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Photocrosslinking locates a binding site for the large subunit of human replication protein A to the damaged strand of cisplatin-modified DNA

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ABSTRACT

The repair proteins XPA, XPC and replication protein A (RPA) have been implicated in the primary recognition of damaged DNA sites during nucleotide excision repair. Detailed structural information on the binding of these proteins to DNA lesions is however lacking. We have studied the binding of human RPA (hRPA) and hRPA–XPA-complexes to model oligonucleotides containing a single 1,3-d(GTG)-cisplatin-modification by photocrosslinking and electrophoretic mobility shift experiments. The 70 kDa subunit of hRPA can be crosslinked with high efficiency to cisplatin-modified DNA probes carrying 5-iodo-2'-deoxyuridin (5-IdU) as crosslinking chromophore. High efficiency crosslinking is dependent on the presence of the DNA lesion and occurs preferentially at its 5'-side. Examination of the crosslinking efficiency in dependence on the position of the 5-IdU chromophore indicates a specific positioning of hRPA with respect to the platination site. When hRPA and XPA are both present mainly hRPA is crosslinked to the DNA. Our mobility shift experiments directly show the formation of a stable ternary complex of hRPA, XPA and the damaged DNA. The affinity of the XPA–hRPA complex to the damaged DNA is increased by more than one order of magnitude as compared to hRPA alone.

INTRODUCTION

The nucleotide excision repair (NER) process is a ubiquitious pathway by which a variety of DNA damages are removed from the genome. Recognition of helix-distorting DNA lesions is followed by an unwinding and incision reaction on both sides of the DNA adduct, releasing a fragment of 24–32 nt in length. The resulting gap is filled in by a DNA polymerase and sealed by a DNA ligase (1).

Replication protein A (RPA), a heterotrimeric protein consisting of subunits of 70, 32 and 14 kDa, participates in DNA-replication, recombination and also in repair processes (2). During NER the human RPA protein (hRPA, HSSB)—similar to other RPA homologs—is not only involved in the DNA polymerase catalyzed gap-filling reaction but also plays a role in the initial damage recognition and incision reactions. hRPA has been shown to act in concert with the XPA protein (3–5) and it interacts with other repair factors such as the nucleases XPG and XPF (6,7). hRPA itself is able to bind various types of DNA lesions (8–10) and has been implicated in stabilizing the opened DNA duplex in cooperation with XPA, TFIIH and XPC (11). XPA complexed to hRPA has long been assumed to be the primary damage recognition protein. Accumulating evidence suggests, however, that this function is fulfilled by XPC in genome-overall repair, leaving the role of a ‘damage verifier’ to XPA (12). Until now only little information about the spatial arrangement of these proteins bound to a DNA lesion has been available. To elucidate this question it is important to define the sites of contact between the damaged DNA and each of the proteins involved. In our approach we used a synthetic 24 bp DNA duplex carrying a single intrastrand 1,3-d(GTG) cisplatin adduct as a model substrate (13). This modification is efficiently removed by NER (14), furthermore its structure has been solved by NMR (15).

In our crosslinking studies we substituted the photocrosslinking reagent 5-iodo-2'-deoxyuridine (5-IdU) for thymidine residues at a number of positions in the damaged and in the non-damaged strand (Fig. 1A and B). The iodine atom of the IdU-chromophore has an only slightly larger van der Waals radius than the corresponding methyl group thereby marginally affecting nucleic acid structure and protein binding. As a zero-length-crosslinker 5-IdU undergoes crosslinking reactions only with amino acid side chains that are in close contact to the photoreactive nucleobase. The reagent exhibits a strong preference for aromatic side chains (16). By substituting only one position at a time we were able to locate a binding site of hRPA70 on the damaged DNA strand. In addition, we could directly show cooperative complex formation between hRPA, XPA and our cisplatin-treated model DNA.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

MATERIALS AND METHODS

Preparation of proteins

hRPA was expressed in *Escherichia coli* BL21 (DE3) pLysS using the expression vector pET11d-thRPA (a generous gift from M. S. Wold) and purified through Affigel-Blue (Bio-Rad), hydroxylapatite (Merck, Darmstadt, Germany) and ResourceQ (Pharmacia) columns as described by Henricksen *et al.* (17). The DNA binding activity was tested by performing EMSA with a single-stranded 43 nt DNA probe; the apparent K_D found was 3 nM.

