Interactions of human replication protein A with single-stranded DNA adducts

Yiyong LIU*, Zhengguan YANG*, Christopher D. UTZAT†, Yu LIU*, Nicholas E. GEACINTOV‡, Ashish K. BASU† and Yue ZOU*¹

*Department of Biochemistry and Molecular Biology, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614, U.S.A., †Department of Chemistry, University of Connecticut, Storrs, CT 06269, U.S.A., and ‡Department of Chemistry, New York University, New York, NY 10003, U.S.A.

Human RPA (replication protein A), a single-stranded DNA-binding protein, is required for many cellular pathways including DNA repair, recombination and replication. However, the role of RPA in nucleotide excision repair remains elusive. In the present study, we have systematically examined the binding of RPA to a battery of well-defined ssDNA (single-stranded DNA) substrates using fluorescence spectroscopy. These substrates contain adducts of (6-4) photoproducts, *N*-acetyl-2-aminofluorene-, 1-aminopyrene-, BPDE (benzo[*a*]pyrene diol epoxide)- and fluorescein that are different in many aspects such as molecular structure and size, DNA disruption mode (e.g. base stacking or non-stacking), as well as chemical properties. Our results showed that RPA has a lower binding affinity for damaged ssDNA than for non-damaged ssDNA and that the affinity of RPA for damaged ssDNA depends on the type of adduct. Interestingly, the bulkier lesions have a

greater effect. With a fluorescent base-stacking bulky adduct, (+)-*cis*-anti-BPDE-dG, we demonstrated that, on binding of RPA, the fluorescence of BPDE-ssDNA was significantly enhanced by up to 8–9-fold. This indicated that the stacking between the BPDE adduct and its neighbouring ssDNA bases had been disrupted and there was a lack of substantial direct contacts between the protein residues and the lesion itself. For RPA interaction with short damaged ssDNA, we propose that, on RPA binding, the modified base of ssDNA is looped out from the surface of the protein, permitting proper contacts of RPA with the remaining unmodified bases.

Key words: adduct, binding affinity, DNA damage recognition, fluorescence spectroscopy, human replication protein A, single-stranded DNA.

INTRODUCTION

Human RPA (replication protein A) is a heterotrimeric protein consisting of three subunits of 70, 32 and 14 kDa [1]. RPA plays an indispensable role in replication, NER (nucleotide excision repair) and homologous recombination of DNA [1,2]. The main activity of RPA is to bind to ssDNA (single-stranded DNA) during DNA metabolism, while RPA also binds to dsDNA with a much lower affinity. The association constant K_a for RPA–ssDNA interaction is in the range of 10^8 – 10^{11} M^{−1} depending on the sequence and length of the ssDNA, the method of analysis and the experimental conditions [2–4]. The affinity of RPA for pyrimidine residues is approx. 50-fold higher than that for purine residues [5]. Structural studies revealed that RPA contains four DBDs (DNA-binding domains). Three of them (DBD-A, -B and -C) are located in RPA70 in tandem with DBD-A and -B in the central region and DBD-C in the C-terminal region [6]. The fourth DBD resides in the central region of RPA32, referred to as DBD-D [7,8]. The binding of RPA to ssDNA has been suggested to occur through a multi-step pathway [2,7]. The binding is initiated by an interaction of the DBD-AB domains with a length of 8–10 nt of the ssDNA [7]. Then, a significant conformational change of the protein allows ssDNA binding between 13 and 22 nt with the DBD-C domain additionally involved [8,9]. Finally, co-operative binding of RPA with all the four DBDs occurs, which involves a region of 30 nt [7]. It is now generally accepted that RPA needs an occluded size of approx. 30 nt for a minimum full and high-affinity binding [1,2,5,10].

RPA has been suggested to be involved in DNA damage recognition of NER owing to its preference for the damaged DNA

relative to undamaged DNA [11–20], although the specific role of RPA in the mechanism is not clear in recent studies [21–26]. Efforts have been made to understand the origin of the recognition and thus the functions of RPA in NER. It has been shown that RPA binds more efficiently to the undamaged ssDNA than to cisplatin-modified ssDNA [13,16,27]. In contrast, the affinity of RPA for UV-damaged ssDNA was up to 60-fold higher compared with that for undamaged ssDNA [12,28]. These apparently inconsistent results have resulted in the unclear notions that RPA binds preferentially to either undamaged ssDNA or damaged ssDNA or both strands of DNA duplex that have been disrupted by the lesion. It has been noted that different methods and adducts were used in these investigations. More importantly, in most of these studies, the ssDNA substrates used were much longer than 30 nt, the occluded binding size of RPA. Use of such long ssDNA substrates appeared to be problematic due to the multiple binding sites available in a single ssDNA molecule and thus to the co-operative binding of RPA molecules to ssDNA. In addition, how RPA interacts with the adduct molecule itself in ssDNA remains unknown. Therefore, to address the controversy and to understand better the mechanism of RPA–ssDNA adduct interactions, a systematic study of RPA binding with well-defined damaged and undamaged ssDNA under strict conditions is necessary.

