Preferential Binding of Human Full-Length XPA and the Minimal DNA Binding Domain (XPA-MF122) with the Mitomycin C−DNA Interstrand Cross-Link†

David J. Mustra,‡ Amy J. Warren,‡ and Joshua W. Hamilton*,‡,§

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755-3835, and Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire 03756-0001

Received December 12, 2000; Revised Manuscript Received April 16, 2001

ABSTRACT: Nucleotide excision repair (NER) is an important cellular mechanism that removes radiation-induced and chemically induced damage from DNA. The XPA protein is involved in the damage recognition step of NER and appears to function by binding damaged DNA and recruiting other proteins to the site. It may also play a role in subsequent steps of NER through interaction with other repair proteins. Interstrand cross-links are of particular interest, since these lesions involve both strands of duplex DNA and present special challenges to the repair machinery. Using 14 and 25 bp duplex oligonucleotides containing a defined, well-characterized single mitomycin C (MMC)−DNA interstrand cross-link, we have shown through gel shift analysis that both XPA and a minimal DNA binding domain of XPA (XPA-MF122) preferentially bind to MMC−cross-linked DNA with a greater specificity and a higher affinity (>2-fold) than to the same undamaged DNA sequence. This preferential binding to MMC−cross-linked DNA occurs in the absence of other proteins from the NER complex. Differences in binding affinity and specificity were observed among the different protein−DNA combinations that were both protein and DNA specific. Defining XPA−MMC−DNA interactions may aid in elucidating the mechanism by which DNA cross-links and other forms of DNA damage are recognized and repaired by the NER machinery in eukaryotic cells.

Nucleotide excision repair (NER)[1] is an important cellular mechanism that removes radiation-induced and chemically induced damage from DNA (*1*−*7*). The stages of NER consist of damage recognition, formation of incisions on either side of the lesion (spanning approximately 26−29 nucleotides), removal of the damaged oligonucleotide, and DNA synthesis followed by ligation to fill in the gap (*4*, *6*). Genetic defects in individual NER proteins can cause severe disease, including Cockayne's syndrome (CS) and xeroderma pigmentosum (XP). Cell fusion studies have identified seven XP genes, initially defined as genetic complementation groups A through G. Of all the complementation groups, XPA is the most common and causes the most severe form of XP disease. Patients with this disorder are deficient in the XPA protein and are therefore unable to efficiently repair DNA damage. They are particularly prone to a high risk of skin cancer from sun exposure and resulting unrepaired UV damage. In addition, cell lines from these patients are particularly sensitive to UV and DNA cross-linking agents, as well as other chemical and physical DNA damaging agents. The XPA protein appears to be involved in the damage recognition step of NER and appears to function by binding damaged DNA and recruiting other proteins to the site. XPA may also play a role in subsequent steps of NER through interaction with other repair proteins (e.g., RPA, ERCC1, TFIIH) (*8*). Thus, XPA is a key component in, and may be the rate-limiting step of, DNA damage recognition and NER (*8*). Despite the fact that numerous chemically and structurally diverse DNA lesions are repaired by NER, the mechanism of the interaction of XPA with damaged DNA is not well characterized.

The human XPA protein is a 273 amino acid (31 kDa) protein that contains a $(Cys)_4$-type "zinc finger" binding motif in its active site. The DNA binding region has been localized to a 122 amino acid between Met98 and Phe219 containing the zinc binding region (*9*), referred to as the XPA-MF122 minidomain, which has previously been characterized (*10*−*14*). XPA has been shown to preferentially bind to UV-irradiated and cisplatin-damaged DNA (*15*−*17*). However, these studies investigated the interaction of the full-length protein with large DNA fragments (>200 bp) that were extensively damaged with UV or cisplatin (*15*−*17*), and thus, the lesions for damage recognition were not well characterized. Limited studies have investigated the use of oligonucleotides containing a single lesion (*18*), and a thorough comparison of the binding characteristics of the full-length

† This work was supported by grants from the National Cancer Institute, NIH (CA49002, J.W.H.), the National Institute of Environmental Health Sciences, NIH (ESO7373, J.W.H.), the Norris Cotton Cancer Center, Dartmouth Cancer Biology Training Grant CA09658 (D.J.M.), and Bristol-Myers Squibb.

* To whom correspondence and requests for reprints should be addressed at the Department of Pharmacology and Toxicology, Dartmouth Medical School, 7650 Remsen, Hanover, NH 03755-3835. Phone: (603)650-1316. Fax: (603)650-1129. E-mail: josh.hamilton@dartmouth.edu.