XPA and its minimal DNA-binding domain MF122 were expressed as histidine-tagged proteins in *E.coli* BL21 (DE3) pLysS using the plasmids pET15b-XPAC (18) (generously provided by R. Wood) and pET16b-$XPA_{98–219}$ (kindly provided by K. Tanaka) and purified by metal chelate chromatography using Ni-NTA fast-flow Sepharose (Qiagen) as described (19).

Preparation of DNA with a single 1,3-intrastrand d(GTG) cisplatin adduct (Pt-DNA)

Oligodeoxynucleotides with and without 5-IdU substitutions at the indicated positions (e.g. position 7 in PT-7 or position 11 in PTB-11, respectively) were purchased from Eurogentech (Seraing, Belgium). Oligodeoxynucleotides of the PT-series (top strand) were allowed to react with a 3-fold molar excess of cisplatin (Sigma) as described (13). After 5'-labeling with T4 polynucleotide kinase (NEB) and [γ-^{32}P]ATP (5000 Ci/mmol; Hartmann Analytics, Braunschweig, Germany) the strands were annealed to the complementary bottom strands of the PTB-series in hybridization buffer [20 mM Tris·AcOH, 50 mM KOAc, 10 mM $Mg(OAc)_2$, 1 mM DTT, pH 7.9]. Quantitative hybridization was checked by native polyacrylamide gel electrophoresis. The degree of platination was determined by denaturing electrophoresis on 20% polyacrylamide gels where the platinated oligonucleotide was found to migrate as a single shifted band compared to the unmodified DNA. Additionally platinated duplex DNA was subjected to restriction by *Apa*LI (NEB) which does not cleave the platinated recognition site.

Photocrosslinking of hRPA to 5-IdU modified DNA

Photocrosslinking was carried out in 96 well microtiter plates using a UV-B light source (four 15 W low-pressure mercury bulbs, λ_{max} = 312 nm, 1.5 mW/cm^2 at a distance of 10 cm). The samples were covered with the polystyrene lid of the microtiter plate to shield them from wavelengths below 300 nm and cooled in an ice-bath during illumination. A typical 25 µl-reaction contained 10 nM 5'-labeled duplex DNA, 250 ng of unrelated plasmid DNA as competitor and 400 nM hRPA in RPA buffer [20 mM HEPES–KOH, pH 7.8, 0.25% (v/v) inositol, 3–150 mM NaCl as indicated]. Reactions were preincubated for 10 min at room temperature prior to illumination. Aliquots were taken at the indicated times and analyzed in 12% SDS–polyacrylamide gels according to Schagger and von Jagow (20). Crosslinking yields were determined by electronic autoradiography using an InstantImager (Canberra Packard).

Electrophoretic mobility shift assays (EMSAs)

Indicated amounts of RPA and XPA were preincubated with 250 ng plasmid competitor in RPA-buffer containing 100 mM KCl and 10 mM $MgCl_2$ for 5 min at room temperature. Labeled DNA was added at a final concentration of 3 nM and samples were loaded on 6% native polyacrylamide gels after 10 min incubation at room temperature. Electrophoresis was done in TAE (40 mM Tris·AcOH, 1 mM EDTA, pH 7.8) for 2 h at 160 V and 4°C.

For antibody supershift experiments two monoclonal antibodies (RBF-4EA, α-hRPA32 and RAC-6D5, α-hRPA70), kindly provided by C. Nasheuer (IMB, Jena, Germany) were used.

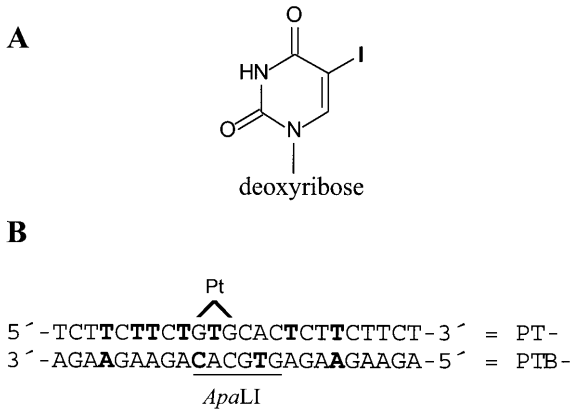

Figure 1. Oligonucleotides used in this study. (**A**) Structure of the photocrosslinking reagent 5-IdU. **(B)** Sequence of the damaged DNA model substrate (Pt-DNA). The PT-strand contains a single 1,3-d(GTG) intrastrand cisplatin adduct (indicated by Pt) within the recognition sequence for *Apa*LI. The positions of 5-IdU substitutions investigated in this article are indicated by bold letters and numbered starting from the 5'-end of each DNA-strand. All changes were conservative except PTB-6 and PTB-15 which formed mismatched base pairs.

