An Affinity of Human Replication Protein A for Ultraviolet-damaged DNA

IMPLICATIONS FOR DAMAGE RECOGNITION IN NUCLEOTIDE EXCISION REPAIR

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Replication protein A (RPA), a heterotrimeric protein of 70-, 32-, and 14-kDa subunits, is an essential factor for DNA replication. Biochemical studies with human and yeast RPA have indicated that it is a DNA-binding protein that has higher affinity for single-stranded DNA. Interestingly, in vitro nucleotide excision repair studies with purified protein components have shown an absolute requirement for RPA in the incision of UV-damaged DNA. Here we use a mobility shift assay to demonstrate that human RPA binds a UV damaged duplex DNA fragment preferentially. Complex formation between RPA and the UV-irradiated DNA is not affected by prior enzymatic photo-reactivation of the DNA, suggesting an affinity of RPA for the (6-4) photoproduct. We also show that Mg$^{2+}$ in the millimolar range is required for preferential binding of RPA to damaged DNA. These findings identify a novel property of RPA and implicate RPA in damage recognition during the incision of UV-damaged DNA.

Communications

Nucleotide excision repair (NER)$^1$ represents the most important cellular mechanism for repairing DNA damaged by ultraviolet (UV) light. Defects in NER result in failure to remove UV lesions from DNA and cause the cancer-prone disease xeroderma pigmentosum (XP) in humans. Cell fusion studies have so far identified seven XP genes, A through G.

Extensive genetic studies in Saccharomyces cerevisiae have indicated the requirement of the RAD1, RAD2, RAD3, RAD4, RAD10, RAD14, and RAD25 genes in the incision step of NER (1). In addition to their role in NER, RAD3 and RAD25 are essential for RNA polymerase II (Pol II) transcription (2–4). The RAD2 and RAD25 homologs of the yeast NER proteins share similar biochemical activities. XPA binds damaged DNA, XPD and XPB are DNA helicases, and XPG and ERCC1-XPF encode the two nuclease (14–18).

Interestingly, in both the yeast and human systems, incision of UV-damaged DNA is absolutely dependent upon RPA (6, 7). However, the precise role of RPA in the incision process remains unclear. RPA is a highly conserved heterotrimeric protein, composed of three subunits of 70, 32, and 14 kDa in humans. In DNA replication, RPA is required for origin-dependent unwinding by the SV40 large T antigen (19). In incision, RPA could function in an analogous manner by binding and stabilizing ssDNA created as a result of unwinding by the two DNA helicases present in TFIIH. Alternatively, RPA could function in another step, such as in damage recognition. Here, we show that RPA binds specifically to UV-damaged DNA, thus identifying RPA as a damage recognition factor.

MATERIALS AND METHODS

RPA Purification—Escherichia coli BL21(DE3) was freshly transformed with pl1d-2RPA, which co-expresses all three subunits of human RPA, and induced as described (20). The 4-g pellet of RPA expressing E. coli was suspended in 30 ml of buffer A (25 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, and 1 mM DTT) containing 100 mM KCl and protease inhibitors (21). Crude cell lysate was prepared by passage of the bacterial suspension through a French Press and subjected to fractionation in columns of Affi-Gel Blue, hydroxyapatite, and Mono Q, as described (20).

DNA Probe—A HindIII-Sall 130-basepair DNA fragment from plasmid pTB402 (8) was purified by electrodialysis from polycyclamide gels. The DNA fragment was labeled at the 3’ end using Klenow polymerase and [$\alpha^{32}$P]dATP (100 Ci; 6000 Ci/mmol) to a specific activity of $2.5 \times 10^9$ cpm/μg. The labeled fragment was purified by phenol-chloroform extraction and gel filtration through a Sephadex G-50 column equilibrated in TE buffer (10 mM Tris-HCl, pH 7.2, 0.2 mM EDTA). The purified DNA fragment was irradiated with 15-watt germicidal lamps emitting at 254 nm at a fluence rate of 10 $\mu$m/s.

DNA Mobility Shift Assay—UV-irradiated DNA or its non-irradiated counterpart, 2 ng each, was incubated with RPA (20 to 100 ng) in 10 μl of reaction buffer (30 mM HEPES-KOH, pH 7.0, 30 mM KCl, 3 mM MgCl$_2$, 1 mM DTT, and 100 μg/ml BSA). After 30 min at 30 °C, samples were mixed with 8 μl of gel loading buffer (0.1 M Tris-HCl, pH 7.0, 5.0% glycerol, and 0.05% Orange G) and electrophoresed at 4 °C for 2 h at 2 mA/cm in 35% polyacrylamide gels, using 40 mM Tris acetate, pH 7.4, 1 mM EDTA as running buffer. Gels were dried and subjected to autoradiography using Kodak XR-5 film. Autoradiograms were subjected to radiography using Kodak XRP film.
image analysis in a Bio-Rad GS-670 Imaging Densitometer to obtain data points for graphical representation of the results.

