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Human HMGB1 directly facilitates interactions between nucleotide excision repair proteins on triplex-directed psoralen interstrand crosslinks

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Abstract

Psoralen is a chemotherapeutic agent that acts by producing DNA interstrand crosslinks (ICLs), which are especially cytotoxic and mutagenic because their complex chemical nature makes them difficult to repair. Proteins from multiple repair pathways, including nucleotide excision repair (NER), are involved in their removal in mammalian cells, but the exact nature of their repair is poorly understood. We have shown previously that HMGB1, a protein involved in chromatin structure, transcriptional regulation, and inflammation, can bind cooperatively to triplex-directed psoralen ICLs with RPA, and that mammalian cells lacking HMGB1 are hyper-sensitive to psoralen ICLs. However, whether this effect is mediated by a role for HMGB1 in DNA damage recognition is still unknown. Given HMGB1's ability to bind to damaged DNA and its interaction with the RPA protein, we hypothesized that HMGB1 works together with the NER damage recognition proteins to aid in the removal of ICLs. We show here that HMGB1 is capable of binding to triplex-directed psoralen ICLs with the dedicated NER damage recognition complex XPC-RAD23B, as well as RPA, and that they form a high molecular weight complex on these lesions. In addition, we demonstrate that HMGB1 interacts with XPC-RAD23B and XPA in the absence of DNA. These findings directly demonstrate interactions between HMGB1 and the NER damage recognition proteins, and suggest that HMGB1 may affect ICL repair by enhancing the interactions between NER damage recognition factors.

INTRODUCTION

DNA repair is a critical cellular function that maintains genomic stability and prevents mutations that can lead to carcinogenesis. The majority of DNA lesions are repaired by either nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), or double strand break repair (DSBR) [1]. However, there is a group of DNA damaging agents that produce lesions that are too complex to be processed by any one of these mechanisms. Agents that cause DNA interstrand crosslinks (ICLs), a very detrimental covalent linkage between the two strands of DNA, represent such a group [2]. The chemotherapeutic agent psoralen is a canonical example of this type of DNA damaging agent. Psoralen DNA ICLs form preferentially at 5'-TpA-3' and 5'-ApT-3' sites in the DNA upon absorption of 2 photons

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of UVA irradiation at 365 nm [3]. Psoralen + UVA (PUVA) is used to treat psoriasis, atopic dermatitis, vitiligo, and cutaneous T cell lymphoma, but due to its formation of ICLs in the DNA, this treatment has been associated with increased risk of squamous and basal cell carcinomas [4]. Despite the obvious clinical significance of these drugs, the mechanism of repair of ICLs is poorly understood in mammals, although it is known to involve proteins from NER [5], MMR [6,7], BER [8,9] and DSBR [10], as well as translesion synthesis (TLS), which is a DNA damage tolerance system [11,12]. Proteins from these repair mechanisms can interact in several different ways to recognize and process ICLs, likely dependent on parameters such as cell cycle status [13,14]. The involvement of some of these pathways, such as NER and TLS, allows errors to occur during the repair process [15–17], whereas others (MMR and DSBR) have been shown to minimize these errors [6,18]. The manner in which proteins from these pathways work together, or which is chosen under what circumstances, has yet to be determined, although multiple models have been proposed [14,19,20]. We and others have shown that both NER and MMR damage recognition proteins are able to bind selectively to psoralen ICLs and signal for repair [5,6,21]. Understanding the proteins involved in ICL repair, and their functions, will allow more effective use of this chemotherapeutic agent.