In the present study, we have systematically examined the interactions of human RPA with a series of modified and unmodified ssDNA substrates with a single protein-binding site (protein/ssDNA stoichiometry = 1) using the rigorous method of fluorescence anisotropy. These substrates contain adducts that are different in molecular size and structure, DNA-interacting structure (e.g. intercalating or non-intercalating), as well as chemical

Abbreviations used: AAF, *N*-acetyl-2-acetylaminofluorene; AP, 1-aminopyrene; BPDE, benzo[*a*]pyrene diol epoxide or 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; C8-AAF-dG, *N*-(deoxyguanosin-8-yl)-AAF; C8-AP-dG, *N*-(deoxyguanosin-8-yl)-AP; DBD, DNA-binding domain; RPA, replication protein A; ssDNA, single-stranded DNA; NER, nucleotide excision repair; (6-4)PP, (6-4)photoproduct.

¹ To whom correspondence should be addressed (email zouy@etsu.edu).

Figure 1 Structures of ssDNA substrates used in the present study

(A) Schematic of ssDNA containing various modifications. F in the sequences stands for a fluorescein modification attached through a C5-linker to the 5'- or 3'-end of the respective ssDNA. X in the sequences represents the single adducted nucleotide. (B) Structures of specific DNA adducts.

properties. Our results showed that RPA has no preference for binding to damaged ssDNA over undamaged ssDNA. Instead, RPA has lower binding affinities for damaged ssDNA than for undamaged ssDNA, and the extent of decrease in binding depends on the type of adduct. Also using BPDE (benzo[*a*]pyrene diol epoxide) as both a DNA adduct and a fluorescent DNA base-interaction and stacking sensor, we have analysed the nature of adduct due to binding of RPA. The results demonstrated that the interactions of the BPDE lesion with neighbouring bases were largely disrupted as a result of the protein binding. However, the results also suggested that there were no obvious direct interactions between the protein residues and the lesion itself.

MATERIALS AND METHODS

DNA substrate preparation

Oligodeoxynucleotides with or without fluorescein modification (except 30mers) were purchased from Qiagen (Alameda, CA, U.S.A.) in HPLC-purified quality. The 5'-fluorescein-labelled 30mer substrates with or without adduct were constructed by ligating the 5'-phosphorylated 11mer (with or without an adduct; Figure 1) with the 5'-fluorescein-labelled 9mer and 5'-phosphorylated 10mer, using T4 DNA ligase. The sequences of oligodeoxynucleotides used in the constructions are as follows: 9mer (GTTACGGCT); 11mer [CCATCXCTACC, X = G or (+)-*cis*-

BPDE-N²-dG or C8-AAF-dG or C8-AP-dG; here, AAF stands for *N*-acetyl-2-acetylaminofluorene, AP for 1-aminopyrene, C8-AAF-dG for *N*-(deoxyguanosin-8-yl)-AAF and C8-AP-dG for *N*-(deoxyguanosin-8-yl)-AP]; and 10mer (GCAATCAGGC). The 5'-fluorescein-labelled 9mers (100 pmol) were incubated with equal moles of the phosphorylated 11mer (with or without an adduct) and 10mer in the presence of a 44mer template strand containing the complementary sequence (GATCTGGCCTGATTGCGGTAGCGATGGAGCCGTAACAGTACGTA) in 100 µl of ligation buffer containing 50 mM Tris/HCl (pH 7.8), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP and 50 µg/ml BSA. The mixture was brought to 85 °C for 5 min and then slowly cooled down to room temperature (25 °C) and finally to 16 °C, followed by the addition of 1 unit of T4 DNA ligase. The ligation was performed at 16 °C for 12 h. The sample was then reheated at 85 °C for 5 min with 8 M urea, followed by rapidly chilling on ice and then purified on a 12 % (w/v) polyacrylamide sequencing gel under denaturing conditions. Using the 5'-³²P-labelled 30mer and 44mer as controls, the band identified as 30mer which migrated much faster than the 44mer in the gel was excised, eluted and precipitated with ethanol. For construction of the 29mer with or without a (6-4)PP [(6-4)-photoproduct] adduct, the same procedures were performed, except that the 11mer was substituted with the 10mer [CGT-AT[^]TATGC, T[^]T = (6-4)PP] and a complementary 35mer (TGG-CCTGATTGCGCATAATACGAGCCGTAACAGTA) was used for the ligation. The single-stranded nature of the constructed substrates was further confirmed by the ssDNA nuclease assays with S1 enzyme. All the substrates used in the fluorescence and anisotropy measurements are depicted in Figure 1.

RPA preparation

Recombinant human RPA was expressed in *Escherichia coli* BL21(DE3)-RP cells and purified as described previously [29]. The concentration of RPA protein was determined using Bio-Rad Protein Assay kit.

Gel mobility-shift assays

Oligonucleotides poly(dT)₈, poly(dT)₃₀, poly(dT)₄₀ and poly(dT)₅₀ were radiolabelled with [γ -³²P]ATP and polynucleotide kinase. The substrate (5 nM) was incubated with the indicated amounts of RPA at 25 °C for 15 min in 20 µl of the binding buffer [40 mM Hepes-KOH, pH 7.9, 75 mM KCl, 8 mM MgCl₂, 1 mM dithiothreitol, 5 % (v/v) glycerol and 100 µg/ml BSA]. After incubation, 2 µl of 80 % glycerol was added, and the mixture was immediately loaded on to a 3.5 % native polyacrylamide gel in TBE running buffer (89 mM Tris/borate, pH 8.3, and 2 M EDTA) and electrophoresed at room temperature.