Electronic autoradiography

The gel images in this paper are reproductions of images obtained by an InstantImager (Canberra Packard) with a linear relationship between signal and image intensity.

RESULTS

The 70 kDa subunit of hRPA contacts the damaged strand on the 5'-side of the lesion

RPA is known to bind DNA in different modes. In addition to the general binding mode where ~30 nt are covered by the protein (21), binding sites of 8–10 nt (6,22,23) or as small as 4 nt bubbles (2) have been reported under specific conditions. The crystal structure of the single-stranded DNA (ssDNA) binding domain of hRPA70 complexed with $(dC)_8$ shows a hydrophobic groove where the ssDNA is accommodated forming close contacts between aromatic amino acid side chains and nucleobases (24). Possibly DNA with helix-distorting modifications such as cisplatin-adducts or (6–4) photoproducts is bound in a similar way (8,9). These modifications lead to a partial unwinding of the DNA duplex at the site of lesion (15,25–27) which should allow hRPA the recognition and binding of unpaired nucleobases. Such π–π-interactions favor high yield photocrosslinking with chromophors like 5-IdU.

We probed 10 positions of 5-IdU substitution in our model DNA substrate (designated as Pt-DNA) for hRPA–DNA

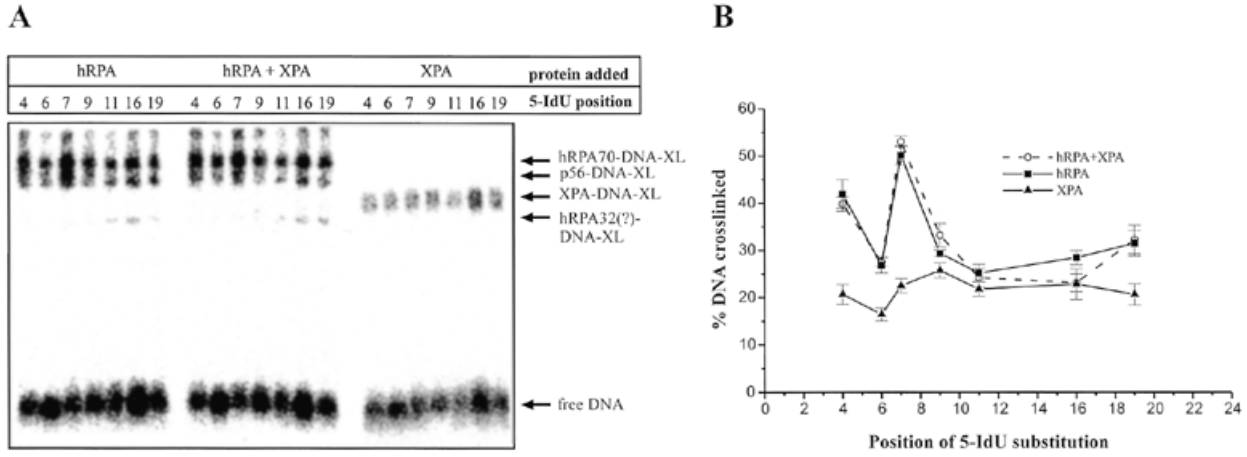

Figure 2. Photocrosslinking of hRPA with cisplatin-modified DNA. (**A**) Crosslinking of hRPA (400 nM), XPA (400 nM) and the hRPA–XPA-complex (400 nM) with the damaged DNA model substrate (10 nM) containing 5-IdU-substitutions at the indicated positions in the PT-strand in the presence of 3 mM NaCl. After 10 min preincubation photocrosslinking was performed for 6 h at 0°C using a transilluminator at λ = 312 nm. Reaction samples were separated by SDS–PAGE and analyzed by electronic autoradiography. (**B**) Crosslinking efficiency from (A) as a function of 5-IdU-position.

photocrosslinking (Fig. 1B). The crosslinking yields were up to 60% when the chromophore was located in the damaged strand, whereas 5-IdU modifications in the undamaged strand resulted in low crosslinking efficiencies of ~10% (data not shown). The preferential crosslinking to the damaged strand is in accordance to the NMR structural model of 1,3-d(GTG) cisplatin modified DNA (15) restricting larger deviations from canonical B-DNA structure to the 5'-side of the cisplatin adduct only in the damaged strand.