Another protein that is capable of binding to psoralen ICLs is the high mobility group protein B1 [HMGB1 [22]]. HMGB1 is a multi-functional protein that mediates a number of processes, both inside and outside the cell. It is involved in transcriptional regulation [23], V(D)J recombination [24], chromatin remodeling [25] and inflammation [26], and can also bind to DNA lesions, such as those induced by cisplatin [27,28], ultraviolet radiation [UV; [29,30]], acetyl aminofluorene (AAF) and benzo/*a*/pyrene diol epoxide [BPDE; [30]]. We have shown that HMGB1 can bind to triplex-directed psoralen ICLs [22] cooperatively with the NER damage recognition/verification complex XPA-RPA. In addition, we have demonstrated that HMGB1-deficient cell lines are hypersensitive to psoralen ICLs, both in terms of mutagenicity and cell survival [31]. However, knowledge of whether the cellular phenotypes observed in HMGB1 knockout cell lines are a result of modifications to chromatin structure, or of direct action of HMGB1 at the lesion, or both, is still lacking.

To study the associations of HMGB1 with the NER damage recognition factors, we induced site-specific psoralen ICLs using triplex technology. This method employs single-stranded triplex-forming oligonucleotides (TFOs) that can bind with sequence specificity to sites in the major groove of purine-rich DNA duplex sequences, forming a three-stranded DNA structure (a triple helix). By conjugating a psoralen molecule to the 5' end of a TFO, a psoralen ICL can be targeted to a single, specific site in the DNA [32]. Here we demonstrate that HMGB1 can enhance the interactions between NER damage recognition proteins on psoralen ICLs. This work establishes HMGB1 as a potential facilitator of DNA damage recognition in the NER mechanism.

MATERIALS AND METHODS

Cell lines

HeLa CCL4 cells were obtained from the American Type Culture Collection (Manassas, VA), and were grown in EMEM (BioWhittaker, Walkersville, MD) with 10% FBS (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Invitrogen). XPA12RO clone 12 cells (SV40-immortalized human XPA fibroblasts complemented with the XPA gene) were obtained from Dr. Richard Wood (UT MD Anderson Cancer Center, Science Park Research Division), and were cultured as described in Koberle *et al.* [33].

Oligonucleotides

The 57-bp synthetic duplex target for TFO binding from the pSupFG1 triplex target site was constructed as described previously [5,21,22]. TFOs were synthesized with a 5'-psoralen derivative [2-[4'-hydroxymethyl)-4,5', 8-trimethylpsoralen] hexyl-1-*O*-(2-cyanoethyl)(*N*, *N*-diisopropyl) phosphoramidite (HMT)] and a 3'-propanolamine by the Midland Certified Reagent Company, Inc. (Midland, TX). Complementary synthetic single-stranded oligonucleotides corresponding to the pSupFG1 sequence were annealed at a 1:1 molar ratio to form the synthetic target duplex. Duplexes were 5'-end-labeled by transfer of ^{32}P from [^{32}P]dATP with T4 polynucleotide kinase, purified by 12% polyacrylamide gel electrophoresis (PAGE), electroeluted, and concentrated by using Centricon centrifugal filter devices (Millipore, Bedford, MA).

TFO-Directed Psoralen ICL Formation

Crosslinked triplex structures were generated by incubating radiolabeled duplexes with psoralen-conjugated TFOs (pAG30) in a triplex binding buffer [10 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 , and 10% (vol/vol) glycerol] at 37°C for 16 h. Samples were then irradiated with 18 kJ/m² UVA light at 365 nm to produce site-specific psoralen ICLs. Efficiency of cross-linking was determined by denaturing PAGE and quantified using a phosphorImager. ICLs were formed in these substrates at the targeted triplex-duplex junction with up to 90% efficiency (data not shown). The triplex-directed ICLs were purified by 12% PAGE, electroeluted, and concentrated using Centricon centrifugal filter devices (Millipore, Bedford, MA).

