for 5 min. The filter was placed in a scintillation vial and 3 ml of CytoScint (ICN) scintillation solution was added. The radioactivities were detected using a Beckman LS 6500 scintillation counter.

### *In vitro* phosphorylation of wt and mutant RPAs

For the phosphorylation of RPA by CDK, the reaction mixture (25 $\mu$l), containing 10 mM $MgCl_2$, 0.5 mM DTT, 40 mM Tris/HCl (pH 7.5), 10 $\mu$M ATP, 4.4 $\mu$M [$\gamma$-$^{32}$P]ATP, p34cdc2 kinase (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and the indicated amounts of wt or mutant RPA, was incubated at 30 °C for 30 min. After the incubation, samples were separated by 12% SDS/PAGE. The gel was dried and exposed to an X-ray film. For the DNA-PK-catalysed phosphorylation reaction, the mixture (20 $\mu$l) contained 50 mM Hepes/KOH (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.25 $\mu$g/$\mu$l BSA, 50 mM KCl, 100 $\mu$M ATP, purified DNA-PK, 20 $\mu$g/ml activated calf-thymus DNA and the indicated amounts of wt or mutant RPA. The reaction mixture was incubated at 30 °C for 1 h, then mixed with an SDS gel loading buffer and boiled for 5 min. The reaction mixture was run on 12% SDS/PAGE. The gel was either subjected to Western blot analysis or subsequent autoradiography.

### RPA–XPA interaction on DNA

Experiments were carried out according to the previously described procedure [27]. Briefly, a 5′-end $^{32}$P-labelled 121 bp fragment (isolated from pBS plasmid digested with *Bst*N1) (3000 c.p.m./fmol) was irradiated with a germicidal UV lamp to prepare UV-damaged DNA. The reaction mixtures (100 $\mu$l) contained 25 mM Hepes/KOH (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, BSA (200 $\mu$g/ml), 0.1 $\mu$g of poly(dI-dC), 50 fmol of either irradiated or non-irradiated 5′-[$^{32}$P]DNA (121 bp) and the indicated amounts of GST–XPA and/or RPA. After a 15 min incubation at 30 °C, NETN buffer [20 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA/0.5% (v/v) NP40] containing 0.5% (w/v) dried-milk and GST beads was added and the reactions were processed as described for the GST-fusion pull-down assay. After the GST beads were washed, the samples were analysed for $^{32}$P-DNA on a 5% polyacrylamide gel containing 0.1% SDS.

### NER *in vitro*

NER of UV-damaged DNA was carried out as described [27,41] with the following modifications. HeLa whole cell extracts (230 $\mu$g) were preincubated on ice with 10 $\mu$g of anti-p70 monoclonal antibody (70 °C) for 30 min, before the addition of the reaction mixtures. Reaction mixtures (50 $\mu$l) contained 0.2 $\mu$g each of UV-irradiated (450 J/m$^2$) pBS (3 kb) and non-irradiated p5A (4.5 kb), 40 mM creatine phosphate-di-Tris salt (pH 7.7), 1 $\mu$g of creatine kinase, 50 mM Hepes/KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 0.5 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 $\mu$M of dGTP, dCTP and TTP, 8 $\mu$M of [$\alpha$-$^{32}$P]dATP (25000 c.p.m./pmol), 5 $\mu$g of BSA and the indicated amounts of either wt or mutant RPA. After incubation for 3 h at 30 °C, DNA was isolated from the reaction mixtures, linearized with *Bam*H1 and separated by 1% agarose gel electrophoresis in the presence of 0.5 $\mu$g/ml ethidium bromide. The DNA and repair products were analysed by both fluorography and exposure to X-ray film.

## RESULTS

### RPA with zinc-finger domain mutation

RPA p70 contains a 4-cysteine-type zinc-finger domain towards the C-terminus (amino acids 481–503). In order to study the role of the zinc-finger domain, we created wt p70, ZFM1, ZFM2 and ZFM4, which contain four, three, two and one cysteine residue (by substituting cysteine for alanine) respectively (Figure 1, upper panel). RPA complexes containing mutant p70 were isolated from insect cells co-infected with recombinant baculoviruses [17] (Figure 1, lower panel) and, judging from immunoprecipitation, mutant p70 forms a stable complex with wt-p34 and wt-p11, which is indistinguishble from that with wt-RPA (results not shown).