Size determination of the crosslinking products by SDS–PAGE showed that the large subunit of hRPA is preferentially crosslinked to the DNA probe with every position of 5-IdU-substitution tested (Fig. 2A). A second band with slightly higher mobility is ascribed to the reaction of a 56 kDa proteolytic fragment of hRPA70 (28). The highest crosslinking yields were obtained at nucleotide positions 4 and 7 on the damaged strand substituted by 5-IdU, which are located 5' to the platination site (Fig. 2B).

The autoradiogram in Figure 2A shows traces of a further crosslinking product for 5-IdU-substitutions lying 3' of the lesion at positions 11, 16 and 19. Its apparent size of ~40 kDa suggests that it may be the hRPA32 subunit crosslinked to DNA. In previous studies crosslinking of hRPA32 to DNA has been already reported (29). Due to the low yield we were not able to prove the identity of this product and we cannot exclude that it represents another proteolytic fragment of hRPA70.

Under the conditions tested XPA alone formed a protein–DNA crosslink only with low efficiency, exhibiting no preference for any 5-IdU-position on the DNA substrate. In samples containing both hRPA and XPA it was only hRPA that underwent significant crosslinking. The presence of XPA affected neither the specificity nor the crosslinking yield after 6 h of irradiation compared to hRPA alone (Fig. 2A and B).

The specificity of hRPA crosslinking is also evident from experiments following the time course of the reaction (Fig. 3). The presence of XPA did not affect the crosslinking of hRPA significantly at all irradiation times investigated. Crosslinking is specific for 5-IdU substituted DNA and occurs only with cisplatin-modified DNA probes. Furthermore, the presence of a high excess of plasmid DNA as a non-specific competitor did not influence the crosslinking with the damaged DNA (data not shown).

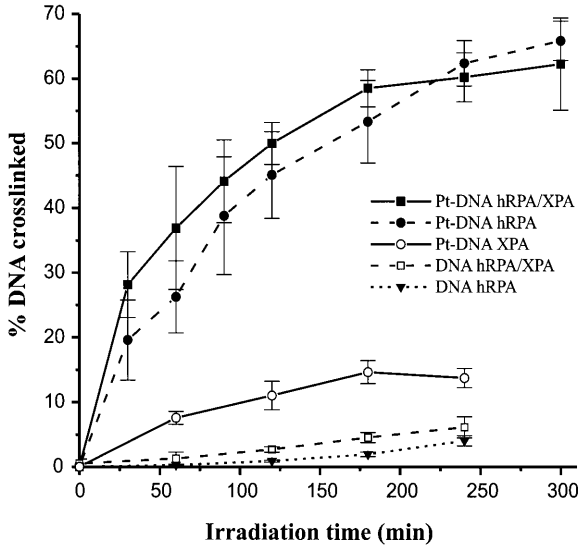

Figure 3. Specificity of hRPA–DNA photocrosslinking tested by crosslinking kinetics. The fraction of crosslinked DNA is plotted as a function of irradiation time. Photocrosslinking was done as described in Materials and Methods with 400 nM hRPA, 400 nM XPA or 400 nM hRPA–XPA complex and either damaged or undamaged DNA with 50 mM NaCl included.

We conclude from these crosslinking data that RPA70 specifically contacts cisplatin modified DNA 5' of the cisplatin adduct on the damaged strand.

hRPA and 1,3-d(GTG)-cisplatin adducted DNA form a complex with defined geometry

From the data presented in Figure 2 the question arose as to why the basal level of photocrosslinking could be as high as 30%. It is known that hRPA exhibits dsDNA unwinding