Expression and Purification of Recombinant Proteins

Histidine-tagged full-length human HMGB1 was expressed in recombinant baculovirus in Sf9 cells and purified by phosphocellulose chromatography as previously described [34,35]. Purified recombinant rat CBP-HMGB1 protein was expressed in *E. coli* using the expression vector pCaln-rHMGB1 [26], kindly provided by Dr. Kevin Tracey (North Shore-Long Island Jewish Research Institute, Manhasset, New York). This protein was purified as specified by Li *et al.* [36]. As in Christensen *et al.* [37], the three-subunit histidine-tagged RPA complex was expressed by co-infection of Sf9 cells at a multiplicity of infection (MOI) of 5 for His-RPA1 and RPA2 baculoviruses and an MOI of 10 for the RPA3 baculovirus. The infected cells were lysed and the RPA complex was purified by Ni^{2+} -chelate chromatography, then further purified by salt gradient elution from a Mono-Q FPLC column as previously described [38]. The MBP-XPC-RAD23B human recombinant protein was expressed in insect Sf9 cells, and purified as previously described [39,40]. The human recombinant XPA-MBP tagged protein was expressed from *E. coli* PR745 cells and purified as in Li *et al.* [41].

Protein-DNA binding assays and antibody super-shift assay

Protein-DNA binding interactions were analyzed by electrophoretic mobility shift assays (EMSAs). Human purified recombinant proteins at a concentration of $\sim 1 \times 10^{-8}$ M (unless otherwise indicated) were incubated in binding buffer [25 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM DTT, 5 mM EDTA, 100 $\mu\text{g}/\text{ml}$ BSA, 0.01% NP-40 (v/v) and 10% glycerol (v/v)] in a 20 μl reaction volume for 10 min at room temperature. Then $\sim 1 \times 10^{-8}$ M radiolabeled duplex or triplex substrates were added and incubated at 30°C for 20 min. For antibody super-shift assays monoclonal antibodies directed against RPA (anti-RPA34; Lab Vision, Fremont, CA), or HMGB1 (anti-HMGB1; BD Pharmingen) or the maltose-binding protein tag on the human recombinant XPC-RAD23B (anti-MBP; New England Biolabs, Beverly, MA) were added to the protein-DNA complexes (1 $\mu\text{g}/20 \mu\text{l}$ reaction) and incubated at 30°C for 10 min. The samples were then electrophoresed on a 6% (37.5:1 acrylamide/bis-acrylamide) native PAGE gel, containing 2.5% glycerol and buffered in 1x TGE (25 mM Tris-HCl, 192 mM glycine and 1 mM EDTA). Electrophoresis was conducted at 4°C, at 170V for 3 h. Gels were

dried and protein-DNA complexes were visualized by autoradiography and quantified using a phosphorImager.

Southwestern Blot Analysis

The protein-DNA complexes were incubated and electrophoresed as for EMSA analysis. Gels were visualized by autoradiography, and then the samples were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Hoefer, San Francisco, CA). The membrane was probed with an antibody specific to the 70 kDa subunit of RPA (NeoMarkers, Fremont, CA), at a 1:1000 dilution.

Detection of proteins in protein-DNA complexes by immunoblotting

The protein-DNA complexes were separated by native PAGE and corresponding bands were excised, electroeluted, and concentrated using Centricon centrifugation filter units according to manufacturer's instructions (Millipore, Bedford, MA). Concentrated proteins were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with antibodies against XPC (Abcam, Cambridge, CA, 1:500 dilution), HMGB1 (Stressgen, San Diego, CA, 1:1000 dilution), or a mixture of monoclonal antibodies against the three RPA subunits (p70 and p34 from NeoMarkers, and p14 from GeneTex, Inc, San Antonio, TX).