‡ Dartmouth Medical School.

§ Dartmouth-Hitchcock Medical Center.

[1] Abbreviations: bp, base pair; EMSA, electrophoretic mobility shift assay; MMC, mitomycin C; NER, nucleotide excision repair; XPA, xeroderma pigmentosum A; XPA-MF122, XPA minimal DNA binding domain.

10.1021/bi002820u CCC: $20.00
Published on Web 05/19/2001

Table 1: Model Duplex Oligonucleotides for MMC−DNA Cross-Link Formation

sequence[a]	length (bp)
GATCTAC**G**TTAGAG CTAGAT**G**CAATCTC	14-mer
AATTAGATCTAC**G**TTAGAGATAATA TTAATCTAGAT**G**CAATCTCTATTAT	25-mer

[a] The boldfaced guanines represent the site for MMC−DNA interstrand cross-link formation.

XPA compared to the minidomain using the same damaged DNA has yet to be described.

We were interested in characterizing the binding of XPA and XPA-MF122 to an interstrand cross-link formed by the genotoxic anticancer drug mitomycin C (MMC). MMC is a bifunctional cross-linking agent that requires sequential chemical or enzymatic reductions to form covalent adducts with DNA; specifically, G-MMC monoadducts and G-MMC-G interstrand and intrastrand cross-links (*19*−*22*). Although a great deal is known about the chemistry of MMC adduction to DNA, little is known about the recognition and repair of the interstrand cross-link. It is currently believed that MMC binding does not significantly disrupt overall DNA helical structure (*23*). However, we had observed by chemical footprinting subtle perturbations in DNA structure due to the MMC−DNA interstrand cross-link (*24*). In addition, using an EMSA analysis, we previously observed preferential binding of nuclear factors to the MMC−DNA interstrand cross-link (*25*). On the basis of the binding profile of various mutant rodent and human cell lines, these studies suggested that the shifted complexes contained XPA and ERCC1.

Therefore, we were interested in comparing the interaction of purified full-length XPA or the XPA-MF122 minidomain to model oligonucleotides containing a single site-specific MMC−DNA cross-link using the gel mobility shift assay. We demonstrate here that both proteins preferentially recognize the MMC−DNA cross-link in duplex DNA as short as 14 bp. The overall goal of this work is to characterize the XPA−MMC−DNA interaction, which may aid in elucidating the mechanism by which DNA cross-links and other forms of DNA damage are recognized and repaired in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Design, Synthesis, and Purification of Model, Duplex Oligonucleotides. A family of duplexes (Table 1) was designed for reaction with mitomycin C. The sequences were designed to favor formation of the interstrand cross-link and to have T_m values high enough for the duplexes to remain fully double stranded at room temperature. Oligonucleotides were synthesized on a 1 or 10 μmol scale and purified by HPLC using a DNAPAC PA-100 (9 × 250 mm) anion-exchange column. DNA probes were annealed with their complementary strands, and 1.94 pmol was 5′-end labeled with [γ-^{32}P]ATP and T4 kinase and purified by NEN20 chromatography (New England Nuclear) as previously described (*25*). For comparisons of XPA binding to cross-linked vs unmodified DNA, the specific activities were made equivalent.

Synthesis and Purification of the MMC−DNA Interstrand Cross-Link. Duplex oligos were reacted with MMC essentially as previously described (*20*, *24*). The key modification involved the use of an atmosphere bag kept under a positive pressure of argon, in which the reducing agent (sodium dithionite) was prepared and the reaction was performed. The interstrand cross-link produced from the reaction was separated from the unreacted duplex by a HiLoad 26/60 Superdex 30PG jacketed column (Pharmacia) (running buffer: 25 mM Tris, pH 8.0, for the 14 bp duplex or 25 mM Tris, pH 8.0, and 25% formamide for the 25 bp duplex) heated to 45 °C using a circulating water bath. The fraction containing the cross-link was confirmed by 20% denaturing polyacrylamide gel electrophoresis (*24*), ^{32}P-postlabeling (*26*), UV−vis (*24*), and T_m analysis (*24*). The cross-linked DNA was then dialyzed against water [2000 molecular weight cutoff (MWCO)].