### Zinc-finger mutants support *in vitro* SV40 DNA replication but not NER

We have shown previously that a zinc-finger domain deletion mutant (RPA:p70ΔTU), containing p70 lacking amino acids 459–507, supported SV40 DNA replication *in vitro* [17]. To investigate further the role of the zinc-finger domain, we examined various zinc-finger mutants (Cys → Ala substitution) for their ability to support DNA replication. In keeping with our previous result, all four zinc-finger domain mutant RPAs efficiently supported SV40 DNA replication (Figure 2), except that the replication activity with mutant RPAs was somewhat lower than that with wt-RPA in the presence of low amounts of RPA.

In an effort to determine the role of RPA in NER, we also examined zinc-finger mutants for their ability to support NER (Figure 3). For this experiment, we first added anti-p70 antibody to neutralize endogenous RPA [18] and then added an increasing amount of either wt or mutant RPA for the reversal of RPA activity in the reaction. In the presence of anti-RPA antibody, repair activity was inhibited by 85%, and this inhibition was almost reversed by the addition of wt-RPA, but not by any of the zinc-finger mutants (Figure 3). This result suggests that RPA has a distinct role in repair, which may be separable from its role in replication.

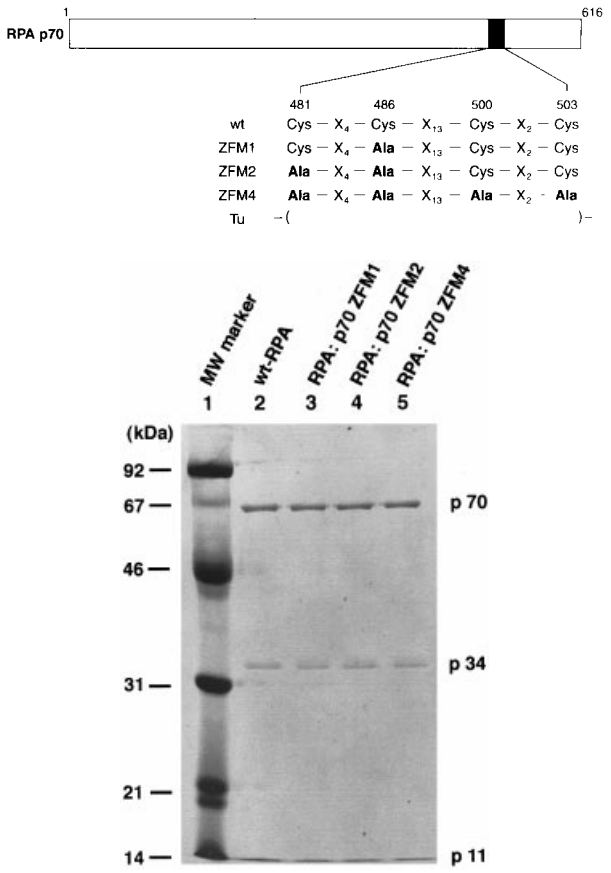


**Figure 1 RPA p70 and zinc-finger domain mutants**

Upper panel: map of RPA p70 zinc-finger domain and its mutants. Lower panel: SDS/PAGE of purified RPA containing either wt or mutant p70. Lysates from 2-litre cultures of Sf-9 cells, co-infected with recombinant baculoviruses encoding p34, p11 and either wt or mutant p70, were used as a source of RPA. RPA was purified by chromatography on a series of columns (ssDNA–cellulose, Affi-Gel Blue, and Q-Sepharose). Proteins were electrophoretically separated by 12% SDS/PAGE and visualized by Coomassie Blue staining.

