Human XPC-hHR23B interacts with XPA-RPA in the recognition of triplex-directed psoralen DNA interstrand crosslinks

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Received February 4, 2005; Revised and Accepted May 3, 2005

ABSTRACT

DNA interstrand crosslinks (ICLs) represent a severe form of damage that blocks DNA metabolic processes and can lead to cell death or carcinogenesis. The repair of DNA ICLs in mammals is not well characterized. We have reported previously that a key protein complex of nucleotide excision repair (NER), XPA-RPA, recognizes DNA ICLs. We now report the use of triplex technology to direct a site-specific psoralen ICL to a target DNA substrate to determine whether the human global genome NER damage recognition complex, XPC-hHR23B, recognizes this lesion. Our results demonstrate that XPC-hHR23B recognizes psoralen ICLs, which have a structure fundamentally different from other lesions that XPC-hHR23B is known to bind, with high affinity and specificity. XPC-hHR23B and XPA-RPA protein complexes were also observed to bind psoralen ICLs simultaneously, demonstrating not only that psoralen ICLs are recognized by XPC-hHR23B alone, but also that XPA-RPA may interact cooperatively with XPC-hHR23B on damaged DNA, forming a multimeric complex. Since XPC-hHR23B and XPA-RPA participate in the recognition and verification of DNA damage, these results support the hypothesis that interplay between components of the global genome repair sub-pathway of NER is critical for the recognition of psoralen DNA ICLs in the mammalian genome.

INTRODUCTION

Most bulky DNA lesions are processed by the nucleotide excision repair (NER) pathway, which is composed of two sub-pathways: transcription coupled repair (TCR) and global genome repair (GGR). Lesions that inhibit DNA transcription are believed to be the principle targets of the TCR pathway. Experimental evidence has shown that RNA Pol II progression along transcribed DNA is sensitive to the presence of DNA damage, leading to the hypothesis that interruption of RNA Pol II processivity could serve as the initiating signal for TCR (1,2). Other constituents of this pathway (such as Cockayne Syndrome Group A and B proteins) may also assist in the identification of DNA damage (3–7). The GGR sub-pathway of NER is believed to be responsible for detecting and repairing bulky DNA lesions over the entire genome with the exception of those genes undergoing active transcription. Although NER has been extensively studied, it is still debatable exactly which proteins are responsible for recognition of specific lesions in GGR and TCR since a number of different proteins, including Xeroderma pigmentosum protein Group C-human homolog of RAD23B (XPC-hHR23B), replication protein A (RPA) and X.pigmentosum protein Group A (XPA), each bind to many DNA lesions that are substrates for NER [reviewed in (8)].

Among the many different kinds of DNA damage, DNA interstrand crosslinks (ICLs) are among the most detrimental to DNA metabolism and are lethal in repair deficient prokaryotic and eukaryotic cells (9). Both NER and homologous recombination (HR) mechanisms are involved in repairing DNA ICLs in bacteria and yeast (10–12), but the mechanisms of repair of crosslinked DNA in mammalian cells are not well characterized. A number of crosslinking agents, including the psoralen family of intercalating, photoactivatable DNA damaging agents, are available for the study of the repair of DNA ICLs. Following irradiation with ultraviolet-A (UVA) light, psoralen molecules form DNA crosslinks between thymines on opposing strands of duplex DNA. Psoralen plus UVA
target and pTFOc is a 19 base scrambled oligonucleotide 19 bp polypurine site in the APRT-derived duplex (37 bp) previously (22). pTFO1 (19mer) binds to the corresponding pSupFG1 triplex target sites were constructed as described Oligonucleotides in mammalian genome. pathway of NER in the recognition of Tdp-ICLs in the lesions. Thus, these data support a role for the GGR sub- XPC-hHR23B and XPA-RPA in the recognition of these ICLs and demonstrate a possible positive interaction between implicate human XPC-hHR23B in the recognition of DNA high molecular weight multimeric complex. Our observations XPA-RPA bound a damaged DNA substrate together to form a purine-rich triplex recognition sequence on a target DNA duplex [reviewed in (17)]. Targeting DNA damage via triplex formation has been used successfully in vitro and in vivo to induce site-specific DNA mutations, DNA recombination and to study DNA–protein interactions at a specific site (17–22). We have previously demonstrated that DNA damage recognition proteins, including the NER proteins RPA and XPA, will bind to triplex DNA substrates containing a single psoralen ICL (22). Although both RPA and XPA likely play roles in detecting DNA lesions (including DNA ICLs), there is very compelling evidence suggesting that XPC-hHR23B is the principle damage recognition factor in GGR [reviewed in (8) and references therein]. Because of the critical role that XPC-hHR23B appears to play in identifying DNA damage, we were interested in whether XPC-hHR23B would recognize a complex lesion such as a TFO-directed psoralen-DNA ICL. In this work the capacity of XPC-hHR23B, or XPC- hHR23B in combination with XPA-RPA, to recognize a unique TFO-directed psoralen-interstrand crosslink (Tdp-ICL) on a DNA substrate was investigated. Previously, others have shown that XPC-hHR23B recognizes substrates containing cis-platin-DNA intrastrand-crosslinks (23–26) and psoralen monoadducted DNA (27). Our results provide the first demonstration that the human recombinant XPC-hHR23B protein complex interacts with a Tdp-ICL with high affinity and specificity. XPC-hHR23B was also observed to bind to complexes formed between psoralen damaged DNA and the XPA-RPA NER recognition complex. At low XPA-RPA concentrations, XPC-hHR23B and XPA-RPA compete for binding to the lesion, but at higher RPA concentrations XPC-hHR23B and XPA-RPA bound a damaged DNA substrate together to form a high molecular weight multimeric complex. Our observations implicate human XPC-hHR23B in the recognition of DNA ICLs and demonstrate a possible positive interaction between XPC-hHR23B and XPA-RPA in the recognition of these lesions. Thus, these data support a role for the GGR sub-pathway of NER in the recognition of Tdp-ICLs in the mammalian genome.