XPA-MF122 and XPA Expression, Purification, and Cleavage. A pET-16b plasmid containing the XPA-MF122 truncated DNA binding domain was transformed into the competent *Escherichia coli* strain BL21(DE3) on the basis of the method described by Kuraoka (*9*) and the Novagen pET manual. XPA-MF122 was isolated from the bacteria as previously described by Kuraoka (*9*) with modifications. Briefly, bacteria were disrupted at 4 °C via a French press, rather than sonication, to avoid excessive heating, and the mixture was centrifuged at 15000*g*, 4 °C, for 30 min. The supernatant from the bacterial lysate was loaded onto a His-bind resin column, charged with 10 mM nickel sulfate, at a flow rate of 0.4 mL/min with storage buffer [20 mM Tris, pH 7.50, 150 mM KCl, 1 mM $MgCl_2$, 20 μM $Zn(OAc)_2$, 500 μM Pefabloc, 1 mM DTT] containing 50 mM imidazole. The protein was eluted with storage buffer containing an imidazole gradient of 50−200 mM. The solution was then dialyzed against storage buffer and concentrated by Macrosep 3K centrifugal concentrators at 2500 rcf, 4 °C, for 60 min to an OD_{280} of 1.00 for storage. Prior to cleavage of the histidine tag, the protein solution was dialyzed to cleavage buffer [50 mM Tris, pH 8.0, 1 mM $CaCl_2$, 150 mM KCl, and 10 μM $Zn(OAc)_2$] at 4 °C under argon to help to prevent the oxidation and precipitation of the protein due to the requirement of removal of DTT prior to enzymatic cleavage. The histidine tag was removed by cleavage with 5 units for XPA-MF122 or 10 units for the full-length XPA protein of factor X_a (Pharmacia) per OD of protein over 24 h at 22 °C. The reaction was halted by the addition of Pefabloc and DTT to final concentrations of 0.5 and 1 mM, respectively. The cleaved protein was concentrated by Macrosep 3K centrifugation at 4 °C and 2500 rcf, 4 °C. The protein solution was further purified on a Sephacryl S-100 column (26/100, Pharmacia) at 4 °C (buffer C, 0.6 mL/min). Full-length XPA protein was prepared in a similar manner using a pET-15b plasmid. After each chromatography step, the fractions containing protein were confirmed by Tris−Tricine SDS−PAGE gels (XPA-MF122, 14.7 kDa; XPA, 31.4 kDa), and protein concentrations were checked by UV−vis (XPA-MF122, $\epsilon_{280} = 17\,585\ M^{-1}\ cm^{-1}$; XPA, $\epsilon_{280} = 29\,295\ M^{-1}\ cm^{-1}$) (*27*). The presence and eventual loss of the histidine tag was also followed by means of Western analysis using an anti-histidine antibody. The fractions containing protein were combined, and DTT was added to the solution to a final concentration of 1 mM. The solution was then dialyzed and concentrated by Macrosep 3K at 4 °C and 3500 rpm.

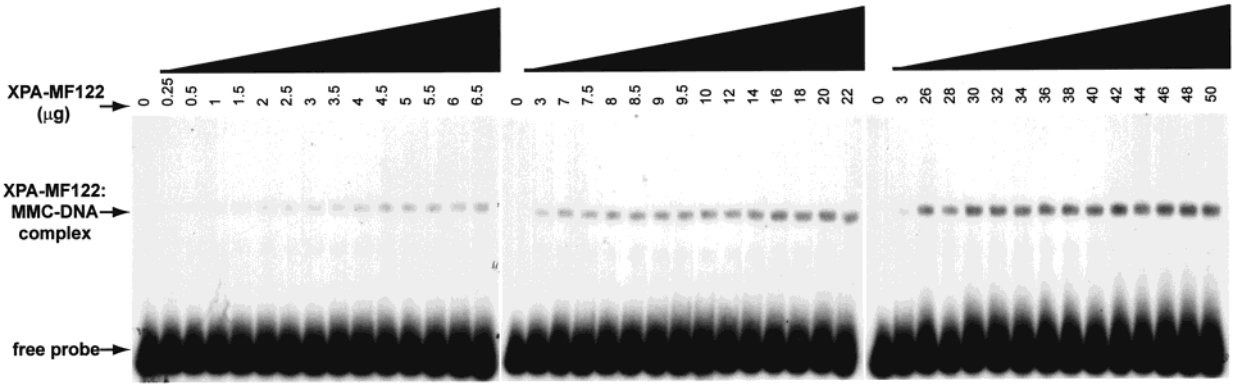

FIGURE 1: Binding of XPA-MF122 to a MMC−cross-linked 14 bp duplex. The radiolabeled MMC−cross-linked 14 bp duplex (100 000 cpm, 10 fmol) was incubated with 17−3400 pmol (0.25−50 μg) of purified XPA-MF122 as indicated, and protein−DNA binding was analyzed by EMSA as described in Experimental Procedures.