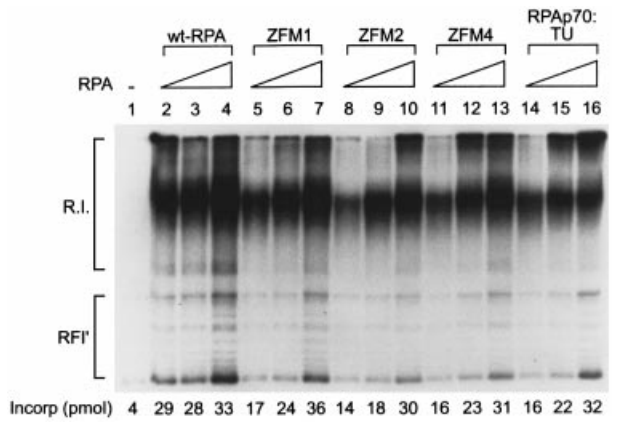


**Figure 2 Effect of zinc-finger domain mutants on SV40 DNA replication *in vitro***

Reaction mixtures (40 $\mu$l) contained 200 $\mu$g of HeLa cell cytosolic fraction lacking RPA (ammonium sulphate 35%–65%), 0.6 $\mu$g of SV40 Tag and increasing amounts (120 ng in lanes 2, 5, 8, 11 and 14; 240 ng in lanes 3, 6, 9, 12 and 15; 480 ng in lanes 4, 7, 10, 13 and 16) of either wt or mutant RPA. After the reaction, one-tenth amounts of reaction mixtures were used for measuring trichloroacetic acid-insoluble radioactivity and the remaining amounts were used to analyse replication products on 1% agarose gel electrophoresis after isolating DNA. R.I., replication intermediates of SV40 origin-containing DNA (pSV01ΔEP); RFI, various topoisomers of relaxed replication form I (closed circular double-stranded) DNA. Incorp. refers to the amount of dAMP incorporated into DNA.

### Zinc-finger domain of RPA is not necessary for its stimulatory effect on the interaction between XPA-damaged DNA, but is required for pol $\delta$ stimulation

RPA is involved in the early stage of NER by interacting with XPA on damaged DNA, which allows the stable RPA–XPA complex to form on damaged DNA [22–25,27]. Since the zinc-finger mutant failed to support NER, we were interested in examining whether the mutation at the zinc-finger domain affects XPA–DNA interaction. Purified GST–XPA fusion protein preferentially interacted with UV-damaged DNA, and this interaction was several-fold stimulated by wt-RPA as well as zinc-finger mutants (Figure 4), suggesting that the failure of zinc-finger mutants to support NER activity is not due to its function in XPA–DNA interaction.

RPA stimulates pol $\delta$ activity [14], which is likely to play a role in NER. We previously reported that a mutant RPA containing p70 with a zinc-finger domain deletion inhibited pol $\alpha$ and $\delta$ activities [17]. Therefore, we examined whether zinc-finger mutant RPA can support the stimulation of pol $\delta$ activity in the presence of two accessory factors, proliferating cell nuclear antigen (PCNA) and RF-C (also known as activator 1, A1) [42], on the elongation of a primed DNA template (Figure 5). In keeping with our previous observation, addition of increasing amount of wt-RPA significantly stimulated pol $\delta$ activity, whereas only marginal stimulation was observed with zinc-finger mutant RPA (Figure 5). This result may explain the failure of zinc-finger mutant RPA in NER.

### Zinc-finger domain of RPA affects its ssDNA-binding activity

RPA p70 has a broad ssDNA-binding domain which is essential for its function in replication and repair [17,30,32]. We and others have reported that RPA's zinc-finger domain is not required for its ssDNA-binding activity [17,30], though it may affect the overall affinity of the ssDNA-binding domain. We therefore examined ssDNA-binding activity of the mutants in the presence of low salt (100 mM NaCl) using 5′-labelled oligo$(dT)_{50}$ as a substrate. ZFM1 with a mutation at Cys-487 (mutated to alanine) showed ssDNA-binding activity similar to that of wt-RPA (Figure 6). The two distict bands seen in Figure 6 represent RPA–DNA complexes with different ratios of RPA:DNA [25,29]. In contrast, ZFM2 and ZFM4 showed 2–3-fold lower ssDNA-binding activity in the presence of 0.1 $\mu$g of RPA (lanes 2 and 5 compared with lanes 8 and 11 in Figure 6). With an increasing amount of RPA, however, the ssDNA-binding activity of zinc-finger mutants was comparable with that of wt-RPA (lanes 4 and 7 compared with lanes 10 and 13). We obtained a similar result in a reaction containing high salt (500 mM NaCl) (results not shown), suggesting that the zinc-finger domain is not directly involved in its ssDNA-binding activity, but affects the overall DNA-binding affinity.

