Functional Analysis of Human Replication Protein A in Nucleotide Excision Repair*

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Human replication protein A (RPA) is a three-subunit protein complex (70-, 34-, and 11-kDa subunits) involved in DNA replication, repair, and recombination. Both the 70- (p70) and 34-kDa (p34) subunits interact with Xeroderma pigmentosum group A complementing protein (XPA), a key protein involved in nucleotide excision repair. Our deletion analysis indicated that no particular domain(s) of RPA p70 was essential for its interaction with XPA, whereas 33 amino acids from the C terminus of p34 (p34<sub>D33C</sub>) were necessary for the XPA interaction. Furthermore, mutant RPA lacking the p34 C terminus failed to interact with XPA, suggesting that p34, not p70, is primarily responsible for the interaction of RPA with XPA. RPA stimulated the interaction of XPA with UV-damaged DNA through an RPA-XPA complex on damaged DNA sites because (i) the RPA mutant lacking the C terminus of p34 failed to stimulate an XPA-DNA interaction, and (ii) the ssDNA binding domain of RPA (amino acids 296–458) was necessary for the stimulation of the XPA-DNA interaction. Two separate domains of p70, a single-stranded DNA binding domain and a zinc-finger domain, were necessary for RPA function in nucleotide excision repair. The mutant RPA (RPA: p34<sub>D33C</sub>), which lacks its stimulatory effect on the XPA-DNA interaction, also poorly supported nucleotide excision repair, suggesting that the XPA-RPA interaction on damaged DNA is necessary for DNA repair activity.

Human replication protein A (also known as human single-stranded DNA-binding protein) is composed of three tightly associated polypeptides (70-, 34-, and 11-kDa subunits). It was originally identified as a factor required for in vitro SV40 DNA replication (1–3). In SV40 DNA replication, RPA<sup>1</sup> interacts with SV40 Tag and the DNA polymerase α-primase complex at the origin, where it participates in the initiation process (4–8). During replication, RPA binds to and stabilizes the ssDNA generated at the replication forks and stimulates DNA polymerase α activity, suggesting it has a role in both initiation and elongation (9–11). Recent mutational analysis, however, indicates that the ssDNA binding activity of RPA, but not its ability to stimulate DNA polymerase, is essential for SV40 DNA replication (12).

The involvement of RPA in DNA repair has been shown in human and yeast nucleotide excision repair systems (13–16). Similar to its function in replication, RPA appears to be involved in multiple stages of the DNA repair process. Its role in the early stage of repair was suggested because the RPA requirement can be bypassed by the presence of Escherichia coli UvrABC, which incises damaged DNA (14). This early stage involvement includes the interaction of RPA with the UV damage recognition factor XPA (17–20). After forming an RPA-XPA complex on damaged DNA, RPA recruits XPG and ERCC1-XPF, two endonucleases that are likely involved in the 3′ and 5′ incision reactions (18, 21) to the damaged site. RPA is also involved in the later stage gap-filling reaction, which requires PCNA, RF-C (A1), and DNA polymerase δ (or ε) (22).

In the mammalian system, RPA p34 phosphorylation is induced by DNA damage (UV or x-ray). DNA-dependent protein kinase (DNA-PK) was shown to be responsible for the hyperphosphorylation of RPA p34 (23, 24); however, recent in vivo observations suggest the possible involvement of other kinases (25). Human and mouse cells deficient in DNA-PK activity are not only defective in site-specific recombination but are also very sensitive to DNA damage, suggesting a role for DNA-PK (and possibly RPA phosphorylation) in these processes (26, 27). In addition, extracts from UV-irradiated HeLa cells poorly support SV40 DNA replication compared with those from non-irradiated cells (23). UV-damage-induced inhibition of replication correlates with RPA phosphorylation and is reversed by the addition of purified RPA to an in vitro system, suggesting a role for RPA phosphorylation in damage-induced inhibition of replication (23). However, no biologic function for RPA phosphorylation has yet been identified.

RPA is involved in recombination through its stimulation of strand exchange reactions in vitro (28) and its requirement in homologous recombination (29). Recently, RPA was shown to interact with Rad52, a protein required for recombination and strand-break repair (30, 31). In addition, the RPA-Rad52 interaction seems to be required for homologous recombination in vivo (32).