Electrophoretic Mobility Shift Assay (EMSA). To study the binding of XPA-MF122 and XPA to the MMC−DNA interstrand cross-link, gel shift assays were performed as described (*25*) with slight modifications. Briefly, radiolabeled duplex (100 000 cpm/lane; average of 10 fmol, depending on specific activity) was incubated with 68 pmol (1 μg) of purified XPA-MF122 or 60 pmol of XPA at 4 °C for 20 min [10 mM Tris, pH 7.5, 20 mM KCl, 0.2 mM DTT, 0.1 mM EDTA, 4 μM $Zn(OAc)_2$, 4% glycerol, 0.2 mg/mL BSA] in a total volume of 25 μL. When appropriate, competitor DNA was added at different molar excesses relative to the radiolabeled probe and incubated simultaneously with the radiolabeled probe. In other experiments, the ability of XPA-MF122 to displace full-length XPA binding to radiolabeled probe was determined by incubating 34 pmol of XPA with increasing molar equivalents of XPA-MF122. Once the binding reactions were complete, the samples were separated by 10% nondenaturing PAGE using high ionic conditions (50 μM Tris, 380 mM glycine, 2.0 mM EDTA, pH 8.5) at 180 V for 4 h using a circulating 4 °C water bath. After electrophoresis, gels were dried and placed on X-ray film for autoradiography, and the images were digitized by a LaCie Silverscanner III or were visualized by phosphorimagery for densitometry. The background was subtracted by using a lane containing probe and no protein. The amount of protein bound to radiolabeled probe was quantified, and the percent binding to MMC-damaged probes relative to the nondamaged sequences was determined. Data from competition experiments were expressed as a percent of binding in the absence of competitor. Data represent an average of three to five repeated experiments.

RESULTS

To investigate the binding of XPA and XPA-MF122 to the MMC−DNA interstrand cross-link, two model duplex oligonucleotides were designed (Table 1) and reacted with MMC. These sequences were designed to specifically provide a single site for MMC adduction, to contain the optimal flanking bases around the site of adduction to increase MMC binding efficiency (*20*, *28*), and to have a normal B-DNA structure. We modified these sequences from those previously reported in our laboratory (*24*, *25*) such that the adenines and thymines were more evenly distributed and several single guanines were added. These changes were predicted to contribute to a more normal B-DNA structure and higher thermal melting temperature (T_m). Despite the presence of guanines outside of the CpG site, which could potentially form MMC monoadducts, we found that guanines on the ends of oligonucleotides did not react well with MMC, probably due to a less double-helical nature at the ends (i.e., "fraying"). In addition, the strong preference of MMC for forming interstrand cross-links at CpG sites (A. J. Warren et al., manuscript in preparation) appears to strongly outweigh the possibility of binding to the other guanines. Thus, although it is statistically possible to form MMC−DNA monoadducts on cross-linked duplexes, we exclusively obtained singly cross-linked duplexes following high-resolution size exclusion chromatography (~25% yield, >97% purity), which was confirmed by ^{32}P-postlabeling (*26*, *29*). Greater yields of cross-links were obtained than previously reported (*24*, *25*) using similarly sized oligonucleotides, primarily due to the use of a glovebag which allows the absolute exclusion of oxygen during preparation of the reducing agent and throughout the MMC reaction. Cross-links were fully characterized by denaturing gel electrophoresis, UV−vis spectroscopy, T_m analysis, and ^{32}P-postlabeling, as previously described by our laboratory (*24*, *26*).

The XPA and the XPA-MF122 proteins were successfully expressed, purified, and characterized according to standard methods (UV−vis, SDS−PAGE, Western analysis, and protein sequencing). Throughout the purification process, Western analysis using an anti-XPA antibody and an anti-histidine antibody was used to verify the presence of XPA and the cleavage of the histidine tag during the final purification step. The molecular weights and sequence analysis were consistent with previous reports and the identity of these proteins (*9*).

To characterize the interactions of purified XPA-MF122 and XPA with MMC-damaged and unmodified duplexes, gel shift analysis was performed with purified 14 and 25 bp duplexes each containing a single MMC−DNA interstrand cross-link. Initially, the cross-linked 14 bp duplex was titrated with 0.25−50 μg (17.0−3400 pmol) of XPA-MF122 (Figure 1). The minimal amount of protein required to give repeatable binding was determined to be approximately 68 pmol (1 μg) of XPA-MF122. Gel shift analysis of binding events is inherently semiquantitative. Thus, although binding was observed, we were unable to use these binding data to calculate an apparent K_d due to the inability to fully saturate