**MATERIALS AND METHODS**

**Oligonucleotides**

Synthetic duplex targets for TFO binding from the APRT and pSupFG1 triplex target sites were constructed as described previously (22). pTFO1 (19mer) binds to the corresponding 19 bp polypurine site in the APRT-derived duplex (37 bp) target and pTFOc is a 19 base scrambled oligonucleotide that does not bind the APRT target (16). pAG30 (30mer) forms a triplex DNA structure on a 30 bp polypurine site on the 57-bp pSupFG1-derived sequence and pSCR30 is a 30 base scrambled control oligonucleotide (22). TFOs were synthesized with a 5’s-psoralen derivative, HMT, (2-[4’ (hydroxymethyl)-4,5’-8-trimethylpsoralen]-hexyl-1-O-(2-cya noethyl)-N,N-diisopropyl)-phosphoramidite) by the Midland Certified Reagent Company, Inc. (Midland, TX). Duplexes were 5’ end-labeled by the transfer of 32P from [γ-32P]ATP with T4 polynucleotide kinase and purified by 12% PAGE, electroeluted and concentrated via Centricon centrifugal filtration devices (Millipore, Bedford, MA). DNA concentration was determined by UV absorbance at 260 nm.

**Tdp-ICL formation**

Triplex substrates were generated by incubating radiolabeled duplex targets with psoralen-conjugated TFOs in a triplex binding buffer [10 mM Tris–HCl, pH 7.6, 10 mM MgCl2, and 10% (v/v) glycerol] at 37°C for 16 h. Samples were then irradiated with 1.8 J/cm2 of UVA light at 366 nm to induce psoralen ICLs. Efficiency of crosslinking at the targeted triplex-duplex junction was as high as 90%, as determined by the quantification of the crosslinked product by denaturing PAGE using a phosphorimager (Figure 1). All Tdp-ICL substrates were gel purified to remove free duplex DNA (unless stated otherwise) prior to use.

**Human recombinant proteins**

The XPC-hHR23B-maltose-binding protein fusion protein was expressed and purified in Sf9 or Hi-5 insect cells as described previously (28,!29). Recombinant XPA-maltose-binding protein fusion protein was expressed in Escherichia coli PR745 and purified as described previously (30). For recombinant RPA, the three subunits were expressed by co-infection of Sf9 insect cells and purified by Ni2+-chelate column chromatography as previously described (31).