Gel-filtration analysis and scintillation counting

Gel-filtration assay was performed on an HR 10/30 Superdex 200 column with an AKTApurifier system (Amersham Biosciences, Uppsala, Sweden) followed by scintillation counting. Poly(dT)₈ and poly(dT)₄₀ were radiolabelled with [γ -³²P]ATP. Binding of RPA to these substrates was conducted the same way as in the gel mobility-shift assays, except that it was performed in a larger volume of 100 µl. After incubation at 25 °C for 15 min, the reaction mixture was loaded on to the column equilibrated with the same binding buffer. Fractions of 0.5 ml were collected at 0.5 ml/min, with the radioactivity of each fraction counted using a liquid-scintillation counter. The column was calibrated with the following molecular-mass standards: RNase A, 13.7 kDa; chymotrypsinogen A, 25 kDa; ovalbumin, 43 kDa; bovine albumin, 67 kDa; aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa; and thyroglobulin, 669 kDa.

Fluorescence measurement of the binding of RPA to ssDNA with a BPDE adduct

Measurements of the fluorescence emission spectra and the fluorescence titration were performed as described previously [30]. Fluorescence of the BPDE-ssDNA substrates was recorded at 25 °C on a SPEX Fluorolog-3 fluorimeter (Jobin Yvon, Edison, NJ, U.S.A.) with the excitation wavelength set at 350 nm, the slit width set at 5 nm for both excitation and emission beams and the integration time set at 0.5 s. Not more than 5 % photobleaching was observed under these conditions. For titration, analysis was performed by measuring the emission at 380 nm with excitation at 350 nm. After sample equilibration, three data points with an integration time of 5 s and S.E.M. of 0.5 % were collected for each titration point. RPA and the DNA substrates were placed in the same binding buffer before the titration to eliminate any change of the background on the addition of protein. All titrations were performed in a micro-quartz cuvette (4 mm × 4 mm) for a minimum sample volume of 200 μ l with a 2 mm × 2 mm stirring bar. Each addition of RPA was 0.5–1 μ l, delivered by a 25 μ l Hamilton syringe using a Hamilton repeating dispenser.

Fluorescence anisotropy measurements

Measurements of fluorescence anisotropy provide information about the rotational behaviour of molecules. On binding of the fluorescently labelled DNA probe to proteins, the fluorescence anisotropy of the probe increased, serving as an indicator of the complex formation [16]. In the present investigation, ssDNA with fluorescein labelling at the 5'-end was used to monitor the RPA-ssDNA binding. The reason for choosing 5'-modification instead of 3'-modification is that, with the ssDNA fluorescein-labelled at the 5'-end, the intensities and shapes of the fluorescence spectra observed before and after the addition of saturating RPA concentrations showed virtually no changes; however, with 3'-labelling, the total fluorescence intensity decreased by approx. 30 % upon binding.

The anisotropy titrations were performed on a SPEX Fluorolog-3 fluorimeter with automated polarizers at an excitation wavelength of 492 nm and monitored at an emission wavelength of 520 nm using a 500-nm cutoff filter, with the slit width set at 14 nm for both excitation and emission beams for reliable signals. Other titrations were performed with the same procedure as described in the above fluorescence titrations of BPDE-DNA substrates.

Data processing

Data obtained from the measurements of fluorescence and anisotropy titrations were processed using a one-site binding model and the non-linear least-squares method as described previously [30].

RESULTS

Stoichiometry of RPA binding to different sizes of ssDNA

RPA needs an occluded binding site of approx. 30 nt for a full and high-affinity binding [1,2]. However, two different modes of complexes of RPA may occur depending on the experimental conditions [10]. To select ssDNA substrates with a binding stoichiometry of one for the present study, we have performed gel mobility-shift assays of RPA with various sizes of ssDNA. As shown in Figure 2, of the substrates poly(dT)₈, poly(dT)₃₀, poly(dT)₄₀ and poly(dT)₅₀, a single shifted band was observed with (dT)₈ and (dT)₃₀ even in the presence of excess protein, suggesting a single RPA binding. In contrast, an additional slower eluting band

Figure 2 Binding of RPA to different sizes of ssDNA

RPA was incubated with 5 nM poly(dT)₈, poly(dT)₃₀, poly(dT)₄₀ or poly(dT)₅₀ at different molar ratios (0, 1 and 5) at 25 °C for 15 min in 20 μ l of the binding buffer. The binding products were analysed on a 3.5 % native polyacrylamide gel. The positions of RPA-ssDNA complexes and free oligonucleotides are indicated.

appeared with (dT)₄₀ and (dT)₅₀ as the concentration of RPA was increased to 25 nM, implying that multiple complexes may form with these longer oligonucleotides. This is not in agreement with the previous report by Kim et al. [5] who noted only one shifting band for the RPA binding to (dT)₅₀, although this may be the result of different conditions used in their assays. It is also worth noting that the electrophoretic mobility of the RPA-ssDNA complex increased as the length of the ssDNA increased from 8 to 40 nt, which was also noted in an earlier study [10]. Although the conformational change of the complex might affect the mobility [10], a more reasonable explanation could be that the ratio of the negative charges to the molecular mass (kDa) of the complex (charge/mass) influenced the mobility as a function of the length of ssDNA. These ratios are consistent with the migration pattern observed with a single bound RPA.