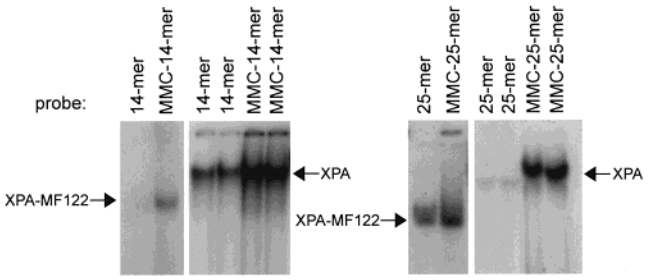

FIGURE 2: Enhanced binding of XPA and XPA-MF122 with MMC−cross-linked DNAs. Purified XPA or XPA-MF122 (68 pmol) was incubated with 100 000 cpm (10 fmol) of either radiolabeled 14 bp duplex, 25 bp duplex, or MMC−cross-linked 14 bp duplex or 25 bp duplex, respectively. Binding events were analyzed by EMSA as in Figure 1, and replicate samples are shown. A dark exposure is shown here to aid in visualization of all the bands. A series of lighter exposures were used to quantify bound protein by densitometry, and data from three to five experiments were averaged.

binding under these conditions. Despite the large excess of protein, a large quantity of free probe was always observed. However, DNA binding events could still be compared in a semiquantitative manner.

Both XPA and XPA-MF122 were capable of binding to the MMC−cross-linked 14 bp duplex and 25 bp duplex (Figure 2). XPA-MF122 bound approximately 11.5-fold better to the cross-linked 14 bp duplex, and 1.5-fold better to the cross-linked 25 bp duplex, than to their corresponding unmodified duplexes. Similarly, full-length XPA bound approximately 2.7-fold better to the cross-linked 14 bp duplex and 2.2-fold better to the cross-linked 25 bp duplex, relative to their respective unmodified duplexes. When the relative affinity of the full-length protein for the two cross-linked sequences was compared, XPA showed a 15-fold greater binding to the longer DNA. Both proteins demonstrated binding to the unmodified duplexes. Full-length XPA appeared to bind approximately 30-fold higher to the unmodified 25 bp duplex than to the unmodified 14 bp duplex. In summary, XPA and XPA-MF122 preferentially bound MMC−cross-linked DNA, and XPA had the highest affinity for the larger oligonucleotide (both with and without MMC modification), which most closely resembles the size of DNA excised from the repair complex in longer DNA.

The binding specificity of XPA-MF122 for the cross-linked 14 bp duplex was further evaluated through competition experiments. XPA-MF122 was incubated with the radiolabeled cross-linked 14 bp duplex in the presence of various molar excesses of unlabeled cross-linked or unmodified 14 and 25 bp duplexes (Figure 3). All unlabeled duplexes demonstrated some competition for binding. However, the cross-linked 14 bp duplex exhibited greater competition at each concentration than did the unmodified 14 bp duplex. For example, addition of a 10-fold molar excess of the unmodified duplex was unable to compete for binding, whereas the same molar excess of unlabeled cross-linked duplex resulted in an approximately 50% decrease in binding (Figure 3). Interestingly, the unmodified 25 bp duplex competed for binding equally well as the unmodified 14 bp duplex, and the MMC−cross-linked 25 bp duplex was substantially less effective at competition for binding than was the cross-linked 14 bp duplex. Thus, these results suggest that the XPA-MF122 minidomain may demonstrate a more facile exchange from the shorter cross-linked duplex.