Affinity Pulldown and Immunoprecipitation Assays

750 ng of purified CBP-HMGB1 was incubated with calmodulin resin (Stratagene, La Jolla, CA) for 1 hour, followed by a 2 hour incubation with either 500 ng purified XPC-RAD23B, XPA and RPA, or with 250 µg cell extracts from the XP12RO clone 12 cell lines [prepared using the Manley protocol [42]]. The samples were washed five times with calmodulin buffer for CBP-HMGB1 bound with purified proteins (10 mM Tris pH 7.6, 500 mM NaCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM imidazole, 0.1% NP-40, 10% glycerol, protease inhibitors), or with CaCl₂ binding buffer for CBP-HMGB1 with cell extracts (50 mM Tris pH 7.8, 150 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM Na acetate, 1 mM imidazole, 2 mM CaCl₂, protease inhibitors), or with NET-NE buffer for MBP-XPA with purified proteins (50 mM Tris pH 7.8, 100 mM NaCl, 6 mM EDTA, 6 mM EGTA, 0.5% NP-40, 1 mM DTT, protease inhibitors), or with NET-N buffer for MBP-XPC with cell extracts (50 mM Tris pH 7.8, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% NP-40, 1 mM DTT, protease inhibitors). The bound proteins were eluted in SDS-PAGE loading buffer at 95°C for 5 minutes. The eluted proteins were separated by SDS-PAGE and transferred to a PVDF membrane, which were then blotted with antibodies specific for HMGB1 (Abcam, 1:500 dilution), XPC (antibody RW028, gift from Richard Wood, UT MD Anderson Cancer Center, Smithville, TX), XPA (Invitrogen 1:1500 dilution), RPA 34 kDa subunit (Invitrogen, 1:500 dilution) or RPA 70 kDa subunit (Invitrogen, 1:500 dilution). The immunoprecipitations were carried out using the Pierce ProFound Coimmunoprecipitation kit (Rockford, IL) according to manufacturers instructions, with an antibody specific for the HMGB1 protein (Abcam), and 200 µg HeLa cell extracts (prepared using the Manley protocol). The eluted proteins were analyzed by Western blotting, as described above.

RESULTS

HMGB1 Binds Psoralen ICLs with XPC-RAD23B

One possible function of HMGB1 at the site of a psoralen ICL would be to directly interact with NER damage recognition proteins. To test this hypothesis, we performed competition experiments between purified human recombinant HMGB1 and the dedicated NER damage recognition protein complex XPC-RAD23B using electrophoretic mobility shift assays (EMSAs). We found that XPC-RAD23B bound the triplex-directed psoralen ICL lesion

(Figure 1, lane 3), as did HMGB1 (Figure 1, lane 4), as we demonstrated previously [21,22]. However, when both proteins were incubated together with the triplex-directed psoralen ICL, a slower-migrating complex was detected, suggesting that the two proteins are capable of binding the lesion together (Figure 1, lane 5). When HMGB1 binds the DNA substrate, the mobility shift is diffuse, rather than a single discrete band. This suggests that HMGB1 can bind to triplex-directed psoralen ICLs as a multimer and/or in multiple orientations. In contrast, when XPC-RAD23B is present in the reaction, a discrete ternary complex is formed, containing HMGB1, XPC-RAD23B, and psoralen-crosslinked DNA. A similar pattern was seen when HMGB1 bound the lesions with RPA [22], suggesting that in the presence of the NER damage recognition factors, HMGB1 binds to the lesions as a monomer and/or with a more uniform orientation. An additional reduction in mobility was observed when the two proteins and DNA substrate were incubated in the presence of an MBP antibody, which targets the MBP tag on XPC, demonstrating the presence of XPC in the complex (Figure 1, compare lanes 5 and 9). A less discrete, but reproducible, shift was also seen when the complex was incubated with an antibody against HMGB1 (Figure 1, compare lanes 5 and 13), demonstrating that both HMGB1 and XPC bind together on triplex-directed psoralen ICLs.