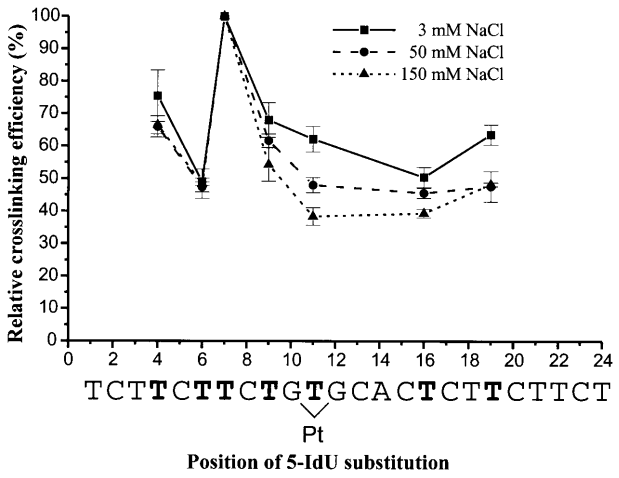

Figure 4. Salt-dependence of hRPA-crosslinking. Crosslinking yields for hRPA70 and Pt-DNA were determined for various positions of 5-IdU-substitution and NaCl-concentrations as indicated. Samples were treated as described in Figure 2. The crosslinking efficiencies are normalized against the value for PT-7 at each salt concentration tested.

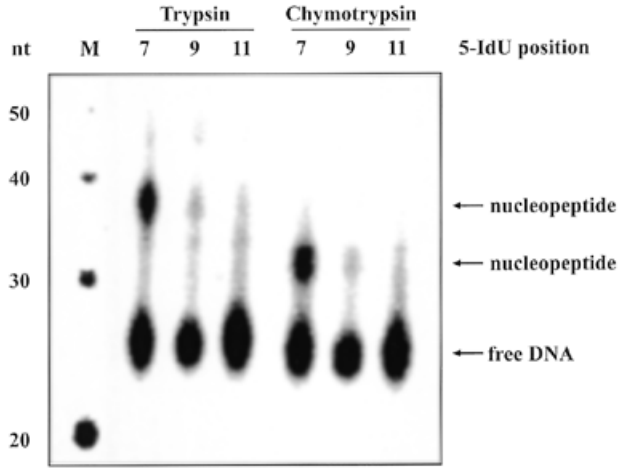

Figure 5. Only the hRPA-PT-7 crosslink yields distinct products upon proteolysis. Photocrosslinking of hRPA and PT-DNA (5-IdU at position 7, 9 or 11) was done as described in Materials and Methods at 150 mM NaCl. 10 µl samples of the crosslinking reactions were subjected to protease digestion with 20 U TPCK-trypsin in 0.1% SDS or 0.3 U chymotrypsin at 37°C for 14 h (both enzymes were from Sigma). Reactions were separated on a 20% PAA gel/8.3 M urea together with a ^{32}P-labeled 10 bp marker (M). Resulting peptides crosslinked to the radioactively labeled DNA were visualized by electronic autoradiography.

activity which can be inhibited by addition of salt (28). To check this point we performed crosslinking experiments in the presence of various salt concentrations. The crosslinking yields decreased with increasing salt concentration down to a level of ~15% for most positions in the presence of 150 mM NaCl (Fig. 4). While crosslinking for position 7 remained nearly unaffected, yields at position 4 and 19 decreased, suggesting that these positions can be easily unwound due to an end-effect and not primarily because of the DNA's cisplatin modification. hRPA obviously was capable of partial unwinding and non-specific binding of the model DNA under low salt conditions. Specificity for binding our Pt-DNA is apparently increased by raising the ionic strength to more physiological values. A similar observation has been reported for the RPA-binding to damaged DNA in EMSAs (9,10).

The X-ray structure of the hRPA70·dC$_8$-complex (24) shows a set of aromatic amino acids in the ssDNA binding groove which can serve as potential acceptor amino acids in photocrosslinking reactions. We subjected aliquots of the crosslinking reaction of hRPA with platinated DNA carrying the 5-IdU at position 7, 9 or 11 to proteolytic digestion by trypsin and chymotrypsin. The digest was then analyzed by denaturing gel electrophoresis (Fig. 5). For both enzymes a distinct nucleopeptide is only found for 5-IdU at position 7 indicating a specific crosslinking of hRPA to this site. Since both proteases have a high number of potential cleavage sites in the hRPA70 primary sequence a non-specific crosslinking of various acceptor amino acids should result in a mixture of nucleopeptides. We conclude from these findings that the complex between hRPA70 and 1,3-d(GTG)-cisplatin adducted DNA has a defined geometry under physiological salt conditions.

hRPA forms a stable complex with XPA on cisplatin-modified DNA

The specific binding of hRPA alone to damaged DNA has been demonstrated by EMSA (8–10). For this reason it should be possible to visualize the ternary complex consisting of hRPA, XPA and Pt-DNA by the same method. In the experiments shown in Figure 6, the platinated model DNA and hRPA were titrated with increasing amounts of either XPA or the DNA binding domain of XPA (MF122, XPA$_{98–219}$). Addition of XPA or MF122 induced a supershift of the hRPA–DNA complex in a dose-dependent manner and increased the fraction of bound DNA. Furthermore, the ternary complex containing hRPA and MF122 migrates faster than that with full-length XPA indicating that XPA and MF122 are present in the respective protein–DNA complexes.