To define the nature and stoichiometry of these complexes, a gel-filtration assay followed by scintillation counting was performed to determine the molecular masses of the complexes formed between radiolabelled (dT)₈ or (dT)₄₀ and RPA at different concentrations. Figure 3(A) shows the elution profile of the RPA-DNA binding reaction mixtures, in which the two major peaks represent two types of DNA-protein complexes. When ten times more RPA (in molar ratio) was incubated with (dT)₈, a single peak appeared at 11.5 ml, which was also the retention volume for the RPA-(dT)₄₀ complex at a protein/DNA ratio of 1:1. The low peak intensity for (dT)₈ was due to the less efficient ³²P-labelling and the much lower binding affinity of RPA for such short substrates compared with (dT)₄₀. Calculations based on mass standards showed that the retention volume of 11.5 ml corresponds to a molecular mass of 130 kDa (Figure 3B), which is consistent with the binding of a single RPA molecule in both cases. In contrast, incubation of the protein with (dT)₄₀ in a 10:1 ratio resulted in an RPA-(dT)₄₀ complex with a retention volume of 10.0 ml, which corresponds to a molecular mass of 238 kDa. This is consistent with a double-bound complex (RPA/DNA = 2). Therefore the (dT)₄₀ ssDNA allows double bindings by RPA, whereas (dT)₈ permits only single binding and the complex has a molecular mass similar to the complex from RPA-(dT)₄₀ single binding. Most probably, the single binding also occurred for (dT)₃₀ ssDNA. Thus we conclude that a length of 30 nt is sufficient for the binding of one RPA molecule under our experimental conditions.

Fluorescence spectroscopic characterization of RPA binding to defined substrates

Little is known about how RPA protein interacts with the adduct molecule of the damaged ssDNA. In the present study, we

Figure 3 Gel-filtration analysis of RPA-ssDNA binding

(A) Scintillation counting of RPA binding reactions with different sizes of ssDNA. The binding reactions of RPA with (dT)₈ and (dT)₄₀ at different molar ratios were constructed and gel-filtrated as described in the Experimental section. Fractions of 0.5 ml were collected and the radioactivity of each fraction was counted in a liquid-scintillation counter. The results for RPA-(dT)₄₀ and RPA-(dT)₈ interactions are presented along the left and right y-axes respectively. (B) The apparent molecular masses of the RPA-ssDNA complexes were determined by gel filtration based on the relationship between the retention volumes of markers versus their molecular masses fitted with the linear regression method. The markers include Rib (RNase A, 13.7 kDa), ChyA (chymotrypsinogen A, 25 kDa), Ova (ovalbumin, 43 kDa), BSA (bovine albumin, 67 kDa), Ald (aldolase, 158 kDa), Cat (catalase, 232 kDa), Fer (ferritin, 440 kDa) and Thy (thyroglobulin, 669 kDa).

examined RPA binding to oligonucleotides containing a single BPDE lesion by monitoring the fluorescence of BPDE. Since aromatic amino acids are probably involved in the interactions of RPA with normal ssDNA through stacking with bases [4,7,31], it is of interest to determine how the pyrene-like residue of the BPDE-N²-dG adduct is affected. The fluorescence of this adduct is a very sensitive probe of its environment and we have previously used this in the study of a protein-DNA interaction system [30]. It is now known that the fluorescence of the pyrenyl residue of BPDE-N²-dG adducts in DNA is strongly quenched [32] by neighbouring T and C bases [33]. In DNA, the fluorescence of BPDE-N²-dG adducts is sensitive to the secondary structure, the aqueous solvent environment [32] and the adduct conformation [34]. Therefore any disruption or weakening of the BPDE-base interactions may lead to an increase in the fluorescence of the adduct, providing evidence for structural alterations in the immediate environment of BPDE-N²-dG lesions. In ssDNA, significant BPDE-base stacking interactions are evident since the absorption spectrum of the pyrenyl residue is significantly red-shifted with an absorption maximum at approx. 352–354 nm

[35]. When excited at 350 nm, the emission spectrum of the BPDE-adducted 11mer oligonucleotides (Figure 1) displays two fluorescence maxima, one at 384 nm and the other at 404 nm, consistent with previous observations [30,36]. After binding to RPA, the λ_{max} at 384 nm blue-shifted to 380 nm, and the fluorescence intensity was enhanced by 8–9-fold (Figure 4A and Table 1). This implies that, on binding of RPA, the interactions between the BPDE molecule and the neighbouring bases were lost due to a local conformational change in the ssDNA. This leads to diminished BPDE-nucleobase stacking interactions as indicated by a significant blue shift in the fluorescence emission maxima and a large enhancement in the fluorescence yield. A (+)-*cis*-BPDE-30mer ssDNA substrate was also subjected to the same measurements. While similar results were observed, the fluorescence enhancement was not as large as with the BPDE-11mer (Table 1). It is probable that the BPDE molecule remained partially stacked in this case. Furthermore, these results suggested that no significant interactions between RPA residues and the BPDE molecule occurred. Otherwise, fluorescence quenching rather than enhancement would be observed upon RPA interaction.

In comparison, emission spectra for a non-stacking fluorescein, site-specifically adducted to the middle of a 9mer ssDNA, F(M)-9mer (Figure 1), were recorded with λ_{ex} = 492 nm in the presence and absence of RPA (Figure 4B). No fluorescence variation was observed, indicating that fluorescence quenching interactions are absent from such adducts. Unlike the (+)-*cis*-BPDE-dG adduct, the fluorescein derivative is covalently attached to the nucleic acids of ssDNA through a long carbon linker.

Additional structural information about the RPA-ssDNA interaction was obtained from fluorescence spectroscopic studies with a fluorescein labelled at either the 5'- or 3'-end ssDNA (Figures 4B and 4C). The fluorescence of the probe labelled at the 3'-end of ssDNA was quenched by approx. 30% on RPA-ssDNA complex formation, which was consistent with the results observed previously [16]. Interestingly, the binding of RPA to the ssDNA with the fluorescein labelled at the 5'-end led to no change in fluorescence, regardless of the length of the substrate. This implies that the 5'-terminus of ssDNA had no direct contact with RPA protein, while the 3'-end did.