HMGB1 Binds Psoralen ICLs in the Presence of the NER Damage Recognition Proteins, XPA-RPA and XPC-RAD23B

The XPA-RPA protein complex also binds to DNA lesions during the NER reaction. Because of this, we wanted to determine if XPC-RAD23B together with XPA-RPA could bind to these DNA lesions in the presence of HMGB1. RPA, XPC-RAD23B, and HMGB1 were all individually capable of binding to the triplex-directed psoralen ICL (Figure 2, lanes 3, 5 and 10), and the DNA-protein complexes were super-shifted by their cognate antibodies (Figure 2, lanes 4, 6 and 11). When RPA and XPC-RAD23B were incubated together at their K_d concentrations, they formed discrete complexes (Figure 2, lane 7), as we have demonstrated previously [21,40]. These proteins are capable of forming a ternary complex on triplex-directed psoralen ICLs, but this only occurs at high protein concentrations [21]. Significantly, when XPC-RAD23B and RPA were incubated in the presence of HMGB1, even at low protein concentrations, a single high molecular weight complex was formed, which led to the complete loss of the discrete single protein-DNA bands (Figure 2, lane 12). This complex could be further super-shifted with an antibody against the MBP tag on XPC (Figure 2, lane 14), and partially by an anti-HMGB1 antibody (Figure 2, lane 15), demonstrating that HMGB1, XPC, and possibly RPA could form a discrete complex on triplex-directed psoralen ICLs, at K_d concentrations. The same pattern was observed in the presence of the XPA protein (data not shown).

RPA and XPC-RAD23B Bind Psoralen ICLs with HMGB1

To determine whether the NER damage recognition proteins (i.e. RPA and XPC-RAD23B) and HMGB1 were all present in the high molecular weight complex formed with ICLs, we attempted to super-shift the protein-DNA complexes with specific antibodies against the individual proteins (Figures 1, 2). This was only partially successful, likely due to antibody epitope sites being blocked by the formation of the complexes. To determine if RPA was present in the high molecular weight complex seen in Figure 2 (lane 12), further EMSA analysis was performed. This showed that a protein-DNA complex formed in the presence of HMGB1, XPC-RAD23B, and RPA with a triplex-directed psoralen ICL (Figure 3A, lane 5) had a slightly lower mobility than the complex containing the damaged substrate and XPC-RAD23B and HMGB1 only (Figure 3A, lane 3). By contrast, both of these complexes had a lower mobility than was observed with the triplex-directed psoralen ICL, HMGB1, and RPA in the absence of XPC-RAD23B (Figure 3A lane 1). Indeed, RPA was present in complexes formed with the ICL-damaged DNA that included HMGB1 and XPC-RAD23B, as confirmed by southwestern analyses (Figure 3A, lower panel).

To further confirm the presence of all three proteins in the shifted complex, we extracted bands from an EMSA gel (Figure 3B), subjected the samples to SDS-PAGE, and conducted immunoblotting assays to identify the proteins present in the protein-DNA complexes (Figure 3C). The presence of HMGB1, RPA, and XPC was detected (Figure 3C, lane 4) in the band corresponding to the high molecular weight protein-DNA complex (Figure 3B, lane 5). The proteins were also incubated in the absence of DNA, subjected to native PAGE, extracted from the gel at the location corresponding to the DNA-protein complex, and immunoblotted. The proteins were not detected at this position in the absence of DNA (data not shown), demonstrating that HMGB1, XPC-RAD23B, and RPA together form a complex on a triplex-directed psoralen ICL.

Order-of-Addition Does Not Affect HMGB1-XPC-RPA Binding to Psoralen ICLs

To determine if there was a competitive interaction between these damage recognition factors, or if there was a change in the interaction depending upon which protein bound first, we conducted order-of-addition experiments. Addition of XPC-RAD23B and RPA to a pre-formed complex of HMGB1 and a psoralen ICL resulted in the formation of a higher molecular weight complex (similar to that shown in Figure 2), and led to a complete loss of the band corresponding to the HMGB1-DNA complex (Figure 4A). Similarly, addition of HMGB1 to pre-formed complexes of RPA, XPC-RAD23B, and ICLs led to the loss of the discrete bands corresponding to the RPA-DNA complex and the XPC-RAD23B-DNA complex (Figure 4B). At higher protein concentrations, no new or different complexes were detected. These results demonstrate that similar complexes were formed with XPC-RAD23B, RPA, and HMGB1 on psoralen ICLs regardless of the order of addition of the proteins to the DNA lesion.