To quantify the effect of XPA addition on the hRPA-damaged DNA interaction we determined apparent dissociation constants by EMSA. The amount of hRPA required for binding 50% of the Pt-DNA was obtained from the titration of hRPA alone or in the presence of saturating XPA concentrations (Fig. 7). The apparent dissociation constant was estimated to be 170 nM in the presence of a 1000-fold excess of double-stranded plasmid DNA for the hRPA·XPA·DNA complex, while the affinity of hRPA for Pt-DNA alone was too low to be determined accurately. We estimate from these data that protein–DNA complex formation is stimulated by more than an order of magnitude by addition of XPA.

The presence of the heterotrimeric hRPA in the hRPA·XPA·Pt-DNA complex was confirmed by supershift experiments using RPA-specific monoclonal antibodies. The α-hRPA70- as well as the α-hRPA32-antibody retarded the protein–DNA complex (Fig. 8). From earlier work (17) it is already known that hRPA32 is insoluble in the absence of hRPA14, suggesting indirectly that the observed complex indeed is composed of heterotrimeric hRPA, XPA and cisplatin modified DNA.

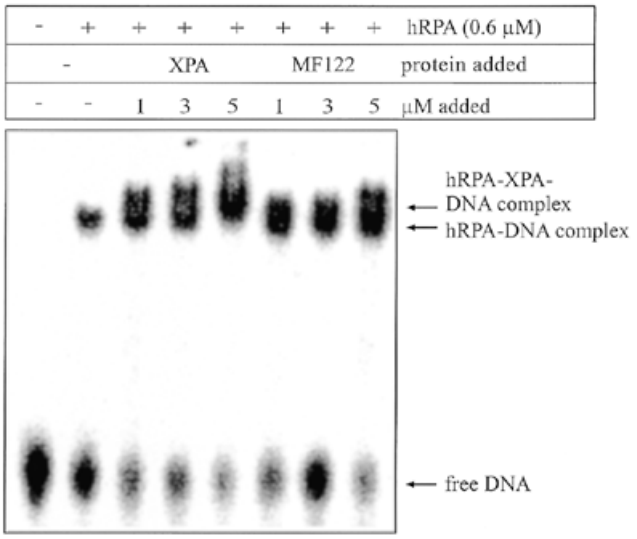

Figure 6. Ternary complex formation between XPA, hRPA and Pt-DNA shown by EMSA. Increasing amounts (0, 1, 3, 5 μM) of either full-length XPA or its DNA-binding domain MF122 were added to 0.6 μM hRPA and 2 nM cisplatinated DNA. Reactions were separated by native PAGE as described in Materials and Methods and analyzed by electronic autoradiography.

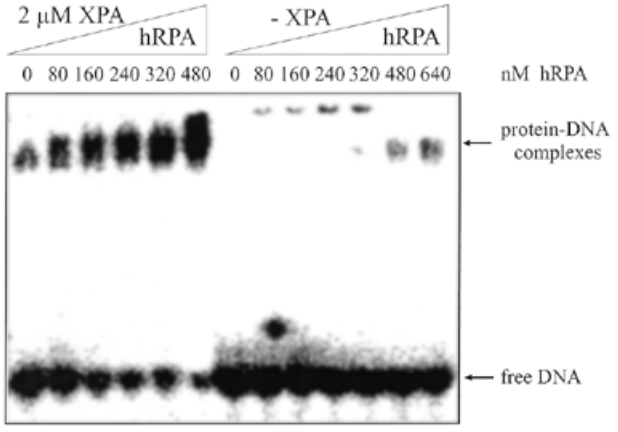

Figure 7. XPA and hRPA bind the 24 bp damaged DNA model substrate cooperatively. Cisplatinated DNA (PT-7, 2 nM) was titrated with increasing amounts of hRPA (as indicated above) in the absence or presence of 2 μM XPA. Binding reactions were subjected to native PAGE as described in Materials and Methods and analyzed by electronic autoradiography. The apparent dissociation constant (hRPA concentration where 50% of DNA is bound) for the hRPA·XPA·Pt·DNA complex was estimated to be ~170 nM.