**DNA–protein binding assays**

DNA–protein binding interactions were analyzed by electrophoretic mobility shift assays (EMSA). Human recombinant proteins, at varying concentrations, were incubated in binding buffer [25 mM Tris–HCl, pH 7.6, 100 mM NaCl, 1 mM DTT, 5 mM EDTA, 100 µg/ml BSA, 0.01% NP-40 (v/v) and 10% glycerol (v/v)] in a 20 µl reaction volume for 10 min at room temperature (unless otherwise stated). Radiolabeled duplex or triplex substrates were then added and incubated at 30°C for 20 min. In the sequential addition experiments, the protein used at the static concentration was incubated with the Tdp-ICL substrate for 10 min, then increasing concentrations of the other protein were added. In the simultaneous experiments, proteins were incubated together for 10 min at room temperature in binding buffer, and then the Tdp-ICL substrate was added. The DNA–protein samples were then electrophoresed through a 6% (37:1 acrylamide/bis-acrylamide) native PAGE, containing 2.5% glycerol and buffered in 1× TGE (25 mM Tris–HCl, 192 mM glycine and 1 mM EDTA). Electrophoresis was conducted at 4°C, 9 mA/cm for 3 h. Gels were dried and DNA–protein complexes were visualized by autoradiography and quantified using a phosphorimager.
Apparent half-maximal binding of the available substrate is defined as $K_{\text{app}}$.

**Kinetic assay**

DNA–protein complexes were formed as described above, except that incubations were carried out for $<10$ s, 1, 2 or 4 min. The shortest time point was defined as the time required to add protein(s) to the DNA substrate immediately prior to loading the reaction on the native PAG at $4^\circ$C (actual incubation time of protein with DNA substrate was $<10$ s at $4^\circ$C).

**Antibody super-shift assay**

DNA–protein complexes were formed as described above. Next, monoclonal antibodies directed against RPA (anti-RPA34; Lab Vision, Fremont, CA) or the maltose-binding protein tag on the human recombinant XPC-hHR23B (anti-MBP; New England Biolabs, Beverly, MA) were added to the DNA–protein complexes (1 μg/20 μl reaction) and incubated at 30°C for 10 min. Following incubation, the reactions were electrophoresed through a 4 or 5% (37.5:1 acrylamide/bis-acrylamide) native PAG in 1× TGE buffer at 4°C, 200 V for 4 h. Gels were dried and the separated complexes were visualized by autoradiography and quantified using a phosphorimager.

## RESULTS

### Formation of Tdp-ICLs

Figure 1A depicts the 37 bp APRT-TA target duplex for the 19 nt psoralen-conjugated oligonucleotides, pTFO1 (the specific TFO), and pTFOc (the control oligonucleotide that does not bind the APRT-TA sequence). Psoralen photomodification efficiency was determined by incubating the psoralen-modified TFOs with the radiolabeled target duplex, APRT-TA, to allow triplex formation, followed by UVA irradiation of the samples at 1.8 J/cm². The samples were then subjected to denaturing PAGE (Figure 1B) and the crosslinked population was quantified by phosphorimage analysis. Psoralen crosslinked product (Tdp-ICL) was formed with high efficiency (>85%), while the amount of crosslinked product formed with the control TFO, pTFOc represented less that 1% of the population, demonstrating that pTFO1 is capable of supporting a high level of site-specific DNA ICL formation.

**Recognition of Tdp-ICL substrate by the human recombinant XPC-hHR23B protein complex**

The XPC-hHR23B complex has been proposed to be the initial damage recognition factor in GGR, although XPA and RPA have also been implicated [recently reviewed in (8)]. Since we have shown previously that the XPA and RPA NER proteins bind to Tdp-ICLs (22), we wanted to determine whether XPC-hHR23B also interacts with these lesions. To address this question, Tdp-ICLs were formed on 32P-end-labeled duplex DNA substrates of different sizes (37 and 57 bp) and incubated with increasing concentrations of human recombinant XPC-hHR23B (0.13, 1.3 and 6.5 nM). XPC-hHR23B bound both Tdp-ICL substrates, except at the lowest protein concentration (data not shown). To determine whether the binding of the Tdp-ICL by XPC-hHR23B was dependent on the triplex structure induced by the TFO, a similar binding experiment was performed on a TFO-directed psoralen ICL (following removal of the third strand TFO). The results indicated that XPC-hHR23B does bind the psoralen ICL in the absence of triplex formation, but with slightly lower affinity (data not shown).