HMGB1, XPC-RAD23B, and XPA Interact in the Absence of DNA

The above experiments demonstrated that HMGB1, XPC-RAD23B, and XPA-RPA interact in the presence of triplex-directed psoralen-damaged DNA. To determine whether these proteins are capable of interacting with each other in the absence of damaged DNA, we conducted affinity pull-down experiments using tags on the purified proteins. While these assays are not meant to provide information regarding the stoichiometry of the interactions between these proteins, they do allow assessment of protein-protein interactions. Using CBP-tagged HMGB1 bound to calmodulin beads incubated in cell extracts from normal human fibroblasts, we observed an interaction between XPC-RAD23B and HMGB1 (Figure 5A). CBP-HMGB1 was also able to interact with purified XPC-RAD23B (Figure 5B). In the reciprocal experiment (MBP-tagged XPC bound to amylose resin), we demonstrated an interaction between XPC-RAD23B and HMGB1 in cell extracts (Figure 5C). Using purified proteins and MBP-tagged XPA protein, we confirmed the known association between XPA and RPA, and identified a new interaction between XPA-RPA and HMGB1 (Figure 5D). By performing immunoprecipitation experiments with HeLa cell extracts, HMGB1 was demonstrated to associate with the XPA protein (Figure 5E). These results suggest that HMGB1 may be acting as a bridging factor at the DNA lesion, helping to bring together these different damage recognition proteins.

DISCUSSION

We have previously demonstrated that HMGB1 is capable of binding to psoralen ICLs cooperatively with RPA [22], and that HMGB1-deficient cells are sensitive to psoralen + UVA irradiation [31]. Here we identified a novel interaction of HMGB1 with XPC-RAD23B, a protein exclusively involved in damage recognition in the NER mechanism, on triplex-directed psoralen ICLs. In addition, HMGB1 facilitated the interactions of XPC-RAD23B and XPA-RPA on the damaged DNA substrate, an interaction that is not seen in the absence of HMGB1. HMGB1 was also capable of binding to XPC-RAD23B and XPA in the absence of DNA, and

this was demonstrated with purified proteins as well as with endogenous proteins from cell extracts. The data presented here provide a mechanistic understanding of our previous work demonstrating that cells lacking HMGB1 are sensitive to psoralen + UVA treatment [31]. Results from the affinity pulldown and immunoprecipitation experiments suggest that HMGB1, XPC-RAD23B, and XPA are capable of interacting with one another, providing support for the hypothesis that these proteins can act together to bind to these DNA lesions. Given this data in addition to our previous findings, we suggest that HMGB1 is acting to enhance the initial recognition of triplex-directed psoralen ICLs.

We were not able to show protein interactions on triplex structures alone, because the DNA triplex structure was not stable under the conditions of our assays. However, we can infer that the structure of the triplex can enhance the recognition of these DNA damage-binding factors. The evidence for this comes from our previously published work showing that HMGB1 [22], XPC-RAD23B [21], and RPA [5] are all capable of binding to psoralen ICLs alone (in the absence of the triplex structure), with binding affinities that are reduced by approximately 10-fold. Thus, the triplex structure itself enhances the recognition of a psoralen ICL, and presumably its downstream processing.