### Zinc-finger domain of RPA is necessary for its p34 phosphorylation by DNA-PK, but not by CDKs

*In vivo* and *in vitro* results indicate that RPA p34 is phosphorylated by at least two protein kinases, cdk2-cyclin A and DNA-PK [6,43–45], even though the recent evidence points to the possibility of other protein kinases [46]. If the zinc-finger

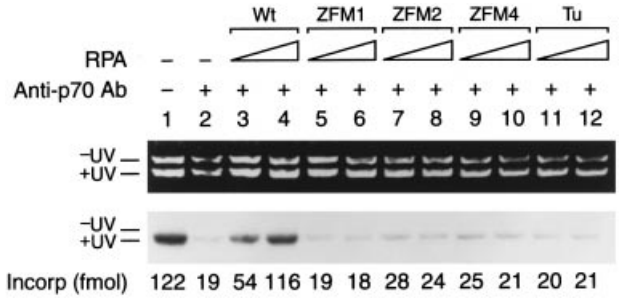


**Figure 3 Analysis of RPA containing a zinc-finger domain mutant p70 in NER *in vitro***

Reaction conditions are described in the Experimental section. In lanes 2–12, HeLa cells nuclear extracts (150 $\mu$g) were preincubated with 2 $\mu$g of anti-p70 monoclonal antibody (Ab) on ice for 30 min. Two different concentrations of either wt or mutant RPAs were added to the reaction mixtures (0.1 $\mu$g in lanes 3, 5, 7, 9 and 11; 0.2 $\mu$g in lanes 4, 6, 8, 10 and 12). After incubation, DNA was isolated, linearized with *Bam*H1 and separated by 1% agarose gel electrophoresis in the presence of 0.5 $\mu$g/ml ethidium bromide. The top panel shows a fluorograph of the gel, and the bottom panel indicates an autoradiogram. −UV and +UV represent the linearized form of non-irradiated p5A DNA (4.5 kb) and UV-irradiated (450 J/m$^2$) pBS (3 kb) respectively. To measure DNA repair activity, regions corresponding to linearized damaged DNA were excised and the radioactivity was quantified. Incorp. refers to the amount of dAMP incorporated into DNA.

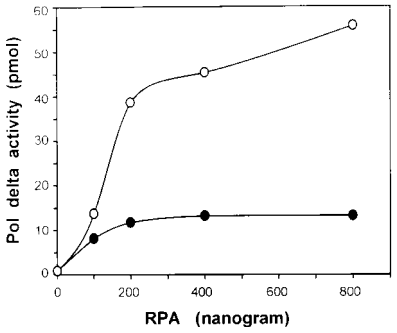


**Figure 5 Effect of wt and zinc-finger mutant RPA on pol $\delta$ activity**

Reaction mixtures (30 $\mu$l) contained 0.15 unit of pol $\delta$ (300 units/ml), 0.4 $\mu$g of PCNA, 0.7 $\mu$g of RF-C, 1 mM [$^3$H]dTTP (400 c.p.m./pmol), 0.1 $\mu$g of poly(dA)$_{4500}$:oligo(dT)$_{12–18}$, and the indicated amount of wt (○) or mutant (ZFM4) (●) RPA. After incubation at 37 °C for 30 min, acid-insoluble radioactivity was measured for pol $\delta$ activity. dTMP (2.2 pmol) was incorporated into DNA in the absence of RPA.

domain plays a role in the structural conformation of the three-subunit complex, the mutation at this site may affect the phosphorylation of the p34 subunit. We therefore examined whether mutation at the zinc-finger domain affects the phosphorylation of RPA p34. Interestingly, the phosphorylation of mutant RPAs (ZFM1 and ZFM2) by DNA-PK was almost completely inhibited (Figure 7B), whereas that by cdk2-cyclin A was comparable with that of wt-RPA (Figure 7A). We observed a similar result with ZFM4 (results not shown). This result suggests that the zinc-finger domain of p70 is necessary for the phosphorylation of the p34 subunit by DNA-PK.