RPA binding to 5'-F-30mer with or without (6-4)PP, BPDE, AAF or AP adduct

Experiments were conducted to assess the binding affinities of RPA interactions with a series of damaged and undamaged ssDNAs of 9mer, 11mer and 30mer nucleotides. Use of such short substrates eliminated the possibility of overlapping binding sites that are usually available with longer ssDNAs. In addition, for RPA interaction with damaged ssDNA, the protein may bind to the flanking undamaged DNA sequence of a lesion if the size of the substrate is too long and direct contact of RPA with the DNA lesion is thermodynamically unfavourable. Since a size of approx. 30 nt is the minimum length of ssDNA required for RPA to have a full functional interaction with ssDNA, we first examined RPA binding to the 30mer ssDNAs containing different types of adduct using fluorescence spectroscopy. All the substrates were 5'-terminally labelled with fluorescein and used as substrates for fluorescence anisotropy measurements. The fluorescence intensity of fluorescein modified either at the 5'-end or in the middle of the sequence of ssDNA remained unaffected by RPA binding, indicating that there was no direct interaction of the protein with the fluorescein. This established the validity of the fluorescence anisotropy measurements. For the 3'-terminally labelled fluorescein, the fluorescence intensity changed due to RPA binding (Figure 4C). Therefore the 3'-fluorescein is not suitable for use

Figure 4 Fluorescence spectroscopic characterization of RPA binding to defined ssDNA substrates

(A) Emission spectra of (+)-cis-BPDE-11mer in the absence and presence of RPA were recorded with excitation at 350 nm in 200 μ l of RPA binding buffer. (B) The fluorescence spectra of a 9mer with fluorescein modification in the middle of the sequence or at the 5'-end in the absence and presence of RPA were recorded with excitation at 492 nm in the RPA-binding buffer. (C) The fluorescence spectra of a 11mer with fluorescein labelling at the 3'-end in the absence and presence of RPA were recorded as in (B).

as a reporter for anisotropy measurements, although it was used previously for the determination of RPA–ssDNA interactions [16]. In addition, although the BPDE, AAF and AP have intrinsic

Table 1 RPA binding to damaged/undamaged ssDNA

ssDNA	$K_{d,obs}$ (nM)	Fluorescence enhancement on RPA binding (fold)
5'-F-9mer	39.1 ± 4.5	
5'-F-abase(M)-9mer	43.0 ± 1.5	
F(M)-9mer	93.7 ± 6.2	
5'-F-11mer	22.9 ± 2.6	
BPDE-11mer	146.0 ± 6.7	8–9
5'-F-30mer	2.5 ± 0.4	
5'-F-BPDE-30mer	11.8 ± 1.5	3
5'-F-AAF-30mer	9.0 ± 1.9	
5'-F-AP-30mer	16.5 ± 3.1	
5'-F-(6-4) PP-29mer	6.1 ± 0.4

Figure 5 Fluorescence anisotropy measurements of RPA binding to 5'F-30mer with or without adducts

5'F-30mer or 5'F-BPDE-30mer (5 nM) was titrated with RPA and the anisotropy was measured at 520 nm with excitation at 492 nm. The binding isotherms were best fitted to obtain the equilibrium dissociation constants ($K_{d,obs}$).

fluorescence when excited at appropriate wavelengths, emissions of these adducts occur at the wavelength range far shorter than 492 nm, the excitation wavelength for producing the anisotropy of fluorescein. Therefore it is unlikely that the adduct fluorophore would interfere with the fluorescence of fluorescein through dipole–dipole coupling. Figure 5 showed the representative isotherms of RPA binding to the 30mers adducted with or without BPDE. As shown in Table 1, RPA binding affinities for the substrates followed the order: ND > (6-4)PP > AAF > BPDE > AP, where ND stands for non-damaged ssDNA, suggesting that RPA binds less efficiently to these adducts than to the non-damaged ssDNA.

To verify that the anisotropy titration and fluorescence titration gave the same results for RPA binding to BPDE-adducted ssDNA, titration was also conducted by measuring the BPDE fluorescence change of 5'F-BPDE-30mer. A non-linear least-squares fit of the data provided a dissociation constant of 14.1 nM. This is evidently in good agreement with the value of 11.8 nM determined from the anisotropy measurement, indicating the reliability of these two approaches used in our binding experiments.

RPA binding to damaged and undamaged 9mer and 11mer ssDNAs

We also examined the RPA binding to the damaged F(M)-9mer and undamaged 5'F-9mer ssDNAs by titrational fluorescence

Figure 6 Typical fluorescence binding isotherms for short ssDNA titrated with RPA

(A) Fluorescence anisotropy measurements of RPA binding to 5'F-9mer and F(M)-9mer. ssDNA (10 nM) was titrated with RPA in 200 μ l of RPA-binding buffer and the anisotropy was monitored at an emission wavelength of 520 nm with an excitation wavelength of 492 nm. The anisotropy data were normalized by subtracting the initial value of respective free ssDNA and best fitted with the procedures described in [29]. (B) Fluorescence titration of BPDE-11mer with RPA. BPDE-11mer (10 nM) was titrated with RPA in the same buffer by measuring the emission at 382 nm (excitation at 350 nm). (C) Fluorescence anisotropy titration of 5'F-11mer with RPA. Titration of 10 nM 5'F-11mer was performed as in (A).