**XPC-hHR23B binds with high specificity to a Tdp-ICL in a concentration dependent manner**

It has been reported that XPC-hHR23B interacts with both damaged and undamaged DNA (24,25,32,33). In order to ascertain specificity of XPC-hHR23B binding to ICL-damaged DNA, XPC-hHR23B’s affinity for the Tdp-ICL substrate was compared to its binding to an undamaged DNA substrate. The Tdp-ICL substrate was incubated with increasing concentrations of XPC-hHR23B (from 0 to 13 nM) and recognition of the Tdp-ICL was detected at concentrations as
low as 1.3 nM (Figure 2, lane 9), with half-maximal binding ($K_{\text{app}}$) at a concentration of 6.5 nM (Figure 2, lane 11). A separate binding titration (to saturation) experiment was also conducted by incubating increasing concentrations of XPC-hHR23B with the Tdp-ICL substrate until essentially 100% of the substrate was bound (at 65 nM XPC-hHR23B; data not shown). Throughout the range of protein concentrations tested, the amount of unbound Tdp-ICL substrate decreased with increasing concentrations of XPC-hHR23B, while the amount of unbound duplex DNA did not change. Recombinant XPC-hHR23B (at concentrations $\geq$ 6.5 nM) was observed to bind the undamaged duplex DNA substrate, however phosphorimaging analysis revealed that $\leq$ 1% of the available substrate was bound at even the highest concentration (13 nM) of XPC-hHR23B (data not shown). Separate experiments revealed that the binding profile of XPC-hHR23B to duplex DNA plus a control psoralen-modified oligonucleotide (that does not bind the duplex target site) was indistinguishable from that seen for protein incubated with duplex DNA only, as expected (data not shown). These results confirm that XPC-hHR23B specifically binds the Tdp-ICL substrate, and supports previous observations by others that XPC-hHR23B binds undamaged duplex DNA, but at a lower level than its binding to a damaged DNA substrate (24,25,32,33).

### Interactions of Human Recombinant XPC-hHR23B and XPA-RPA on Tdp-ICLs

We have previously demonstrated that both RPA and XPA can bind to the Tdp-ICL substrate used in the experiments herein and that XPA appeared to modify RPA’s interaction on these lesions (22). Unique interactions among XPC-hHR23B, TFIIH and XPA on different types of DNA damage have also been demonstrated (34–38). Thus, we were interested in the potential interactions of RPA and/or XPA with XPC-hHR23B on a Tdp-ICL substrate. In addition, we wanted to investigate whether the order in which the proteins were added to the reaction would influence any interactions of the proteins on these lesions. In these experiments the concentration of XPC-hHR23B was held constant at 6.5 nM ($K_{\text{app}}$ concentration) while increasing concentrations of RPA (0.45–23.0 nM) and/or XPA (1.6–160 nM) were added to the reaction. In other experiments, the amount of XPC-hHR23B was varied (from 0.26 to 13.0 nM) while RPA and/or XPA were held constant at concentrations of 4.5 and 160 nM, respectively. As reported previously (22), XPA alone did not appear to shift the Tdp-ICL substrate in gel-shift assays (Figure 6, lane 2). Furthermore, recognition of the Tdp-ICL substrate by XPC-hHR23B did not appear to be affected by XPA at any of the concentrations tested, regardless of when it was introduced into the reaction with XPC-hHR23B and DNA substrate (data not shown). Interestingly, RPA and XPC-hHR23B did appear to influence each other when both proteins were present in the reaction (Figure 3A and B), with a biphasic dependence on RPA concentrations. At low concentrations of RPA and XPC-hHR23B, they each form simple complexes with the

![Figure 2](image-url)  
**Figure 2.** Human recombinant XPC-hHR23B recognizes Tdp-ICLs with high specificity and affinity. The psoralen-conjugated specific TFO, pTF01, was UVA-crosslinked to a $^{32}$P-end-labeled 37 bp APRT-TA DNA target duplex to form a site-specific Tdp-ICL. Varying concentrations of human recombinant XPC-hHR23B (at concentrations of 0, 0.13, 1.3, 3.3, 6.5 and 13.0 nM) were incubated with $^{32}$P-end-labeled APRT-TA duplex (10 nM, lanes 1–6) or Tdp-ICL (10 nM, lanes 7–12) for 20 min at 30°C. DNA–protein complexes were electrophoretically separated on a 6% native PAGE in 1 x TGE buffer for 3 h at 9 mA/cm at 4°C. The gel was dried and the bands were visualized by autoradiography and quantified via phosphorimaging to determine the apparent dissociation constant ($K_{\text{app}}$).