RPA forms a complex in an orderly manner such that p34 and p11 form a stable subcomplex to which p70 is then added (33, 34). The p70 subunit has multiple functional domains, an N-terminal domain for interacting with pol α (12, 35), p53 (36), two middle subdomains with DNA binding activity (12, 37), four cysteine-type zinc finger domains (amino acids 481–503),
and a C-terminal domain for interacting with other RPA subunits (12, 37). The p34 phosphorylation sites for DNA-PK and cyclin-dependent kinase have recently been mapped to its N-terminal domain (38, 39).

In this report, we analyzed RPA function in nucleotide excision repair by examining various RPA mutants. We found that the C-terminal domain of RPA p34 is responsible for RPA interaction with XPA. This interaction is necessary for the formation of a stable RPA-XPA complex on DNA. An RPA mutant lacking the p34 C terminus poorly supported DNA repair, which strongly suggests that the XPA-RPA interaction is necessary for efficient DNA repair. We also found two p70 domains (a dsDNA binding domain and a zinc finger motif) that were essential for nucleotide excision repair.

MATERIALS AND METHODS

DNA, Recombinant Baculoviruses, and Antibodies—A plasmid expressing glutathione S-transferase (GST)-Rad52 was described previously (32), and a plasmid harboring the GST-XPA fusion gene (17) was kindly provided by Dr. K. Tanaka (Osaka, Japan). Random oligonucleotides (90-mer, 5\(^{\text{d}}\)GGCCGAGGAT-3\(^{\text{r}}\) and 3\(^{\text{r}}\)-GATCGATCGAGCGGCCGCCA-5\(^{\text{d}}\)) were synthesized by the Molecular Resource Center at St. Jude Children’s Research Hospital, Memphis, TN.

The DNA plasmid, p5A (4.5 kbp), represents pBluescript (pBS, 3 kbp) in which the 1.45-kb full-length cDNA for RPA p34 had been inserted. This plasmid was prepared for the nucleotide excision repair assay by two rounds of CsCl gradient centrifugation (40) followed by 5–20% sucrose density gradient centrifugation at 25,000 rpm for 19 h at 2 °C. pBS (3 kbp) was prepared by two rounds of CsCl centrifugation from the E. coli strain DH5\(\alpha\), which contains a plasmid harboring the GST-XPA fusion gene (17) described previously (33). Glutathione-Sepharose 4B and protein A-Sepharose CL 4B were kindly provided by Dr. C. Anderson (Brookhaven National Laboratory, Long Island, NY) (44).

Cell Extracts and Their Fractions—HeLa whole cell extracts were prepared as described previously (41). Briefly, asynchronously grown HeLa cells (5 \(\times\) 10\(^6\)) were washed twice with phosphate-buffered saline and resuspended in hypotonic buffer (20 mM Hepes-KOH, pH 7.5, 5 mM KCl, 1.5 mM MgCl\(_2\), 1 mM DTT). Protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM/ml antipain and leupeptin) were then added, and the swollen cells were broken by 15–20 strokes in a dounce homogenizer. An equal volume of buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl\(_2\), 2 mM DTT, 25% sucrose, and 50% glycerol) was added to the dounce-homogenized extracts followed by one-eighth volume of saturated ammonium sulfate solution, pH 7.5. The mixture was then stirred for 30 min on ice. After centrifugation at 41,000 rpm for 3 h at 2 °C, the supernatant was collected, and proteins were precipitated by the addition of ammonium sulfate (0.33 g/ml). After centrifugation, the precipitates were resuspended and dialyzed against buffer (25 mM Hepes-KOH, pH 7.9, 0.1 mM KCl, 12 mM MgCl\(_2\), 1 mM EDTA, 2 mM DTT, and 17% glycerol).

To prepare the DNA substrate, E. coli endonuclease III-overproducing strain was originally obtained from Dr. J. Cunningham (State University of New York, Albany, NY) and purified according to the published procedure (45). DNA-PK was kindly provided by Dr. C. Anderson (Brookhaven National Laboratory, Long Island, NY) (44).