Enzymatic Photoreactivation—UV-irradiated 32P-labeled DNA fragment (0.25 μg) was incubated with 6 μg of E. coli photolase in 100 μl of reaction buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM NaCl, 1 mM DTT, 1 mM EDTA, and 300 μg/ml BSA) for 1 h at 25 °C under black light emitting at ~360 nm. After photoreactivating treatment, reaction mixtures were extracted twice with phenol, and the DNA was precipitated with 3 volumes of ethanol at −70 °C and redissolved in 50 μl of TE buffer. The removal of cyclobutane pyrimidine dimers was verified by incubating 5 ng of the photoreactivated DNA with T4 pyrimidine-dimer endonuclease in 10 μl of reaction buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM NaCl, 4 mM EDTA, and 100 μg/ml BSA), as described previously (8). Reaction mixtures were incubated at 35 °C for 3 min, chilled on ice, and electrophoresed in a 7.5% polyacrylamide gel in TAE buffer (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA). After drying, the gel was exposed to an x-ray film to detect the products generated as a result of the T4 endonuclease action.

RESULTS

For examining the DNA damage binding properties of RPA, it was purified from E. coli strain BL21(DE3) harboring plasmid p11d-trPa, which co-expresses the three RPA subunits (20). RPA thus purified is fully active in the in vitro replication of SV40 DNA (20), in nucleotide excision repair in a reconstituted system (7), and exhibits an affinity for ssDNA indistinguishable from that observed with RPA purified from HeLa cell extract (20). Based on the chromatographic procedures described by Henricksen et al. (20) that yield biologically active RPA protein, we subjected clarified bacterial extract to fractionation in columns of Affi-Gel Blue, hydroxyapatite, and Mono Q. As shown in Fig. 1A, the RPA pool (fraction IV) from the final step of purification on Mono Q contained stoichiometric amounts of the 70-, 32-, and 14-kDa subunits of RPA, and a minor species migrating below the largest subunit, which, as has been noted before (20), is a proteolytic product of the 70-kDa RPA subunit. The fraction IV RPA (Fig. 1A) was used in all of the DNA binding experiments described below.

The lesions induced by UV light occur predominantly at pyrimidine residues in DNA. As DNA probe for damage binding, a 130-base pair DNA fragment high in pyrimidine content was therefore chosen, and it was generated by restriction digest of plasmid DNA, purified, and 3'-end-labeled with 32P, as described under “Materials and Methods.” The radiolabeled DNA fragment was then irradiated with an ultraviolet source for increasing lengths of time, giving final dosages ranging from 0.5 to 15 kJ/m2. In the UV damage binding reaction, RPA was incubated with the DNA fragment which had received increasing UV dosage in the presence of 3 mM MgCl2 and 30 mM KCl at 30 °C for 30 min, and the reaction mixtures were mixed with loading buffer and then applied onto 3.5% polyacrylamide gels. After electrophoresis at 4 °C, the gels were dried and exposed to x-ray films to visualize the free DNA probe and any retarded form of the DNA probe, which would be indicative of an RPA-DNA complex. As shown in Fig. 1B, a slow migrating form of the UV irradiated DNA fragment, designated as C, was seen upon incubation with RPA, indicating that RPA binds UV damage. The amount of the RPA-DNA complex increased with the UV dose, and densitometric scanning of the autoradiogram indicated that, at the highest dose (15 kJ/m2) used, greater than 85% of the input DNA probe was bound by RPA (Fig. 1, B and C). We next examined complex formation as a function of RPA concentration, using DNA fragment that had been irradiated with 15 kJ/m2 of UV. As expected, the amount of RPA-damaged DNA complex increased with the RPA concentration (Fig. 2A and B). In these (Figs. 1 and 2) and other experiments (data not shown), only a low level (~2%) of binding of the nonirradiated counterpart of the DNA fragment was detected, even at the highest amount of RPA used (Fig. 2A, lane 3). Taken together, our results indicate that RPA binds preferentially to duplex DNA containing UV-induced lesions.