anisotropy measurements. For the substrate F(M)-9mer, the fluorescein also was considered as a lesion in the ssDNA. Figure 6(A) showed the RPA-binding isotherms of titration data with sub-

strates 5'F-9mer and F(M)-9mer. The anisotropy data were normalized by subtracting the initial value of the respective free DNA and best fitted with the procedures described previously [30]. The results indicated that the affinity of RPA for the 5'F-9mer was significantly higher than that for the F(M)-9mer (Table 1), as demonstrated by the values of dissociation constants 39.1 ± 4.5 and 93.7 ± 6.2 nM for 5'F-9mer and F(M)-9mer respectively (Table 1). Since the centrally located fluorescein was not attached to a base, as a control, the same anisotropic measurement was conducted also for RPA binding to a 9mer (5'-end labelled with fluorescein) containing an abasic site in the middle of the same sequence (CATC[abasic]CTAC). The binding affinity of RPA for this abasic substrate is very close to that for the undamaged 5'F-9mer (Table 1), indicating that the lack of base in the middle nucleotide had no effect on RPA binding. Thus the decrease in the affinity for the F(M)-9mer was attributed to the presence of the fluorescein in the middle of the 9mer, although there was no direct interaction between the protein and the aromatic moiety of the fluorophore.

To determine how a base-stacking aromatic lesion, adducted directly to a base, influences RPA binding, BPDE, a different type of adduct with a larger aromatic ring system, was introduced into the 11mer (Figure 1). As described earlier, binding of RPA to BPDE-adducted ssDNA led to a large fluorescence enhancement. This fluorescence change can be used as a signal to monitor RPA-ssDNA interactions and to generate binding isotherms. As shown in Figures 6(B) and 6(C), titrations of RPA binding to the BPDE-ssDNA 11mer and the normal 11mer with the same sequence were performed and the binding affinities were determined for comparison. The dissociation constant for the damaged 11mer was 146.0 ± 6.7 nM compared with 22.9 ± 2.6 nM for the undamaged 11mer ssDNA, indicating a much tighter binding with the undamaged DNA.

DISCUSSION

In the present study, we have systematically analysed the interactions of RPA with a group of damaged and undamaged ssDNA substrates by fluorescence spectroscopy under strict and comparable experimental conditions. Unlike most previous studies, the substrates used in the present study were in length no longer than 30 nt, which kept the binding stoichiometry at one RPA per ssDNA to avoid potential binding complications.

Our experiments designed to probe the nature of the adduct of ssDNA on RPA binding indicated that the protein-bound ssDNA involved a significant local structural alteration around the lesion. This structural change probably caused the disruption of BPDE-ssDNA base stacking and the exposure of the BPDE molecule on RPA binding, which was particularly evident for the short substrate of BPDE-11mer. The results also suggested that the exposed BPDE lesion may be in no or little direct contact with amino acids of RPA. For RPA binding to the BPDE-11mer, it is probable that the modified base resides in the DBDs-AB [2] and is probably flipped out of the protein-ssDNA interaction interface, permitting better contacts of RPA with the unmodified bases. For BPDE-30mer, two additional DBDs, DBD-C and DBD-D of RPA, are involved in the binding. Since binding of a protein to its substrate is a dynamic equilibrium process and RPA has a higher affinity (at least 50-fold) for ssDNA of 30 nt or longer than that for ssDNA of 8–10 nt [3], at equilibrium almost all RPA molecules bind to ssDNA starting from the 5'-end of the substrates. Thus the modified base that is centrally located in the substrate sequence may reside in the entrance region of DBD-C in the DBDs-ABCD tandem [2], which may result in

different and smaller structural changes at adduct. Since both the RPA–ssDNA binding modes of 8–10 nt and 30 nt are probably biologically important in cells (although the 8–10 nt binding mode is not a full-length binding) [2], our results provide valuable information on RPA–ssDNA adduct interactions in the two cases.

The present study on RPA binding to various ssDNA substrates showed that RPA binds to undamaged ssDNA more favourably than adducted ssDNA, indicating that the presence of lesions in the ssDNA interrupts RPA binding. Two factors might be attributed to this observation: space restriction and the potential repulsion between the exposed aromatic lesion and non-hydrophobic or non-aromatic amino acids. Comparison between RPA binding to the AAF and AP (both being C8-guanine adducts) adducted ssDNAs indicated that AP has an apparent lower affinity than AAF (Table 1). This may be an indication that the larger the aromatic rings system of the adduct, the stronger the inhibition on RPA binding (Figure 1). This was also true when a comparison was made between the BPDE and fluorescein ssDNA. The affinity for BPDE-11mer was lower than that for F(M)-9mer, although it is generally believed that, for undamaged ssDNA, the longer ssDNA binds to RPA more tightly. It is conceivable that, unlike the fluorescein, which contains a long arm stretching the aromatic rings far away from the DNA, the large BPDE ring system is directly attached to the base.