The DNA plasmid, p5A (1.2 kbp), represents the plasmid prepared by two rounds of CsCl centrifugation from the E. coli strain DH5\(\alpha\), without chloramphenicol amplification. The purified pBS plasmid was digested with BstN1 and a 121-bp fragment isolated (9). The purified 121-bp fragment was used as a probe to isolate recombinant baculoviruses containing either wild-type or mutant RPA subunits described previously (8, 12, 33). Anti-p70 and p34 polyclonal antibodies were also described previously (33). Glutathione-Sepharose 4B and protein A-Sepharose CL 4B were purchased from Amersham Pharmacia Biotech.

Metabolic Labeling of SJ-9 Cells with \(^{35}\)SMethionine and Immunoprecipitation—\(^{35}\)SMethionine-labeled cell lysates were prepared as described previously (35). SJ-9 cells (2.0 \(\times\) 10\(^6\)) plated on a 60-mm dish and infected with individual recombinant baculoviruses encoding RPA subunits at a multiplicity of infection of 15 for approximately 40 h at 27 °C. The infected cells were then labeled with trans\(^{35}\)S-labeled methionine at 200 \(\mu\)Ci/ml (1200–1600 Ci/mmole) for 4 h in methionine-free medium containing 5% dialyzed fetal calf serum. Cells were briefly washed with phosphate-buffered saline and lysed for 1 h on ice in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 1 mM EDTA, 0.1 mM NaF, 10 mM \(\beta\)-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 M/g/ml leupeptin, and 0.2 M/g/ml antipain). For immunoprecipitation, cleared cell lysates (50 \(\mu\)l) were incubated with the indicated polyclonal antibody (5 \(\mu\)l) in the presence of bovine serum albumin (200 \(\mu\)g/ml) at 4 °C. After centrifugation (13,000 rpm, 1 min), the mixture was incubated for 1 h at 4 °C. Immunoprecipitates were collected by centrifugation, washed 5 times with cell lysis buffer, and analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE).

GST Fusion Pull-down Assay—Unless otherwise specified, aliquots (0.4–1.0 ml) of GST fusion protein containing lysates were adjusted to 0.5% nonfat dried milk and add to 25 \(\mu\)l of glutathione-Sepharose beads that had been washed three times and resuspended (1.1 (v/v)) in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 0.5% dried milk. The aliquots were then rocked for 30 min at 4 °C. After being washed 3 times with NETN containing 0.5% dried milk, the beads were incubated with lysates containing target protein (or purified protein) for 1 h at 4 °C on a rocker and then washed for 3 times with NETN. For the analysis of GST-XPA proteins bound to the beads, the beads were mixed with SDS-PAGE sample buffer, and proteins were resolved on SDS-PAGE. Proteins were then visualized either by fluorography or immunoblotting. To purify bound GST-XPA, proteins bound to the GST beads were eluted with elution buffer (40% ethylene glycol, 10% glycerol, 1.5 mM NaCl, 0.01% Nonidet P-40, 1 mM EDTA, 1 mM DTT, and 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 100 \(\mu\)g/l antipain and leupeptin) overnight.

SDS-PAGE and Western Blot Analysis—Protein samples were separated by 12% SDS-PAGE as described previously (42). Proteins were then transferred to nitrocellulose (BAA5; Bio-Rad), immunoblotted with an anti-p70 (or anti-p34) rabbit polyclonal antibody (33), and detected by \(^{125}\)I-protein A (Amersham).

Preparation of Proteins—Wild-type RPA and RPA mutants, PCNA, and XPA were isolated by the procedures described previously (12, 20). The E. coli endonuclease III-overproducing strain was obtained from Dr. J. Cunningham (State University of New York, Albany, NY) and purified according to the published procedure (45). DNA-PK was kindly provided by Dr. C. Anderson (Brookhaven National Laboratory, Long Island, NY) (44).

Cell Extracts and Their Fractions—HeLa whole cell extracts were prepared as described previously (41). Briefly, asynchronously grown HeLa cells (5 \(\times\) 10\(^6\)) were incubated at 4°C for 1 h with 0.5% sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM/ml leupeptin, and 0.2 M/ml antipain) for 1 h at 4 °C. After centrifugation (13,000 rpm, 1 min), the mixture was incubated for 1 h at 4 °C. Immunoprecipitates were collected by centrifugation, washed 5 times with cell lysis buffer, and analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE).