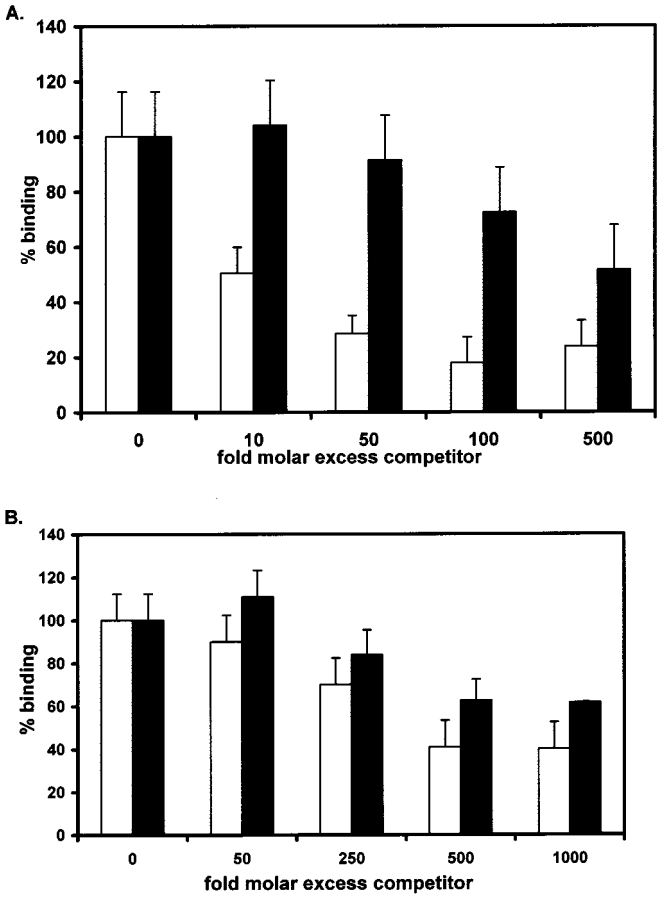

FIGURE 3: Competition for binding of XPA-MF122 to MMC−cross-linked 14 bp duplex using MMC−cross-linked and unmodified duplexes. Specificity for XPA-MF122 binding (68 pmol) to the radiolabeled 14 bp duplex (100 000 cpm, 10 fmol) was examined by EMSA as in Figures 1 and 2, using various molar excesses of unlabeled cross-linked (open bars) or unmodified (black bars) 14 bp duplex (panel A) or 25 bp duplex (panel B) duplexes. Densitometry was performed as described in Experimental Procedures, and data were expressed as a percent of binding in the absence of competitor (0). Each bar represents the mean ± SEM of three to four experiments.

The binding specificity of XPA-MF122 for the cross-linked 25 bp duplex was also evaluated through competition experiments (Figure 4). The MMC−cross-linked 25 bp duplex was able to reduce binding by 1.6-fold at a 500-fold molar excess, while no appreciable competition was observed with the unmodified 25 bp duplex. Similarly, the unmodified 14 bp duplex was unable to effectively compete at any concentration (Figure 4B), and the MMC−cross-linked 14 bp duplex was able to reduce binding by 1.3-fold at a 500-fold excess. Although the MMC−cross-linked 25 bp duplex was able to further reduce binding by 1.8-fold at a 1000-fold excess, this same excess of the unmodified 14 bp duplex did not show any additional competition than observed at a 500-fold excess. These results suggest that XPA-MF122 has a preference for the larger MMC−cross-linked duplex.

Similar competition experiments were performed with the full-length XPA protein. The highest level of XPA binding was observed for MMC−cross-linked 25 bp duplex, and unlabeled cross-linked 25 bp duplex was the most effective competitor for this binding (Figure 5). For example, unmodified 25 bp duplex was able to reduce binding of XPA only at a 1000-fold excess (40-fold decrease in binding), whereas

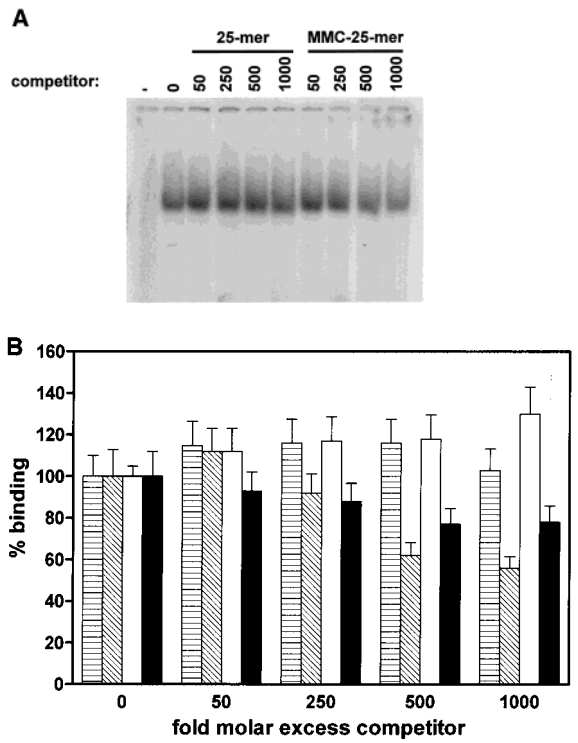

FIGURE 4: Competition for binding of XPA-MF122 to MMC−cross-linked 25 bp duplex using cross-linked and unmodified DNAs. Specificity for XPA-MF122 binding (68 pmol) to the radiolabeled MMC−cross-linked 25 bp duplex (100 000 cpm, 10 fmol) was examined by EMSA (panel A) as in Figures 1 and 2, using various molar excesses of unlabeled cross-linked or unmodified 25 bp duplex as shown. Autoradiographs were analyzed by densitometry as described in Figure 3 (panel B), with unlabeled cross-linked 25 bp duplex (diagonal striped bars), unmodified 25 bp duplex (horizontal striped bars), cross-linked 14 bp duplex (black bars), and unmodified 14 bp duplex (open bars). Each bar represents the mean ± SEM of three to four experiments.