The first step in the NER pathway, DNA damage recognition, is thought to be rate limiting, and is still poorly understood despite the large amount of work that has been done to determine the exact step-by-step coordination of proteins on DNA lesions [40,43,44]. At one time it was debated whether the XPA-RPA protein complex [40,45], or the XPC-RAD23B protein complex [43,46] is the first to bind DNA lesions. However, Volker *et al.* [43] showed conclusively in 2001 that XPC-RAD23B was required for colocalization of all other NER factors to UV irradiation-induced DNA lesions. The XPA and RPA proteins are now thought to be components of the DNA damage verification/pre-incision complex [47], but the order and exact function of these early-acting repair factors is still under debate. We have provided one of the few demonstrations of all three of the NER damage recognition/verification factors binding together to a DNA lesion (a triplex-directed psoralen ICL), although this only occurs at protein concentrations above their dissociation constants for this particular DNA lesion in the absence of HMGB1 [21]. Other studies have shown that the XPC-RAD23B and RPA proteins cannot bind to a DNA lesion at the same time [48], and that they do not act together to facilitate DNA damage incision [40]. Here we showed that RPA and XPC-RAD23B were capable of binding together on a triplex-directed psoralen ICL at K_d concentrations in the presence of HMGB1. This suggests that other “non-DNA repair proteins” may play a role in the NER process in a cellular context, an association that cannot be detected in purified, *in vitro* repair systems. We also demonstrated that HMGB1, XPC-RAD23B, and XPA were capable of interacting in the absence of DNA. It has been demonstrated previously that, in the absence of DNA, there are certain core NER factors that are capable of interacting in a defined manner [49]. TFIIH is a central player in this core of proteins, with XPA, XPC-RAD23B, and the 3' NER endonuclease XPG interacting directly with it. The 5' NER endonuclease, XPF-ERCC1, binds more weakly through an association with the XPA protein, and RPA does not interact with this complex at all in the absence of DNA. Given our data, it is possible that HMGB1 may also be a player in this complex, enhancing the recruitment of the NER apparatus to sites of DNA ICLs. It is known that HMGB1 has an affinity for damaged DNA, and that once it binds to the lesion, it induces a further bend in the DNA [28]. This increased distortion in the DNA could enhance the recognition of the site by the DNA damage recognition proteins, because these proteins generally bind more robustly to DNA that is more highly distorted [50,51]. In addition, HMGB1 could facilitate the binding of these proteins as a complex to the lesion, and therefore increase the kinetics of this rate-limiting step.

The significance of this work is two-fold. Psoralen is used to treat several types of skin diseases, but in the long-term this treatment can have carcinogenic outcomes. Other ICL-producing

agents are also used as chemotherapeutic agents, such as mitomycin C and cisplatin, both of which are used as treatments for cancer. These agents can also have detrimental long-term consequences. A more thorough understanding of these agents and the repair of the ICLs they induce will lead to an increase in the efficacy of these treatments, as well as a moderation of their carcinogenic consequences. Any agent, such as HMGB1, that increases the repair of DNA lesions acts to protect against mutations that can lead to carcinogenesis. This is particularly relevant given that another role of HMGB1 is as a pro-inflammatory and pathogenic factor in diseases such as sepsis and rheumatoid arthritis [26,52]. Because of this role, anti-HMGB1 therapies are being developed to treat these diseases. However, if HMGB1 is acting to enhance the activity of DNA damage recognition proteins, as our data suggests, then removing the protein could have long-term consequences on DNA repair, and therefore on carcinogenesis. For this reason it is critical to understand the functions of the HMGB1 protein, and how using it as a therapeutic target could affect its other roles in the cell.

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Figure 1. Interactions of human HMGB1 and XPC-RAD23B proteins on triplex-directed psoralen ICLs

Human purified recombinant HMGB1 and XPC-RAD23B proteins ($\sim 1 \times 10^{-8}$ M) were incubated with $\sim 1 \times 10^{-8}$ M radiolabeled triplex-directed psoralen ICLs in binding buffer for 20 min at 30°C. Antibodies were added to indicated samples and incubation was continued for an additional 20 min. Protein-DNA complexes were subjected to EMSA on a 6% native PAGE gel and visualized by autoradiography. Lane 1 contains non-damaged duplex DNA, and the remaining lanes contain triplex-directed psoralen ICLs (Tdp-ICL substrate).