## DISCUSSION

The three-subunit RPA complex is the bona fide ssDNA-binding protein that has multiple functions in replication, repair and recombination. All eukaryotic RPAs contain a conserved putative metal-binding motif toward the C-terminus of the p70 subunit, whose role in DNA metabolism is unclear. In an effort to define the structure–function relationships for RPA, we prepared various zinc-finger domain mutants and examined their activities in a number of assays requiring RPA function. In this study we show, by mutational analysis, that RPA's zinc-finger motif is necessary for pol $\delta$ stimulation, which is probably essential for NER, but not for DNA replication. We also showed that the zinc-finger domain of RPA p70 is necessary for phosphorylation of p34 by DNA-PK and affects the stablility of the RPA–ssDNA interaction (Figures 6 and 7).

All three zinc-finger domain mutants with Cys → Ala substitution efficiently supported SV40 DNA replication *in vitro*, which is consistent with our previous observation that mutant RPA with zinc-finger domain deletion supported DNA replication [17]. This result is contradictory to a previous report [32] that the zinc-finger domain is necessary for SV40 DNA replication *in vitro* [32]. At present, we do not know how this discrepancy in the role of the zinc-finger domain has arisen; however, the source of mutant RPA might be responsible for it (we prepared mutant RPA using a recombinant baculovirus

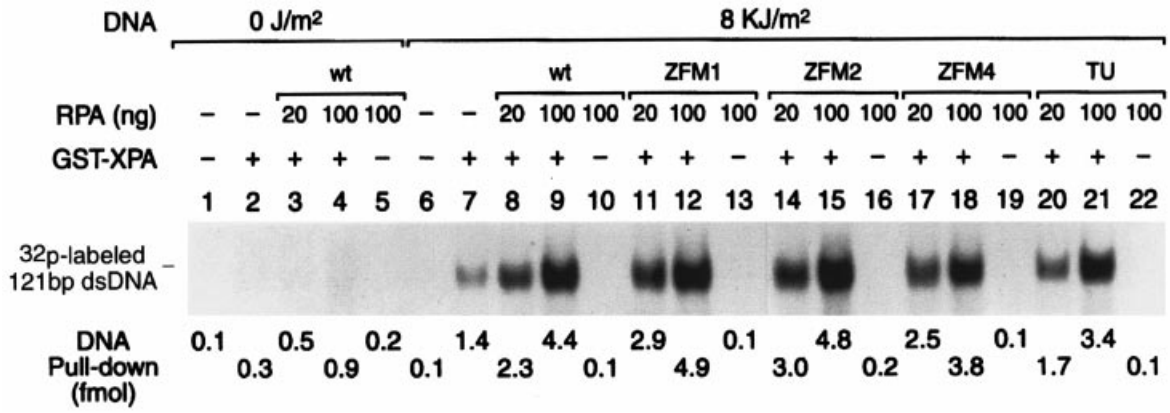


**Figure 4 Zinc-finger domain of RPA is not involved in its stimulation of XPA–damaged DNA interaction**

Reaction mixtures (100 $\mu$l) contained 25 mM Hepes/KOH (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, BSA (200 $\mu$g/ml), 1.0 $\mu$g of poly(dI.dC), 50 fmol of either irradiated (8 kJ/m$^2$) or non-irradiated 5′-$^{32}$P-labelled double-stranded DNA (121 bp of *Bst*NI fragment isolated from pBS) and, where indicated, 100 ng of purified GST–XPA was used. To this, the indicated amounts of either wt or mutant RPA were added. After incubation for 15 min at 30 °C, the mixtures were incubated with glutathione–Sepharose beads and analysed for GST–XPA–DNA complexes by 5% PAGE.