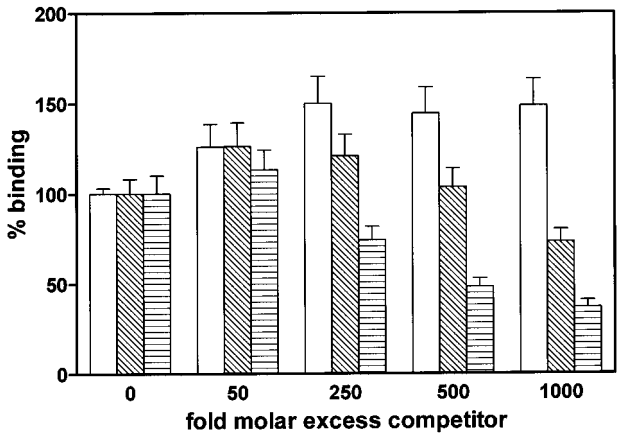

FIGURE 5: Competition for binding of full-length XPA to the MMC−cross-linked 25 bp duplex with MMC−cross-linked and unmodified DNAs. Specificity for full-length XPA binding (68 pmol) to radiolabeled MMC−cross-linked 25 bp duplex (100 000 cpm, 10 fmol) was examined by EMSA as described in Figure 4, using various molar excesses of unlabeled MMC−cross-linked 14 bp duplex (open bars), 25 bp duplex (horizontal striped bars), and unmodified 25 bp duplex (diagonal striped bars). Each bar represents the mean ± SEM of two to three experiments.

cross-linked 25 bp duplex caused a similar decrease in XPA binding by 40-fold at a 250-fold excess. The MMC−cross-linked 14 bp duplex was unable to reduce binding at any concentration, demonstrating the preference of the full-length XPA protein for binding to the larger cross-linked DNA.

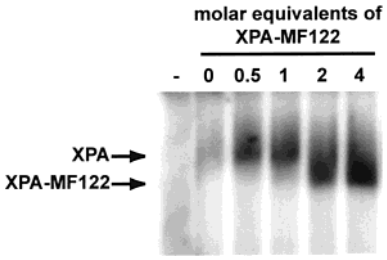

FIGURE 6: Competition for binding of full-length XPA to the MMC−cross-linked 25 bp duplex with XPA-MF122. XPA (34 pmol) was incubated with radiolabeled MMC−cross-linked 25 bp duplex (100 000 cpm, 10 fmol) in the presence of various molar equivalents of XPA-MF122, and binding was analyzed by EMSA as described in Figure 2.

Finally, the relative binding affinities of the two proteins for the MMC−cross-linked 25 bp duplex were investigated. As shown in Figure 6, when XPA was incubated with the radiolabeled cross-linked 25 bp duplex in the presence of a 2-fold excess of XPA-MF122, a shift in mobility was observed, consistent with formation of the XPA-MF122 complex. Thus, XPA-MF122 is capable of displacing full-length XPA protein from the cross-linked duplex, and the shift between a 1- and 2-fold excess of XPA suggests that they have similar affinities for this duplex.

DISCUSSION

Elucidating the pathways for the cellular recognition and repair of DNA damage is important both for understanding the processes of mutagenesis and carcinogenesis and for understanding and advancing the use of genotoxic agents in cancer chemotherapy. Interstrand cross-links are of particular interest, since these lesions involve both strands of duplex DNA and present special challenges to the repair machinery. Unrepaired interstrand cross-links are potentially lethal to dividing cells, which is the basis for the use of agents such as MMC and cisplatin in cancer chemotherapy. Unlike DNA damaging agents such as UV, cisplatin, and psoralen described in previous studies of DNA damage recognition proteins (*15*, *17*, *30*−*35*), which are believed to form adducts that significantly distort DNA helical structure, MMC induces only a minor degree of DNA helix distortions (*23*). However, despite the subtle perturbations in DNA structure caused by the MMC−DNA interstrand cross-link, such lesions appear to be readily recognized and repaired. Our laboratory has observed rapid kinetics of MMC interstrand cross-link removal in cell culture and in vivo (*26*, *29*, *36*). Therefore, it is possible that large alterations of DNA structure alone may not be required for effective recognition and repair.