One of the objectives of the present work was to understand better the biochemical basis for RPA binding to ssDNA and dsDNA and to determine the roles, if any, of RPA in damage recognition in the NER. Although RPA binds to both ssDNA and dsDNA, the binding affinity for duplex DNA is much lower than that for ssDNA. This can be attributed to three factors. First, the binding to ssDNA is facilitated by stacking and other interactions between DNA bases and the residues of RPA [4,7,31], while formation of dsDNA eliminates most of the intermolecular interactions due to the base-pairing and stacking. Secondly, the dsDNA is more rigid and thus more resistant to being bent than the ssDNA. At least local DNA bending could be a result of efficient RPA binding. Finally, dsDNA has a larger helix diameter relative to ssDNA, which may make the binding to dsDNA spatially less favourable. Our results for the unfavourable binding of RPA to damaged ssDNA compared with undamaged ssDNA suggested no direct adduct recognition power from RPA. Several other groups have suggested that the function of RPA in DNA damage recognition in NER is to recognize the local single-stranded character induced by lesions in the duplex [13,16,19,27,37]. However, efficient binding of RPA to the ssDNA requires a minimum length of approx. 8 nt [10,31] and, in most of the cases, the local DNA denaturation induced by bulky DNA lesions is much smaller than 8 nt. Based on the results of the present study, we therefore, propose that base stacking disruption and DNA strand flexibility induced by the lesions may play a role in the binding of RPA to damaged DNA. The base stacking disruption and strand flexibility would allow the exposure of the DNA bases to specific amino acid residues of the RPA. The strand flexibility for bending could in turn structurally facilitate intermolecular interactions. Our hypothesis was also supported by the fact that, even without any lesions, RPA discriminates the ssDNA of different sequences and this discrimination is probably based on the strength of base stacking in the sequences. Previous studies [3,5] reported that affinity of RPA for pyrimidine sequences was approx. 50-fold higher than that for purine sequences. It is conceivable that this preference for pyrimidine residues is the result of a weaker base stacking between the relatively smaller aromatic rings of the pyrimidines compared with those of the purine residues.

This work was supported by an NCI grant CA86927 (to Y.Z.) and National Institute of Environmental Health Sciences grants ES09127 and ES00318 (to A. K. B.).

REFERENCES

- Wold, M. S. (1997) Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem.* **66**, 61–92
- Iftode, C., Daniely, Y. and Borowiec, J. A. (1999) Replication protein A (RPA): the eukaryotic SSB. *Crit. Rev. Biochem. Mol. Biol.* **34**, 141–180
- Kim, C., Paulus, B. F. and Wold, M. S. (1994) Interactions of human replication protein A with oligonucleotides. *Biochemistry* **33**, 14197–14206
- Kim, C. and Wold, M. S. (1995) Recombinant human replication protein A binds to polynucleotides with low cooperativity. *Biochemistry* **34**, 2058–2064
- Kim, C., Snyder, R. O. and Wold, M. S. (1992) Binding properties of replication protein A from human and yeast cells. *Mol. Cell. Biol.* **12**, 3050–3059
- Brill, S. J. and Bastin-Shanower, S. (1998) Identification and characterization of the fourth single-stranded-DNA binding domain of replication protein A. *Mol. Cell. Biol.* **18**, 7225–7234
- Bochkareva, E., Korolev, S., Lees-Miller, S. P. and Bochkarev, A. (2002) Structure of the RPA trimerization core and its role in the multistep DNA-binding mechanism of RPA. *EMBO J.* **21**, 1855–1863
- Bastin-Shanower, S. A. and Brill, S. J. (2001) Functional analysis of the four DNA binding domains of replication protein A. The role of RPA2 in ssDNA binding. *J. Biol. Chem.* **276**, 36446–36453
- Kolpashchikov, D. M., Weissbart, K., Nasheuer, H. P., Khodyreva, S. N., Fanning, E., Favre, A. and Lavrik, O. I. (1999) Interaction of the p70 subunit of RPA with a DNA template directs p32 to the 3'-end of nascent DNA. *FEBS Lett.* **450**, 131–134
- Blackwell, L. J. and Borowiec, J. A. (1994) Human replication protein A binds single-stranded DNA in two distinct complexes. *Mol. Cell. Biol.* **14**, 3993–4001
- Burns, J. L., Guzder, S. N., Sung, P., Prakash, S. and Prakash, L. (1996) An affinity of human replication protein A for ultraviolet-damaged DNA. *J. Biol. Chem.* **271**, 11607–11610
- Lao, Y., Gomes, X. V., Ren, Y., Taylor, J. S. and Wold, M. S. (2000) Replication protein A interactions with DNA. III. Molecular basis of recognition of damaged DNA. *Biochemistry* **39**, 850–859
- Patrick, S. M. and Turchi, J. J. (1999) Replication protein A (RPA) binding to duplex cisplatin-damaged DNA is mediated through the generation of single-stranded DNA. *J. Biol. Chem.* **274**, 14972–14978
- Sancar, A. (1996) DNA excision repair. *Annu. Rev. Biochem.* **65**, 43–81
- He, Z., Henriksen, L. A., Wold, M. S. and Ingles, C. J. (1995) RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. *Nature (London)* **374**, 566–569
- Hey, T., Lipps, G. and Krauss, G. (2001) Binding of XPA and RPA to damaged DNA investigated by fluorescence anisotropy. *Biochemistry* **40**, 2901–2910
- Clugston, C. K., McLaughlin, K., Kenny, M. K. and Brown, R. (1992) Binding of human single-stranded DNA binding protein to DNA damaged by the anticancer drug cis-diamminedichloroplatinum (II). *Cancer Res.* **52**, 6375–6379
- Lao, Y., Lee, C. G. and Wold, M. S. (1999) Replication protein A interactions with DNA. 2. Characterization of double-stranded DNA-binding/helix-destabilization activities and the role of the zinc-finger domain in DNA interactions. *Biochemistry* **38**, 3974–3984
- De Laat, W. L., Appeldoorn, E., Sugasawa, K., Weterings, E., Jaspers, N. G. J. and Hoeijmakers, J. H. J. (1998) DNA-binding polarity of human replication protein A positions nucleases in nucleotide excision repair. *Genes Dev.* **12**, 2598–2609
- Missura, M., Buterin, T., Hindges, R., Hubscher, U., Kasparkova, J., Brabec, V. and Naegeli, H. (2001) Double-check probing of DNA bending and unwinding by XPA-RPA: an architectural function in DNA repair. *EMBO J.* **20**, 3554–3564
- Reardon, J. T. and Sancar, A. (2002) Molecular anatomy of the human excision nuclease assembled at sites of DNA damage. *Mol. Cell. Biol.* **22**, 5938–5945
- Reardon, J. T. and Sancar, A. (2003) Recognition and repair of the cyclobutane thymine dimer, a major cause of skin cancers, by the human excision nuclease. *Genes Dev.* **17**, 2539–2551
- Riedl, T., Hanaoka, F. and Egly, J.-M. (2003) The comings and goings of nucleotide excision repair factors on damaged DNA. *EMBO J.* **22**, 5293–5303
- Volker, M., Mone, M. J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., Hoeijmakers, J. H., van Driel, R., van Zeeland, A. A. and Mullenders, L. H. (2001) Sequential assembly of the nucleotide excision repair factors *in vivo*. *Mol. Cell* **8**, 213–224
- Sugasawa, K., Ng, J. M., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P., Hanaoka, F., Bootsma, D. and Hoeijmakers, J. H. (1998) Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol. Cell* **2**, 223–232
- Wakasugi, M. and Sancar, A. (1999) Order of assembly of human DNA repair excision nuclease. *J. Biol. Chem.* **274**, 18759–18768