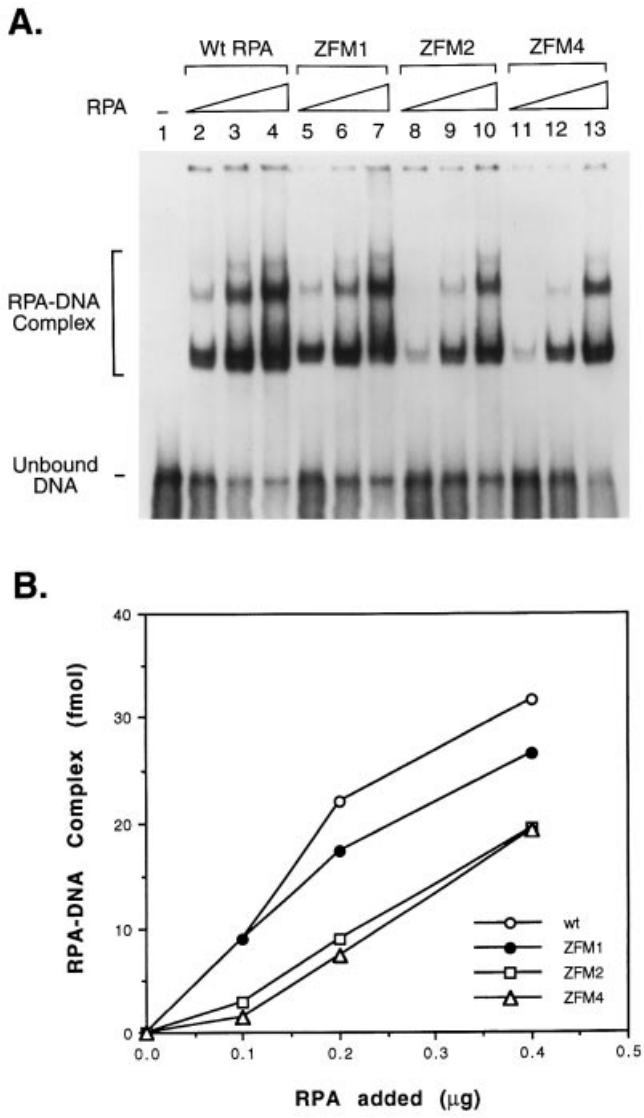


**Figure 6 ssDNA-binding activity of wt and zinc-finger domain mutants**

(**A**) Increasing amounts (0.1, 0.2 and 0.4 $\mu$g) of either wt or mutant RPA, as indicated at the top of the Figure, were incubated with 100 fmol of 5′-$^{32}$P-labelled oligo(dT)$_{50}$ for 5 min at room temperature. RPA–DNA complexes were analysed by 5% PAGE (acrylamide/bisacrylamide = 79:1). (**B**) Regions of RPA–DNA complex shown in (**A**) were excised and quantified.

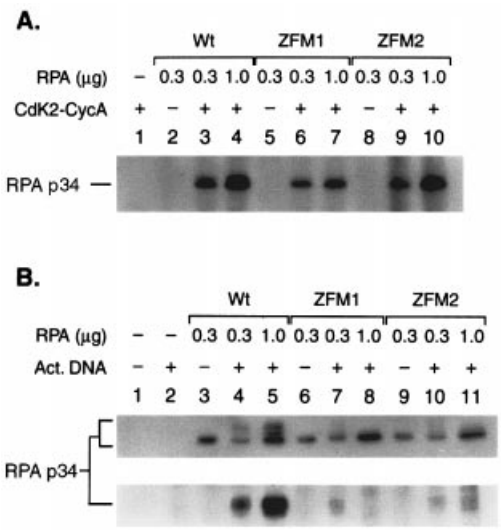


**Figure 7 Zinc-finger domain is essential for RPA phosphorylation by DNA-PK, but not by CDK**

(**A**) Phosphorylation of wt and mutant RPAs by cdk2-cyclin A (CdK2-CycA). Reaction mixtures (20 $\mu$l) contained 50 mM Hepes/KOH (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.25 $\mu$g/$\mu$l BSA, 50 mM KCl, 100 $\mu$M [$\gamma$-$^{32}$P]ATP (15000 c.p.m./pmol), and the indicated amounts of wt or mutant RPA. Where indicated, 20 ng of cdk2-cyclin A was used. The reaction mixtures were separated by 12% SDS/PAGE and analysed by exposure to X-ray film. (**B**) Phosphorylation of wt and ZFM mutants by DNA-PK. Reaction conditions were as described in the Experimental section. Where indicated, 0.2 $\mu$g of DNA-PK and 0.1 $\mu$g of activated calf-thymus DNA (Act. DNA) were used. The reaction mixtures were incubated for 30 min and were separated by 12% SDS/PAGE. The gel was either subjected to Western blot analysis using anti-p34 polyclonal antibody (upper panel) or subsequent autoradiography (lower panel).

expression system, whereas others have obtained it from an *Escherichia coli* expression system [47]). Also, we cannot completely rule out the possibility that the 10% impurities in these RPA preparations may have affected their biochemical activities. Further investigation is necessary to clarify the role of the zinc-finger domain in DNA metabolism.