In the experiments described thus far, wherein preferential binding of RPA to UV damaged DNA occurred, 3 mM MgCl2 was present during incubation of DNA with RPA. Interestingly, when MgCl2 was omitted from the reaction, the undamaged
DNA fragment was bound by RPA nearly as well as the UV irradiated (15 kJ/m²) DNA (Fig. 3, compare lanes 2 and 8). Consistent with the results obtained in prior experiments (Figs. 1 and 2), nonspecific binding of RPA to undamaged DNA was essentially eliminated by the inclusion of 3 mM Mg²⁺, such that only <2% of the unirradiated DNA was bound by RPA in the absence of Mg²⁺ (Fig. 3, lanes 5–8). As indicated, at 30 °C for 30 min. F, unbound DNA; C, RPA-DNA complex. B, graphical representation of the results in A.

whereas the yield of the (6-4) photoproducts continues to increase linearly with the UV dose above 4 kJ/m² (22, 23). An examination of the RPA damage binding profile as a function of the UV dose (Fig. 1, B and C) revealed that the amount of RPA-DNA complex is relatively insignificant at low UV doses where the CPD represents the predominant lesion, suggesting that the primary target for RPA in UV-damaged DNA is the (6-4) photoproduct. We used enzymatic photoreactivation, a procedure that selectively and quantitatively removes CPDs from UV-damaged DNA without affecting the content of the (6-4) photoproducts, to test the notion that RPA has little affinity for the former class of UV lesion. To do this, DNA which had been irradiated with a UV dose of either 3 kJ/m² or 15 kJ/m² was incubated with E. coli photolyase under photoreactivating light, which provides the energy for photolyase to catalyze the monomerization of the pyrimidine dimers (24). To verify that CPDs in the UV-irradiated DNA had been removed, after incubation with photolyase, the DNA was treated with the bacteriophage T4 pyrimidine-dimer endonuclease, which nicks DNA at CPD sites. As shown in Fig. 4A, incubation of UV-irradiated DNA with photolyase resulted in the removal of T4 endonuclease-sensitive sites, indicating that the UV-damaged DNA had now been freed of CPDs. We then examined the ability of the photoreactivated, UV-irradiated DNA to form a complex with RPA. As shown in Fig. 4, B and C, complex formation between UV irradiated DNA and RPA was refractory to enzymatic photoreactivation, indicating that RPA does not detectably bind CPD and suggesting that RPA binds the (6-4) photoproduct in target DNA. At the UV dose of 12 kJ/m², where we observed ~50-fold preferential binding to UV-damaged DNA (Fig. 1), there are ~1.8 (6-4) photoproducts per DNA probe. From these results, it can be estimated that RPA binds the UV damage with an affinity ~3600-fold over undamaged nucleotides.

**DISCUSSION**

In this work, we demonstrate a role for human RPA in DNA damage recognition. RPA binds specifically to UV-damaged DNA in a UV dose- and protein concentration-dependent manner. Enzymatic photoreactivation experiments suggest that RPA has affinity for (6-4) photoproducts. It remains to be determined whether RPA recognizes the UV damage per se or the single-strandedness resulting from UV lesions. The damage binding ability of RPA may explain the absolute dependence of the damage-specific incision reaction on RPA.

RPA binds tightly to ssDNA with an apparent binding constant of ~10⁰ (25, 26), and the ss binding activity of RPA is necessary for both replication and recombination. In SV40 origin-dependent DNA replication, RPA assists in DNA unwinding...
RPA Is a DNA Damage Recognition Protein

**Figure 4.** Removal of CPDs by enzymatic photoreactivation does not affect damage binding by RPA. A, DNA irradiated with 3 kJ/m² (lanes 1, 3, and 5) or 15 kJ/m² (lanes 2, 4, and 6) of UV was incubated with lanes 1–4) or 15 kJ/m² (lanes 1–4) or 15 kJ/m² in the absence (lane 1) and presence (lanes 2–4) of 100 ng of RPA at 30°C for 30 min. Photoreactivated UV-irradiated DNA (3 kJ/m² in lane 5) was also incubated with 100 ng of RPA at 30°C for 30 min. C, the autoradiogram was subjected to image analysis to determine the amount of DNA-DNA complex formed before and after photoreactivation of the UV-irradiated DNA. −PRE, no photoreactivation; +PRE, enzymatic photoreactivation.

Catalyzed by T antigen by binding and stabilizing the unwound, single-stranded DNA, RPA also functions as a single strand binding protein in DNA chain elongation, and interaction with RPA stimulates the activity of various enzymes assembled at the replication fork (reviewed in Ref. 27). Additionally, RPA functions as an important component in the homologous DNA pairing and strand exchange reaction catalyzed by the S. cerevisiae Rad51 protein (28). In this reaction, as a single strand DNA-binding protein, RPA enhances the efficiency of Rad51 filament formation on ssDNA (29). Our study now implicates RPA as a damage recognition factor in NER.

The structure and function of RPA have been conserved among eukaryotes; the 70-kDa subunit binds ssDNA and interacts directly with the 32- and 14-kDa subunits (30 and references therein). Between residues 481 and 503, human RPA70 contains a putative C4 type zinc finger motif, and this motif may have a role in specific DNA-binding activity (30) because a protein deleted for the carboxyl-terminal portion of RPA, including the C4 zinc finger motif, retains normal ssDNA binding activity (30). The C4 motif may have a role in specific binding of RPA to DNA damage sites.

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