GST Fusion Pull-down Assay—Unless otherwise specified, aliquots (0.4–1.0 ml) of GST fusion protein containing lysates were adjusted to 0.5% nonfat dried milk and add to 25 \(\mu\)l of glutathione-Sepharose beads that had been washed three times and resuspended (1.1 (v/v)) in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 0.5% dried milk. The aliquots were then rocked for 30 min at 4 °C. After being washed 3 times with NETN containing 0.5% dried milk, the beads were incubated with lysates containing target protein (or purified protein) for 1 h at 4 °C on a roller and then washed for 3 times with NETN. For the analysis of GST-XPA proteins bound to the beads, the beads were mixed with SDS-PAGE sample buffer, and proteins were resolved on SDS-PAGE. Proteins were then visualized either by fluorography or immunoblotting. To purify bound GST-XPA, proteins bound to the GST beads were eluted with elution buffer (40% ethylene glycol, 10% glycerol, 1.5 mM NaCl, 0.01% Nonidet P-40, 1 mM EDTA, 1 mM DTT, and 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 100 \(\mu\)g/l antipain and leupeptin) overnight.
mm EDTA, 2 mM ATP, 20 μM each dGTP, dCTP, TTP, 8 μM [α-32P]dATP (25,000 cpm/pmole), 5 μg of bovine serum albumin, and 150 μg of the PC-1.0 fraction of HeLa whole cell extracts. After incubation for 3 h at 30 °C, DNA was isolated from the reaction mixtures, linearized with BamHI, and separated by 1% agarose electrophoresis in the presence of 0.5 μg/ml ethidium bromide. The DNA and repair products were analyzed by both fluorography and exposure to x-ray film.

RESULTS

The C Terminus of p34 Is Primarily Responsible for Interaction of RPA with XPA—RPA interacts with SV40 Tag, XPA, and human Rad52, key proteins involved in replication, repair, and recombination, respectively. These interactions are essential to the function of RPA in DNA metabolism. We and others have shown that both p70 and p34 interact with XPA, Rad52 (17, 18, 32), and SV40 Tag (data not shown) and have suggested that RPA may function in all three DNA metabolic events by interacting with key proteins in replication, repair, and recombination.

To identify the domain(s) of RPA p70 and p34 involved in these protein-protein interactions, we examined p70 and p34 deletion mutants for their ability to interact with XPA. An GST-XPA fusion protein was used to pull down the metabolically labeled RPA p70 or p34 (Fig. 1, panel A). Compared with the amount of mutant p70 immunoprecipitated by the anti-p70 antibody, only a fraction of the p70 in lysates was pulled down by the GST fusion protein (Fig. 1, panel B). In addition, no particular domain for p70 binding to XPA was identified. In contrast, p34 interacted efficiently with XPA (Fig. 1, panel C). Of interest, XPA interacted with a p34 mutant that had an N-terminal deletion (p34Δ2-30) as well as a mutant that had a serine-to-alanine substitution in its cdk consensus sites (amino acids 23 and 29); however, XPA did not interact with a p34 mutant that lacked 33 amino acids at its C terminus (Fig. 1, panel C).

To further analyze the RPA-XPA interaction, we examined purified RPA complexes containing either p34 that lacked the C terminus (p34Δ33C) or p70 that lacked the ssDNA binding domain (ARS) for their interaction with XPA. Mutant RPA that lacked the p34 C terminus failed to interact with XPA, whereas RPA containing the p70 mutant (ΔARS) interacted with XPA (Fig. 2). All other RPA mutants that failed to interact with XPA contained deletions at the N terminus (p34Δ23-30, 33C, and Wt). In contrast, p34Δ2-30 interacted efficiently with XPA (Fig. 1, panel C). Of interest, XPA interacted with a p34 mutant that had an N-terminal deletion (p34Δ2-30) as well as a mutant that had a serine-to-alanine substitution in its cdk consensus sites (amino acids 23 and 29); however, XPA did not interact with a p34 mutant that lacked 33 amino acids at its C terminus (Fig. 1, panel C).