- 27 Patrick, S. M. and Turchi, J. J. (2001) Stopped-flow kinetic analysis of replication protein A-binding DNA: damage recognition and affinity for single-stranded DNA reveal differential contributions of $k(\text{on})$ and $k(\text{off})$ rate constants. *J. Biol. Chem.* **276**, 22630–22637
- 28 Schweizer, U., Hey, T., Lipps, G. and Krauss, G. (1999) Photocrosslinking locates a binding site for the large subunit of human replication protein A to the damaged strand of cisplatin-modified DNA. *Nucleic Acids Res.* **27**, 3183–3189
- 29 Yang, Z. G., Liu, Y., Mao, L. Y., Zhang, J. T. and Zou, Y. (2002) Dimerization of human XPA and formation of XPA2-RPA protein complex. *Biochemistry* **41**, 13012–13020
- 30 Zou, Y., Bassett, H., Walker, R., Bishop, A., Amin, S., Geacintov, N. E. and Van Houten, B. (1998) Hydrophobic forces dominate the thermodynamic characteristics of UvrA-DNA damage interactions. *J. Mol. Biol.* **281**, 107–119
- 31 Bochkarev, A., Pfuetzner, R. A., Edwards, A. M. and Frappier, L. (1997) Structure of the single-stranded-DNA-binding domain of replication protein A bound to DNA. *Nature (London)* **385**, 176–181
- 32 Geacintov, N. E., Zinger, D., Ibanez, V., Santella, R., Grunberger, D. and Harvey, R. G. (1987) Properties of covalent benzo[a]pyrene diol epoxide-DNA adducts investigated by fluorescence techniques. *Carcinogenesis* **8**, 925–935
- 33 Geacintov, N. E., Solntsev, K., Johnson, L. W., Chen, J. X., Kolbanovskiy, A. D., Liu, T. M. and Shafirovich, V. Y. (1998) Photoinduced electron transfer and strand cleavage in pyrenyl-DNA complexes and adducts. *J. Phys. Org. Chem.* **11**, 561–565
- 34 Huang, W., Amin, S. and Geacintov, N. E. (2002) Fluorescence characteristics of site-specific and stereochemically distinct benzo[a]pyrene diol epoxide-DNA adducts as probes of adduct conformation. *Chem. Res. Toxicol.* **15**, 118–126
- 35 Cosman, M., Ibanez, V., Geacintov, N. E. and Harvey, R. G. (1990) Preparation and isolation of adducts in high yield derived from the binding of two benzo[a]pyrene-7,8-dihydroxy-9,10-oxide stereoisomers to the oligonucleotide d(ATATGTATA). *Carcinogenesis* **11**, 1667–1672
- 36 Geacintov, N. E., Gagliano, A. G., Ibanez, V. and Harvey, R. G. (1982) Spectroscopic characterizations and comparisons of the structures of the covalent adducts derived from the reactions of 7,8-dihydroxy-7,8,9,10-tetrahydrobenzo [a] pyrene-9,10-oxide, and the 9,10-epoxides of 7,8,9,10-tetrahydrobenzo [a] pyrene and 9,10,11,12-tetrahydrobenzo [e] pyrene with DNA. *Carcinogenesis* **3**, 247–253
- 37 Mu, D., Hsu, D. S. and Sancar, A. (1996) Reaction mechanism of human DNA repair excision nuclease. *J. Biol. Chem.* **271**, 8285–8294

Received 7 July 2004/19 August 2004; accepted 14 September 2004

Published as BJ Immediate Publication 14 September 2004, DOI 10.1042/BJ20041151