DISCUSSION

It is still poorly understood how DNA lesions are recognized by the NER complex. In this study we report on the interaction between damaged DNA and two proteins, hRPA and XPA, that are assumed to be involved in primary recognition of DNA lesions. The major experimental approach of our work is the site-specific photocrosslinking of hRPA to DNA containing a single cisplatin-modification. Photocrosslinking was mediated

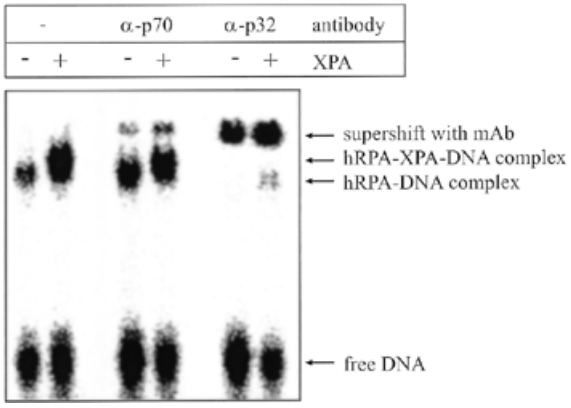

Figure 8. Antibody supershift experiments. hRPA (0.6 μM) and platinated DNA (2 nM) were incubated with or without XPA (5.5 μM) for 5 min. Saturating amounts of the indicated antibody were added and the binding reactions were subjected to native PAGE. Unbound DNA, shifted and supershifted complexes were visualized by electronic autoradiography.

by 5-IdU introduced at specific sites of the model DNA which allows to probe for intimate protein–DNA contacts.

5-IdU (Fig. 1A) is a thymidine-analog with specific properties that make it well suited for the crosslinking of protein–DNA complexes. The van der Waals-radius of iodine is very close to that of the methyl group of thymidine allowing its substitution with a minimal distortion of the DNA helix. Upon UV-irradiation the carbon–iodine bond undergoes homolysis to generate a uridyl radical with an extremely short lifetime. Crosslinking of the radical with nearby amino-acid side chains is only possible if the reaction partners are in close contact rendering 5-IdU a zero-length-crosslinker. Due to this property 5-IdU-substituted oligonucleotides have been successfully used to uncover close contact points in protein–nucleic acid complexes (30). A further advantage of the 5-IdU-chromophore is the excitation by UV-light at wavelengths above 300 nm which minimizes photodamaging of protein and nucleic acid as well as unspecific photocrosslinking (16).

Proteins binding ssDNA often stabilize unstacked nucleobases by interactions with hydrophobic, mainly aromatic, amino acid side chains. For T4 gp32 this type of residue could be efficiently crosslinked to $(dT)_8$ and the close contacts revealed by this approach have been confirmed by the crystal structure of the complex (31,32). This mode of DNA binding is conserved in the group of ssDNA binding proteins and we speculated that hRPA might bind damaged DNA in a similar way. 5-IdU was the reagent of choice to probe for hRPA–DNA contacts, since several studies have shown that π-stacking interactions between the unpaired photoreactive nucleobase and aromatic acceptor amino acids facilitate excitation and result in high crosslinking yields (33).

We report that hRPA70 undergoes specific and efficient photocrosslinking with our 5-IdU substituted Pt-DNA. The high crosslinking yield of up to 60% suggests that hRPA indeed binds the damaged DNA via aromatic amino acids within its ssDNA binding domain. The Pt-DNA used in our study contains a single 1,3-d(GTG) cisplatin adduct that is

efficiently repaired by NER (14). In addition the structure of such an cisplatin-adducted DNA has been solved by NMR (15). One of the major findings from the NMR structure model is the unwinding of the duplex by 19° in the region just 5' to the platination site. Most intriguingly, we observed preferential crosslinking in this region of the DNA. Since hRPA70 also displays helicase activity (34) and is able to bind unpaired regions as small as 4 bases (2) one could envision a model where hRPA first penetrates and then extends the region of unwinding initially induced by bulky DNA damages such as the 1,3-d(GTG)-cisplatin adduct.