RPA has multiple functions in repair [24], one of which is to form a complex with XPA at damaged DNA sites and stimulate XPA–DNA interaction [23,24,27]. RPA's ssDNA-binding activity is essential for XPA–RPA interaction and NER activity [27]. Even though ZFM has no significant impact on XPA–RPA interaction on damaged DNA (Figure 4), it showed significantly reduced stimulation of pol $\delta$ activity compared with that with wt-RPA (Figure 5), suggesting that ZFM may affect the later stage of DNA repair, i.e. the filling-in reaction by pol $\delta/\epsilon$. It has been shown previously that the deletion of the zinc-finger domain abolished RPA's function in stimulating DNA pol $\alpha$ and $\delta$ activities in the elongation of a primed DNA template [17]. Requirement of a zinc-finger domain in DNA repair, but not in replication, strongly suggests a differential role for RPA in DNA metabolism. This may be useful in the future in elucidating the role of RPA in DNA repair.

RPA's ssDNA-binding domain resides in the middle of p70 [17,30,32] and the structural analysis revealed that this domain consists of two homologous subdomains in tandem positions [48]. RPA's zinc-finger domain may not be directly involved in its ssDNA-binding activity, since a mutant RPA lacking the zinc-finger motif retained its ssDNA-binding activity. In this study, however, we have demonstrated that mutation at the zinc-finger domain lowered its ssDNA-binding activity, particularly in the presence of low RPA. It is likely that a mutation at the zinc-finger domain inhibits the conformational change in p70 that is necessary for the switch from an unstable and weak RPA–ssDNA interaction to a stable and strong RPA–ssDNA interaction. This is also supported by recent biochemical studies [29], which indicate that human RPA combines with ssDNA in at least two binding modes: one is a relatively unstable and compact complex and the other is an elongated and stable complex.

The zinc-finger domain of adenovirus DNA-binding protein has previously been shown to be involved in ssDNA-binding activity, and a mutation in this region abolished its ssDNA-binding activity [34]. Several eukaryotic transcription factors for RNA pol II, such as SP1, contain zinc-finger domains which have been shown to be essential for their sequence-specific DNA-binding activity. In yeast, RPA binds to regulatory elements of the genes involved in DNA repair and other DNA metabolism

[49]. This sequence-specific DNA-binding activity might be related to the role of RPA in transcriptional activation; RPA p34 has two distinct transcriptional activation domains which are potentially involved in transcription or transcriptional activation necessary for the initiation of DNA replication. It would be interesting to see whether RPA's zinc-finger domain is involved in its sequence-specific DNA binding.

RPA phosphorylation occurs at the beginning of the S-phase and upon DNA damage, suggesting its role in the regulation of DNA metabolism. Our studies from this work suggest that the phosphorylation sites for DNA-PK are likely to be hindered by p70 and are not available when RPA is not associated with DNA. Upon DNA binding, the structural change in p70 allows p34 (and its phosphorylation sites) access to DNA-PK for phosphorylation. In keeping with this notion, others have shown that formation of the extended hRPA30nt complex, the stable form of RPA–DNA interaction, correlates with increased phosphorylation of RPA p34 [29]. In this way, the phosphorylation of p34 by DNA-PK is controlled by the p70 subunit in a zinc-finger-domain dependent manner. There are a number of cases where the zinc-finger domain controls the biological activity of RPA, for example, in aspartate transcarbamoylase a Zn(II)-binding small regulatory subunit forms a stable complex with the catalytic subunit of RPA and regulates the enzyme activity [50]. It should be pointed out that, at present, there is no direct evidence that the zinc-finger motif actually binds zinc. At any rate, these studies provide an interesting insight into the structure and function relationship of a multiprotein complex, such that the role of one subunit (or a specifc domain) is regulated by the other subunit. This zinc-finger domain mutant may be useful in the future to identify the the role of RPA phosphorylation *in vivo*.

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