Figure 2. Binding of human purified recombinant HMGB1, XPC-RAD23B, and RPA proteins on triplex-directed psoralen ICLs

Human purified recombinant HMGB1, XPC-RAD23B, and RPA proteins ($\sim 1 \times 10^{-8}$ M) were incubated with $\sim 1 \times 10^{-8}$ M radiolabeled triplex-directed psoralen ICLs in binding buffer for 20 min at 30°C. Antibodies were added to the indicated samples and incubation was continued for an additional 20 minutes. Protein-DNA complexes were subjected to EMSA on a 6% native PAGE gel and visualized by autoradiography. Lane 1 contains non-damaged duplex DNA, and the remaining lanes contain triplex-directed psoralen ICLs (Tdp-ICL substrate).

Figure 3. Identification of proteins in the protein-DNA complexes

(A) Human purified recombinant HMGB1, XPC-RAD23B, and RPA proteins ($\sim 1 \times 10^{-8}$ M) were incubated with $\sim 1 \times 10^{-8}$ M radiolabeled triplex-directed psoralen ICLs (lanes 1, 3 and 5), or in the absence of DNA (lanes 2, 4 and 6) in binding buffer for 20 min at 30°C. Protein-DNA complexes were subjected to EMSA on a 6% native PAGE gel and visualized by autoradiography, followed by semi-dry blotting to transfer the protein-DNA complexes to a membrane. The membrane was then blotted with an α -RPA-70 antibody; (B, C) Human purified recombinant HMGB1 (lane 1), XPC-RAD23B (lane 2) and RPA (lane 3) were incubated separately, or together (lane 4), on a triplex-directed psoralen ICL substrate for 20 min at 30°C, followed by EMSA analysis using a 6% native PAGE gel (B). The electrophoretic bands

corresponding to the protein-DNA complexes were excised, subjected to SDS-PAGE, and immunoblotted with antibodies against XPC, RPA and HMGB1 (C).

Figure 4. Sequential addition of human purified recombinant HMGB1, XPC-RAD23B, and RPA to triplex-directed psoralen ICLs

(A) Purified recombinant human HMGB1 (10 nM) was incubated for 20 minutes at 30°C with radiolabeled triplex-directed psoralen ICLs, after which increasing concentrations of XPC-RAD23B and RPA proteins (10, 20, or 30 nM) were added, incubated for a further 20 minutes, and then subjected to EMSA analysis using a 6% native PAGE. (B) Recombinant human XPC-RAD23B and RPA proteins (10 nM) were incubated for 20 minutes with the radiolabeled ICL-containing substrate (Tdp-ICL substrate), followed by incubation with increasing concentrations of HMGB1 (10, 20, or 30 nM) for 20 minutes, then analyzed using EMSA.

Figure 5. Interactions between HMGB1 and NER DNA damage recognition factors

(A, B) Calmodulin resin was bound with CBP-tagged HMGB1 protein, then incubated with cell extracts (A) or human purified XPC, HMGB1, and RPA proteins (B), and the bound proteins were electrophoresed by SDS-PAGE, transferred to membranes and immunoblotted with antibodies against HMGB1 or XPC. Calmodulin-resin (CaM resin) alone was used as a specificity control, and cell extracts (Cell Ext, A) or purified proteins (Prot Mark, B) were run on the gels as protein-size markers. (C, D) Amylose resin was bound with MBP-tagged XPC-RAD23B protein (C), or XPA protein (D) then incubated with cell extracts (C) or human purified HMGB1 and RPA proteins (D), and then immunoblotted as in A. MBP peptide (C) or amylose-resin (Amyl resin, D) alone were used as specificity controls, and cell extracts (Cell Ext, C) or purified proteins (Prot Mark, D) were run on the gels as protein-size markers. (E) Representative immunoblots of human XPA and HMGB1 proteins immunoprecipitated from cell extracts using antibodies against rabbit IgG (control) and HMGB1.