![Figure 3](image-url)  
**Figure 3.** XPC-hHR23B and RPA interact to recognize a Tdp-ICL substrate to form a higher-order protein–DNA complex. Proteins were tested by both (A) sequential and (B) simultaneous addition to the Tdp-ICL. For these experiments XPC-hHR23B (lanes 1–5) or RPA (lanes 6–10) were held steady at $K_{\text{app}}$ concentration while the concentration of the other protein was varied. All DNA–protein reactions were incubated and separated by EMSA as described in the experimental procedures. Lane 1: XPC-hHR23B (6.5 nM) only. Lanes 2–5: XPC-hHR23B (6.5 nM) and RPA at 0.45, 4.5, 9.0 or 23.0 nM respectively. Lane 6: RPA (4.5 nM) only. Lanes 7–10: RPA (4.5 nM) and XPC-hHR23B at 0.26, 1.3, 6.5 or 13.0 nM, respectively. (*): indicates position of the higher order protein complex.
damaged DNA, and compete with one another for binding to DNA. At higher RPA concentrations (>9 nM), higher order complexes are formed, when either XPC-hHR23B or RPA is added sequentially, or when they are added simultaneously. For example, a unique higher-order complex was detected with a slower mobility than that of the XPC-hHR23B-DNA complex [indicated by an asterisk, Figure 3A and B, compare lanes 3–5] in the presence of both XPC-hHR23B and RPA. This higher-order complex was initially observed when the concentration of XPC-hHR23B was held constant (6.5 nM) and RPA was added at a concentration of 11.3 nM (*, Figure 3A and B, lane 4). At protein concentrations of 6.5 and 23.0 nM for XPC-hHR23B and RPA, respectively, the higher-order complex (*, Figure 3A and B, lane 5) was calculated to represent nearly 50% of the bound substrate. A higher-order complex was also seen when RPA was held steady at 4.5 nM and XPC-hHR23B was present at 6.5 and 11.3 nM, but under these conditions it represented only a minor amount of the total shifted substrate (*, Figure 3A and B, lanes 9 and 10). These results were observed regardless of whether the proteins were added to the reaction sequentially (Figure 3A) or simultaneously (Figure 3B). When the experiments described above were performed with XPC-hHR23B and the XPA-RPA complex (rather than RPA alone), the results were indistinguishable from those presented in Figure 3.

Since the observations made in Figure 3 were based on changes in the protein concentration relative to each component’s K_app concentration, the binding of these proteins to the Tdp-ICL substrate was also tested at equimolar concentrations. Proteins were added (in 10-fold increments) at concentrations ranging from 0.01 to 10 nM and allowed to incubate with the damaged DNA substrate. Similar to the results shown in Figure 3, the higher-order complex was only observed in the presence of high concentrations of RPA (10 nM) (data not shown).

Kinetic analysis of XPC-hHR23B and XPA-RPA recognition of a Tdp-ICL substrate

In order to determine the kinetic properties of XPC-hHR23B with XPA and RPA on Tdp-ICLs, RPA (4.5 nM) and XPC-hHR23B (6.5 nM) were incubated, independently or together, for varying lengths of time with the radiolabeled Tdp-ICL substrate. As shown in Figure 4, the results indicate that RPA and XPC-hHR23B both interact with the damaged DNA substrate rapidly, with apparent half-maximal binding being achieved in less than four minutes for both XPC-hHR23B (Figure 4, lane 7) and RPA (Figure 4, lane 4). XPC-hHR23B bound ~25% of the available damaged substrate even at the <10 s time point (Figure 4, lane 5), compared to RPA which bound <5% (Figure 4, lane 1). These results suggest that XPC-hHR23B can bind to the Tdp-ICL substrate more rapidly than is detectable by this assay. As expected, based on the results shown in Figure 3, these concentrations of RPA (4.5 nM) and XPC-hHR23B (6.5 nM) resulted in only a minor formation of the higher-order product, as seen by the presence of a faint band migrating above damaged DNA substrate bound by XPC-hHR23B alone (Figure 4, lanes 9–12). Surprisingly, incubation of both proteins with the DNA substrate resulted in a slight increase in complex formation at the <10 s time-point, compared to the amount of substrate bound when incubated with the individual components (Figure 4, compare lane 9 to lanes 1 and 5). The mechanism of this interaction is not clear and further investigations using techniques with better time resolution are warranted.