RPA-XPA Interaction on UV-damaged DNA—Previous reports demonstrated that XPA binding to damaged DNA is enhanced by the presence of RPA (18, 19), even though both XPA and RPA can independently bind to damaged DNA (45, 46). We examined the effect of RPA on the interaction of XPA with DNA by using purified GST-XPA in a pull-down assay to measure the interaction of XPA with a 5′-labeled 121-bp fragment of damaged and nondamaged dsDNA. The purified GST-XPA used in this experiment was described previously (17) and was functionally active in nucleotide excision repair (data not shown). Consistent with a previous study (45), XPA alone showed a strong interaction with damaged DNA (8 kJ/m2) but not with nondamaged DNA (Fig. 3, panel A). The XPA-damaged DNA interaction was significantly stimulated (up to 4-fold) by the addition of RPA (Fig. 3, panel B). The weak interaction between XPA and nondamaged DNA was also stimulated by RPA (Fig. 3, panel B, lanes 2–4), which may be explained by the fact that RPA binds to dsDNA in a nonspecific manner (9, 11). We examined whether the stimulatory effect of RPA-XPA interaction on UV-damaged DNA—

FIG. 1. Interaction of RPA subunits with XPA. A, interaction of GST-XPA with p70 and p34 subunits of RPA. [35S]Methionine-labeled lysates from insect (Sf-9) cells infected with recombinant baculoviruses encoding p70, p34, or p11, or all three, is shown. Aliquots of precleared lysates were incubated with glutathione-Sepharose loaded with GST (first five lanes) or GST-XPA protein (last five lanes). After washing, bound proteins were analyzed by electrophoresis in a 12% polyacrylamide gel and visualized by fluorography. B, interaction of various RPA p70 deletion mutants with XPA. [33S]Methionine-labeled lysates from insect cells infected with recombinant baculoviruses encoding either wild-type or mutant p70 were immunoprecipitated with anti-p70 polyclonal antibody (upper panel) or incubated with glutathione-Sepharose loaded with GST-XPA (lower panel) and analyzed for their interaction with p70 by 12% SDS-PAGE. B, interaction of various RPA p70 deletion mutants with XPA. [33S]Methionine-labeled lysates from insect cells infected with recombinant baculoviruses encoding either wild-type or mutant p70 were immunoprecipitated with anti-p70 polyclonal antibody (upper panel) or incubated with glutathione-Sepharose loaded with GST-XPA (lower panel) and analyzed for their interaction with p70 by 12% SDS-PAGE. C, interaction of RPA p34 mutants with XPA. [33S]Methionine-labeled lysates from insect cells infected with recombinant baculoviruses encoding either wild-type or mutant p34 were immunoprecipitated with anti-p34 polyclonal antibody (upper panel) or incubated with glutathione-Sepharose loaded with GST-XPA (lower panel) and analyzed by 12% SDS-PAGE. The p34(S/A:23,29) is a double point mutant in which alanine is substituted for serine at amino acids 23 and 29. The p34Δ2-30 and the p34Δ33C represent p34 mutants lacking amino acids 2–30 (at the N terminus) and 33 amino acids at the C terminus, respectively. Wt, wild type.
RPA on the XPA-DNA interaction occurs through the interaction of RPA with XPA on DNA. For this, we compared the XPA-DNA interaction of wild-type RPA with that of mutants lacking either the N-terminal or C-terminal domain of p34 (RPA:p34Δ2-30 and RPA:p34Δ33C, respectively) (Fig. 4). The wild-type RPA and RPA:p34Δ2-30 showed similar stimulatory effects on XPA-DNA, whereas RPA:p34Δ33C had virtually no effect. We also examined the effects of mutant RPAs on the XPA-damaged DNA interaction. As shown in Fig. 5, mutant RPAs lacking the N-terminal domain (RPAp70Δ2-100 and RPAp70JK (Δ101-169)) showed a 25–50% reduction in their ability to stimulate the XPA-DNA interaction, whereas mutant RPAs lacking the ssDNA binding domain (RPAp70PQ and RPAp70RS; see Ref. 12 for details) were totally inactive. Our results strongly indicate that RPA forms a complex with XPA on damaged DNA and thereby stabilizes the interaction of XPA with the DNA.