Our laboratory previously investigated whether mammalian nuclear proteins can recognize and bind to a model oligonucleotide containing a single MMC lesion (*25*). EMSA analysis identified multiple complexes from both rodent and human cell lines that appeared to specifically recognize the MMC interstrand cross-link. In addition, nuclear extracts from normal and excision repair-defective mutant Chinese hamster ovary (CHO) cell lines and from human XP complementation groups A−E were examined and suggested that XPA and ERCC-1 were required for formation of these complexes. The current work demonstrates that purified XPA and the minimal DNA binding domain of XPA are fully

capable of preferentially binding to MMC−DNA cross-links in the absence of other proteins from the NER complex.

Both the full-length and the truncated XPA protein were capable of binding a MMC−cross-linked 14 bp duplex, which is half the size of the excised oligonucleotide in mammalian NER. However, both proteins demonstrated enhanced binding to the larger 25 bp MMC−cross-linked duplex. These data suggest that the larger size may be less important for damage recognition than for subsequent steps involving the other proteins of the NER complex. Competition experiments and direct comparisons of binding events suggested that full-length XPA has only a slightly greater affinity than XPA-MF122 for the same cross-linked duplex. These results support the model that the $(Cys)_4$-type zinc-binding motif-containing domain is the main region of XPA that interacts with DNA and is sufficient for initial binding and damage recognition. It should be noted, however, that unlike the full-length protein, the minidomain cannot participate in protein−protein interactions with other members of the NER complex. It is highly likely that other proteins (e.g., RPA, ERCC1) in the NER complex stabilize the interaction of XPA with damaged DNA, leading to a much higher affinity than that of XPA alone. Therefore, if XPA-MF122 can compete with XPA for initial binding, it might serve in a dominant negative role, thus suppressing or diminishing repair capacity. This might be particularly relevant in cases where drug-resistant chemotherapy patients have enhanced repair function. However, future studies will be required to further examine this possibility.

XPA has been shown to preferentially bind to UV-irradiated and cisplatin-damaged DNA as compared to undamaged DNA (*15*−*17*). However, these studies investigated the interaction of the full-length protein with large DNA fragments (>200 bp) that were extensively damaged with UV or cisplatin (*15*−*17*). Recently, the interaction of the XPA minidomain to short oligonucleotides containing a single cisplatin adduct was investigated by electrospray ionization (*18*). In contrast to previous studies with larger DNA fragments, Xu et al. (*18*) reported relatively weak protein−DNA interactions when using the XPA minidomain and short single site-modified oligonucleotides. The reported affinity of XPA for various substrates varies widely in the literature: values for the K_d of XPA to damaged DNA range from 10^3 to 10^8 M, depending on the size of the DNA and the method used, and probably other factors that are yet to be determined (*15*−*17*, *37*). Thus, it is difficult to directly compare the affinities of XPA for different forms of DNA damage from these studies. We were not able to accurately estimate a K_d in these studies due to the inherently semi-quantitative nature of EMSA analysis (*37*). However, we observed that XPA appears to bind generally 2−3-fold greater to MMC−cross-linked DNA than to the corresponding undamaged DNA. Xu et al. reported that XPA-MF122 had an overall low affinity for damaged DNA and that this affinity was much lower for single site-modified oligos than for extensively modified DNA fragments (*18*). Studies are under way using more quantitative approaches for determination of a K_d, including isothermal titration calorimetry, differential sedimentation, and surface plasmon resonance analysis.

A low level of binding of both proteins to the undamaged oligonucleotides was observed. We hypothesize that XPA adopts a different conformation when binding to undamaged DNA than when binding to damaged DNA. This would argue that initial binding affinity alone is not the critical or rate-limiting step in DNA damage recognition but rather the subsequent induction of altered protein−DNA conformation by DNA damage recognition. Circular dichroism (CD) studies support this hypothesis (Mustra et al., manuscript in preparation). Further, such a change in conformation of the damaged DNA−protein complex might be important in recruiting the other proteins required in the NER complex. A more detailed structural and biophysical analysis would be useful to further characterize the interaction XPA with a MMC−DNA interstrand cross-link. These studies may aid in elucidating the mechanism by which DNA cross-links and other forms of DNA damage are recognized and repaired by eukaryotic cells.

ACKNOWLEDGMENT

The authors thank Dean Wilcox (Dartmouth College, Department of Chemistry) and Alan Eastman (Dartmouth Medical School, Department of Pharmacology and Toxicology) for their scientific advice and critical review of the manuscript.

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BI002820U