XPC-hHR23B and XPA-RPA interact in the recognition of Tdp-ICLs

The higher-order complex seen in Figure 3 migrates in the gel at a position above that seen for either RPA or XPC-hHR23B when bound to the Tdp-ICL substrate. The presence of both XPC-hHR23B and RPA was confirmed by antibody super-shift assays using monoclonal antibodies directed against the 34 kDa subunit of RPA or to the maltose-binding protein tag present on the purified human recombinant XPC-hHR23B protein complex. Both the anti-RPA34 and anti-MBP antibodies were able to super-shift essentially 100% of the RPA-DNA or XPC-hHR23B-DNA complex (Figure 5, lanes 2 and 4, respectively). The presence of both XPC-hHR23B (at 6.5 nM) and RPA, at both low (4.5 nM; Figure 5, lanes 5–8) and high

Figure 4. XPC-hHR23B demonstrates rapid binding to a Tdp-ICL substrate. RPA (4.5 nM, lanes 1–4), XPC-hHR23B (6.5 nM, lanes 5–8) or both proteins (Lanes 9–12) were incubated with the Tdp-ICL substrate (10 nM) and subjected to EMSA analysis. Incubation times were carried out for <10 s (lanes 1, 5 and 9), 1 (lanes 2, 6 and 10), 2 (lanes 3, 7 and 11) or 4 min (lanes 4, 8 and 12).
(23.0 nM; Figure 5, lanes 9–12) concentrations, on the Tdp-ICL substrate (10 nM) and protein, with or without monoclonal antibodies specific for the 34 kDa subunit of RPA or the MBP tag on the human recombinant XPC-hHR23B protein complex. All conditions were carried out as described in the experimental procedures. RPA: 4.5 nM (lanes 1, 2, 5–8) or 23 nM (lanes 9–12). XPC-hHR23B: 6.5 nM (lanes 3–12). Anti-RPA34: 1 µg (lanes 2, 7, 8, 11, 12). Anti-MBP: 1 µg (lanes 4, 6, 8, 10, 12). †: RPA-Tdp-ICL super-shifted complex also migrated at the same position as when Tdp-ICL was bound by XPC-hHR23B. (*, #1, #2 and #3): see text for explanation.

**DISCUSSION**

While unrepaired DNA damage threatens cell survival and proliferation, repair can introduce deleterious mutations.
The nature and frequency of these mutations are functions of the type of damage and the repair mechanisms that operate on them. DNA repair pathways are conveniently categorized according to the types of lesions with which they are associated, but there is growing evidence that there is considerable overlap and ‘repair crosstalk’ between these pathways [recently reviewed in (39)].

For example, both HR and NER participate in the removal of DNA ICLs in bacteria and yeast (10–12,40), however the mechanism of ICL repair in mammalian cells is not clear. Liu et al. (41) demonstrated that repair of mammalian DNA ICLs required members of the HR repair pathway since cells deficient in some of these proteins are highly susceptible to DNA ICLs in bacteria and yeast (10–12,40). In addition, Wang et al. (44) demonstrated that constituents of the NER pathway facilitate the repair of ICLs in mammalian cells, but that the process occurred in a recombination-independent, error-generating fashion. Many different lines of evidence implicate the XPC-hHR23B protein complex as the primary initiating factor for DNA damage recognition in the GGR sub-pathway of NER [reviewed in (8) and references therein].

A number of studies have demonstrated that the XPC-hHR23B protein complex is the first to bind damaged DNA and that XPA is not localized to DNA damage unless XPC-hHR23B is already present at the site of the lesion (24,45,46). The findings from these studies implicate XPC-hHR23B as a crucial component needed to initiate the repair of DNA damage that falls within the purview of the GGR pathway.