DNA damage induces phosphorylation of RPA and inhibits DNA replication in S-phase cells (23, 24, 47), suggesting a possible role for RPA phosphorylation in repair and replication. However, both phosphorylated and nonphosphorylated RPA efficiently interacted with XPA, indicating that the interaction of RPA with XPA does not require phosphorylation of RPA and can occur in the absence of DNA (Fig. 6).

The C-terminal Domain of p34 Is Necessary for the Function of RPA in Repair—Because the C-terminal domain of RPA p34 is responsible for the interaction of RPA with XPA (Figs. 2 and 3), we examined whether this interaction was also necessary for the function of RPA in DNA repair. It has previously been suggested that the RPA-XPA interaction is required for DNA repair (19). We, therefore, examined the ability of RPAs containing different p34 mutants to support nucleotide excision repair activity. The RPAp34Δ33C mutant poorly supported nucleotide excision repair in vitro, whereas an RPA mutant lacking phosphorylation sites (Δ2-30) efficiently supported repair activity (Fig. 7). This result is in keeping with our results on the stimulation of the interaction between XPA and damaged DNA and suggests that the interaction of RPA p34 with XPA is necessary for nucleotide excision repair. These results also support the previous observation that RPA phosphorylation is not required for nucleotide excision repair (48).

![Fig. 2. The p34 but not p70 is responsible for the RPA-XPA interaction.](Image)

![Fig. 3. RPA stimulates the interaction of XPA with UV-damaged DNA.](Image)

![Fig. 4. The RPA-XPA interaction is necessary for the effect of RPA on the XPA-damaged DNA interaction.](Image)
Fig. 8, the DNA binding domains of p70 were essential for DNA repair activity, but the N-terminal polymerase α stimulation domain was not. Of interest, mutant RPA that lacked a zinc finger domain of p70 (RPAp70ΔD1TU) was unable to support nucleotide excision repair, suggesting a role for the zinc finger domain in this activity. In contrast, zinc finger domain deletion and substitution mutants efficiently support SV40 DNA replication in vitro (12).

DISCUSSION

RPA plays an essential role in multistaged nucleotide excision repair. To better understand the roles of the individual RPA subunits in repair, we analyzed a series of RPA mutants for their ability to repair DNA and to interact with XPA.

As reported previously (18), both the p70 and the p34 subunit of RPA interacted with XPA. Our GST fusion pull-down assay demonstrated that p70 interacted weakly with XPA and that no particular p70 domain was involved in this interaction. On the other hand, the interaction of p34 with XPA was very strong and required the C-terminal domain of p34, suggesting that the p34 C terminus is primarily responsible for the RPA-XPA interaction. In studies by others (19), however, deletion at conserved motifs in XPA abolished binding to p70 but not binding to p34. It is still possible that other p70 deletions or mutations that were not used in this study would cause loss of binding to XPA. Nonetheless, the C-terminal domain of p34 is highly acidic and conserved among eukaryotes (49, 50), suggesting that this domain has an essential role in the regulation of DNA metabolism. In fact, the C-terminal domain of p34 is also involved in the RPA-Rad52 interaction (data not shown).

The RPA-XPA interaction is likely involved in recognition of damaged DNA because the stimulatory effect of RPA on the DNA binding activity of XPA requires the RPA-XPA interaction. Furthermore, the RPA mutant that lacks ssDNA binding domain was not able to stimulate the interaction between XPA and damaged DNA. These results indicate that both RPA and XPA likely interact with each other on the damaged DNA to form a stable complex.

Fig. 5. The ssDNA binding activity of RPA is necessary for its effect on the interaction of XPA with UV-damaged DNA. Reaction mixtures contained 50 fmol of irradiated (8 kJ/m²) 32P-labeled dsDNA (121-bp fragment), and where indicated, 100 ng of purified GST-XPA and 20 ng (lanes 3, 6, 9, 12, 15, and 18) or 100 ng (lanes 4, 5, 7, 8, 10, 11, 13, 14, 16, 17, 19, and 20) of either wild-type (wt) or mutant RPA were included. All other conditions were the same as those described for Fig. 3.