A major result of our crosslinking studies is the specific crosslinking of hRPA to the damaged DNA. High efficiency crosslinking is dependent on the presence of the DNA lesion and is highly position dependent. We observe a strong preference for crosslinking at position 7 of the damaged strand. This finding indicates specific contacts of hRPA to the region 5' to the site of DNA damage and it supports a binding mode where hRPA is bound in a distinct orientation with respect to the lesion. The specificity of complex formation is also supported by proteolytic digestion of the crosslinked complex where we observed a distinct band of digested, crosslinked nucleoprotein of rather low molecular weight. If hRPA would bind in a non-specific manner to the Pt-DNA one would expect a smear of digested nucleoproteins with a rather broad size distribution. Our data clearly show that hRPA is able to recognize and bind a distorted DNA helix in a specific way.

The presence of XPA has a distinct effect on the interaction between hRPA and the Pt-DNA. The two proteins have been shown to interact specifically in the absence of DNA (5). Our data show that the hRPA–XPA-complex binds more strongly to the Pt-DNA as compared to hRPA alone. Most interestingly we could not detect crosslinking of the XPA component to the damaged DNA strand indicating that only the 70 kDa subunit of hRPA makes photoreactive contacts to the damaged strand. However, in the absence of hRPA, XPA can be crosslinked to the Pt-DNA with moderate efficiency. This crosslinking is still dependent on the presence of the cisplatin-modification (data not shown). However, under the conditions tested, we could not observe a position preference suggesting a rather non-specific binding of XPA to the damaged DNA strand (Fig. 2A and B). Therefore in the binary complex the XPA protein is quite differently arranged with respect to the platination site as compared to the situation in the ternary complex.

Formation of the ternary hRPA·XPA·Pt-DNA-complex could be directly observed in our EMSA experiments. So far we know of no report showing the cooperative binding of hRPA and XPA to singly modified DNA using this assay. Using a TC[6–4] photoproduct as model substrate, Wakasugi and Sancar (35) have not been able to detect efficient ternary complex formation by EMSA.

XPA enhances the affinity of hRPA to the Pt-DNA in a cooperative way by more than one order of magnitude. The minimal DNA binding domain of XPA is sufficient to bring about the enhancement of affinity indicating that this domain mediates most of the protein–protein contacts within the ternary complex. These contacts have also been uncovered by (^{15}N-^{1}H)-HSQC-NMR spectra in solution without DNA (36).

Until now only one group (37,38) reported on the positioning of hRPA within the excision nuclease complex bound to damaged DNA. In this work hRPA was found to influence the permanganate footprint at the 5'-side of the DNA damage, the very region where we observe high efficiency crosslinking of hRPA70. Based on the observation that hRPA protected the undamaged strand from the XPG and XPF nucleases the authors however located the hRPA-binding site to the non-damaged strand. Our crosslinking data identify major contact points for hRPA70 to the 5'-side of the DNA lesion on the damaged strand. We do not believe that this result is in conflict with the data reported by Evans *et al.* (37,38) where the complete NER complex was investigated. The positioning of hRPA may vary depending on the complexity of the system. Furthermore the large size of hRPA makes it difficult to interpret footprinting experiments in terms of distinct contact points. De Laat *et al.* (39) also located hRPA to the undamaged strand. However, these authors used partially ssDNA constructs and the results thus cannot be easily compared to our data.

The exact binding mode of XPA within the ternary complex could not yet be determined from our experiments. Given the cooperativity in binding we suggest that XPA binds close to hRPA in the immediate vicinity of the damage but in a manner that does not result in productive photocrosslinking. XPA may not act primarily within damage recognition but rather in recruiting other repair factors to the lesion. This interpretation is in line with the 'damage verifier' concept proposed by Sugasawa *et al.* (12) who ascribed XPC the function of the initial damage sensor.

Our data assign the hRPA–XPA complex an important role in primary damage recognition. In our ternary system the hRPA70 subunit mediates important contacts to the damaged DNA strand and is thought to help in further unwinding at the damaged site. The efficiency of hRPA–XPA binding may strongly depend on the extent of initial helix destabilization at the site of DNA damage and thus may be different for various types of DNA damage. In addition to targeting the other components of the NER to the damaged DNA the XPA-protein seems to act as an enhancer of hRPA binding.

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