The XPA-RPA complex was previously observed to recognize psoralen-crosslinked triplex DNA substrates (22). In the work described here, we have presented the first biochemical evidence that XPC-hHR23B recognizes psoralen-crosslinked triplex DNA (Tdp-ICL) with high specificity and affinity (Figure 2). The recognition of the Tdp-ICL by XPC-hHR23B occurred within seconds of encountering the substrate, suggesting a very fast mechanism of recognition (Figure 4). A novel finding in the work presented here was that XPC-hHR23B (6.5 nM) and XPA (160 nM)-RPA (at concentrations >9 nM) were able to form a unique ternary complex (Figure 3) on the Tdp-ICL substrate, confirmed by antibody super-shift assay (Figure 5). Notably, incubation of both proteins with antibodies specific to either human recombinant XPC-hHR23B or RPA resulted in a complete loss of the higher-order complex (*, Figure 5) and the formation of a super-shifted complex with a lower mobility (●, Figure 5, lanes 8 and 12) than was observed with incubation of the higher-order complex with either antibody alone. The observed antibody-antigen specificity strongly suggests that the uppermost super-shifted complex is indeed a complex of XPC-hHR23B-XPA-RPA on the damaged DNA substrate, representing a possible positive interaction between these proteins. These results implicate the GGR sub-pathway of NER as a possible mechanism for the removal of DNA ICLs in mammalian genomes. Although no direct protein–protein interaction has been demonstrated between XPC-hHR23B and RPA, it has been proposed that binding of XPC-hHR23B near a site of the lesion (24,47). Consistent with these findings, Reardon and Sancar (48,49) recently proposed that cooperation between NER pre-incision proteins (XPC-hHR23B, RPA and XPA) plus a kinetic proofreading function, supplied by TFIH, could explain the ability of the NER pathway to detect and repair thymine dimers. They further hypothesized that increased specificity is accomplished through the interplay of proteins on neighboring or overlapping regions of DNA.

Experiments using XPC-hHR23B and XPA (without RPA) demonstrated that XPA by itself was not enough to influence the recognition of the Tdp-ICL substrate by XPC-hHR23B (Figure 6, lane 4). The interaction of XPC-hHR23B with RPA (in the presence or absence of XPA) on ICL-containing substrates was also seen in experiments involving combinations of XPC-hHR23B, XPA and RPA at different concentrations (Figure 6). Interestingly, our results differ from those reported by You et al. (34) who showed that XPC-hHR23B physically interacted with XPA but that addition of both XPA and RPA displaced XPC-hHR23B from a substrate containing a cis-platin intrastrand crosslink. In our work, XPC-hHR23B and RPA competed for binding to the lesion at low concentrations of RPA but formed a complex on the damaged DNA including both XPC-hHR23B and XPA-RPA at high concentrations of RPA; the concentrations of RPA required are not inconsistent with a physiological role for this complex, given the high levels of RPA in the nucleus. It is possible that the types of complexes formed on the intrastrand crosslinks studied by You et al. (34) may differ from those formed on the ICLs studied here.

Wakasugi and Sancar (28) performed an experiment similar to ours using high concentrations of RPA (100 nM) with XPC (37 nM) on a single (6–4) photoproduct, but did not observe any evidence of a positive interaction between these proteins. Some possible reasons for this apparent discrepancy can be given. First, the lack of a higher-order complex could be due to their use of a substrate containing a different type of lesion, the (6–4) photoproduct. Second, they used XPC at a concentration that was <3-fold below that of RPA. In our experiments, formation of the ternary complex was most favorable when there was a 5-fold difference between the protein concentrations, although moderate formation of the higher-order complex could be seen with as low as a 3-fold difference in protein concentrations (*, Figure 3A and B, compare lanes 3 and 5).

There have been a number of studies that have confirmed the formation of an XPC-RPA-DNA complex (22,28,34,50,51), but to our knowledge, no one has reported the formation of a ternary complex between ICL-damaged DNA, XPC-hHR23B, and RPA. The cellular concentrations of XPC, RPA and XPA have been estimated at $4\times10^4$ (52), $2.4\times10^5$ (53) and $5\times10^4$ (54) molecules per cell, respectively. We believe our experimental conditions reflect the presumed ratio of these NER factors in eukaryotic cells, thus supporting the possibility of an in vivo cooperative interaction between XPC-hHR23B and XPA-RPA in the recognition of psoralen-ICLs. The abundance of RPA suggests that it may play a role in determining the sensitivity of cells to DNA damage.
ACKNOWLEDGEMENTS

We thank Dr Theodore G. Wensel and Dr Rick A. Finch for advice and critical reading of the manuscript. We thank Sarah Henninger for her assistance in preparing this manuscript. This work is dedicated to the memory of Dr Peter Snow. This work was supported by a NCI Training Grant CA09480 (to BST) and NIH/NCI Grants CA93729 and CA97175 (to KMV). Funding to pay the Open Access publication charges for this article was provided by NIH/NCI Grant CA93729.

Conflict of interest statement. None declared.

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