Fig. 6. RPA phosphorylation has no effect on the RPA-XPA interaction. Reaction mixtures (60 μl) contained 7 mM MgCl₂, bovine serum albumin (200 μg/ml), 4 mM ATP, and 1.0 μg of purified wild-type (wt) RPA. Where indicated, 1.0 μg of purified DNA-PK or 0.2 μg of φX174 (ssc) was added. After a 1-h incubation at 30 °C, the mixtures were directly analyzed by 12% SDS-PAGE and Western blot analysis (panel A) or incubated with glutathione-Sepharose beads loaded with GST-XPA before Western blot analysis (panel B). An anti-p34 polyclonal antibody (from rabbit) was used for immunoblotting.

Fig. 7. Analysis of RPA containing mutant p34 in nucleotide excision repair. A, reaction conditions were described under "Materials and Methods" Where indicated, 0.2 μg of PCNA and increasing amounts (0.15, 0.3, and 0.45 μg) of either wild-type (wt) or mutant RPA were used. After incubation, DNA was isolated, linearized with BamHI, and separated by 1% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide. The top panel indicates a fluorograph of the gel, and the bottom panel indicates an autoradiogram. B, regions corresponding to linearized damaged DNA were excised, and the radioactivity was quantitated.

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is essential for nucleotide excision repair (18, 19). Our result that an RPA mutant lacking the ability to interact with XPA poorly supports nucleotide excision repair in vitro is consistent with these previous observations. Furthermore, an RPA mutant that lacks the RPA phosphorylation sites supported nucleotide excision repair (Fig. 7), suggesting that RPA phosphorylation is not necessary for DNA repair (48). Consistent with this finding, our results also show that RPA phosphorylation has no effect on the interaction between RPA and XPA (Fig. 6).

We previously reported that XPA interacts with RPA and interferes with RPA function in DNA replication (20). In this report, however, we have demonstrated that the RPA-XPA interaction is necessary for nucleotide excision repair. Therefore, it is possible that the RPA-XPA complex, once formed, is used for repair and not for replication, which suggests some mechanism exists to differentially regulate RPA activity upon DNA damage.

The ssDNA binding domain of RPA is localized in the middle of the p70 subunit and is essential for DNA replication (12). Mutant RPA that lacks ssDNA binding activity does not support nucleotide excision repair (Fig. 8), suggesting that the ssDNA binding domain of RPA is essential for its function in DNA metabolism. Both XPA and RPA need to contact the DNA ssDNA binding domain of RPA is essential for its function in nucleotide excision repair (Fig. 8), suggesting that the ssDNA binding domain of RPA is essential for its function in primed DNA templates (12). Similarly, the zinc finger mutant could affect DNA polymerase activity during the gap-filling reaction, thereby inhibiting repair. Despite being involved in repair, this zinc finger mutant may not have a significant impact on DNA polymerase activity at the replication fork. Another possibility is that the zinc finger mutant affects the ability of RPA to bind DNA, particularly damaged DNA (46). We observed that the zinc finger RPA mutant (cysteine-to-alanine substitution) had reduced ssDNA binding activity compared with wild-type RPA. It is not clear, however, whether this reduction in ssDNA binding activity contributes to the inability of the mutant to support nucleotide excision repair. Further characterization will be necessary to clarify the role of zinc finger domain of RPA in repair.

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5. The addition of increasing amounts of zinc finger mutant RPA inhibits DNA polymerase α and δ activities on primed DNA templates (12). Similarly, the zinc finger mutant could affect DNA polymerase activity during the gap-filling reaction, thereby inhibiting repair. Despite being involved in repair, this zinc finger mutant may not have a significant impact on DNA polymerase activity at the replication fork. Another possibility is that the zinc finger mutant affects the ability of RPA to bind DNA, particularly damaged DNA (46). We observed that the zinc finger RPA mutant (cysteine-to-alanine substitution) had reduced ssDNA binding activity compared with wild-type RPA. It is not clear, however, whether this reduction in ssDNA binding activity contributes to the inability of the mutant to support nucleotide excision repair. Further characterization will be necessary to clarify the role of zinc finger domain of RPA in repair.
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