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Biochemical and Biophysical Research Communications 356 (2007) 219–225
BBRC
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Domains in the XPA protein important in its role as a processivity factor

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Received 20 February 2007
Available online 2 March 2007

Abstract

XPA is a protein essential for nucleotide excision repair (NER) where it is thought to function in damage recognition/verification. We have proposed an additional role, that of a processivity factor, conferring a processive mechanism of action on XPF and XPG, the endonucleases, involved in NER. The present study was undertaken to examine the domain(s) in the *XPA* gene that are important for the ability of the XPA protein to function as a processivity factor. Using site-directed mutagenesis, mutations were created in several of the exons of *XPA* and mutant XPA proteins produced. The results showed that the DNA binding domain of XPA is critical for its ability to act as a processivity factor. Mutations in both the zinc finger motif and the large basic cleft in this domain eliminated the ability of XPA to confer a processive mechanism of action on the endonucleases involved in NER.

Keywords: Xeroderma pigmentosum; XPA protein; DNA repair; Processive mechanism of action

Though the XPA protein is a key component in NER, its precise function in this repair pathway is not clear. There is evidence that it plays a role in the initial steps of NER, where it is involved in damage recognition/verification [1–3]. We have proposed an additional important function for XPA, that of a processivity factor needed to confer a processive mechanism of action on the endonucleases, XPF and XPG, involved in NER [4,5]. Proteins can locate target sites on DNA by two distinctive mechanisms: (1) a processive mechanism in which a protein first binds to a random site on DNA and then translocates to a specific site by a facilitated-diffusion process in which it slides or hops along the DNA or (2) a distributive mechanism, in which a protein has no affinity for non-target DNA and locates target sites by a random, three-dimensional diffusion process [6–9]. The mechanism utilized by DNA-targeting proteins is extremely important in determining the ability of the protein to properly interact with its target sites on DNA and, for many of these proteins, a processive mechanism of action is essential [6,8]. A number of proteins involved in DNA repair have been shown to act processively. These include DNA helicases [10], T4 endonuclease V [11,12], *Micrococcus luteus* UV endonuclease [13], UvrABC nuclease [14], DNA glycosylases [15,16], and AP endonucleases [17].

We have previously shown that during repair of UVC light-induced cyclobutane pyrimidine dimers in normal human cells, the endonucleases, XPG and XPF, incise DNA at sites of damage using a processive mechanism of action [4,5]. In contrast, these endonucleases in cells from patients with the repair-deficient genetic disease, xeroderma pigmentosum complementation group A (XPA), do so by a distributive mechanism of action [4,5]. We have additionally shown that the mechanism of location of sites of damage is of critical importance in determining the ability of these repair proteins to interact with damaged nucleosomal DNA [4]. When there is a loss in ability of these endonucleases to act processively, as occurs in XPA cells, there is a deficiency in their ability to incise damaged nucleosomal DNA [4]. There is thus a correlation between a processive mechanism of action and ability of these endo-

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doi:10.1016/j.bbrc.2007.02.125

nucleases to incise damaged DNA when it is present in nucleosomes [4].

Using a recombinant XPA protein, we have additionally shown that the defect in the ability of these endonucleases in XPA cells to incise UVC-irradiated DNA using a processive mechanism of action is corrected by addition of the XPA protein to our *in vitro* assay system [5]. We have thus proposed that the XPA protein may also function as a processivity factor. The current studies were carried out to determine which exon(s) in the *XPA* gene are important for the ability of the XPA protein to show this function and confer a processive mechanism of action on the endonucleases involved in NER. Using site-directed mutagenesis, the results show that the DNA-binding domain of XPA is critical for its role as a processivity factor.

Materials and methods

Chromatin-associated proteins. Normal (GM 3299B) and XPA [GM 02250C (XP12BE)] lymphoblastoid cell lines were obtained from the Coriell Institute for Medical Research (Camden, NJ). The cells were grown in suspension culture in RPMI 1640 medium and the cell nuclei isolated and chromatin-associated proteins extracted as previously described [5,18].

Analysis of the mode of recognition of sites of damage on DNA. The method of analysis for determination of the mode of recognition of sites of damage involved time course experiments of endonuclease activity on UVC-irradiated DNA either in the presence or absence of a UVC-irradiated competitor DNA, as previously described [4,5]. Briefly, plasmid pWT830/pBR322 and competitor pGEM-3zf(+) DNA (Promega, Madison, WI) (0.05 μg/μl) were irradiated with UVC (254 nM) light at 245 J/m^2 from a germicidal lamp (American Ultraviolet Co., Chatham, NJ) to generate approximately 25 cyclobutane pyrimidine dimers per DNA molecule [4,5]. The plasmid DNA (0.15 μg) was then incubated for 50 min at 37 °C with the XPA chromatin-associated proteins (0.1 μg), either with or without recombinant XPA protein (0.06 μg). At the end of this period, the UVC-irradiated competitor DNA (0.25 μg) was added and the incubation continued. At various time points, the enzymatic reactions were terminated and the samples electrophoresed on 1.1% agarose gels, which were then stained with 0.5 μg/ml ethidium bromide and visualized on a Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA) using Image Quant 5.2 software (Molecular Dynamics) [4,5]. Endonuclease activity, expressed as the number of enzyme-mediated breaks per DNA molecule, was determined as previously described [4,5]. Enzyme activity on undamaged DNA was subtracted from these values.

Site-directed mutagenesis of XPA cDNA. Site-directed mutagenesis was carried out on exons 2 (E2 mutation: deletion of Gly 72–Phe 75), 3 (E3 mutation: Cys 105 → Ser), and 5 (E5 mutation: Arg 207 → Stop) of the XPA gene using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenesis reactions utilized plasmid pET15b vector containing XPA cDNA (pET15b-XPA) [5] and the mutagenic primers. At the end of each reaction, the region of the plasmid containing the XPA cDNA was sequenced to verify that the desired mutation was present.

Extraction and purification of recombinant XPA protein. Plasmids containing the normal or mutated XPA cDNAs were transformed into the *Escherichia coli* expression strain Rosetta (DE3) pLysS (Novagen, Madison, WI) [5]. The XPA protein was extracted and purified by passage through a Ni^{2+}–NTA–agarose column (Qiagen, Valencia, CA) and a DNA–cellulose column (Amersham Biosciences, Piscataway, NJ) as previously described [5]. Purity of the XPA protein was ascertained by electrophoresis on a 12% SDS–polyacrylamide gel, electroblotting the protein onto nitrocellulose and staining with colloidal gold (Bio-Rad, Hercules, CA). Confirmation of this protein was made by Western blot analysis in which blots were probed with HisProbe-HRP (Pierce Biotechnology, Rockford, IL), incubated with Supersignal West Pico Substrate (Pierce) and developed.

DNA binding assays. A 93-bp substrate was synthesized that contained a centrally located hotspot for the formation of pyrimidine dimers (TTTTTT). The top strand, (5′-GCGAGCCGACGACCGCGCCC TACTGATGAGACCACTACTGATGTTTTTTCCGACGACGACGA GCCTACCACTGACTGAGCACTGAGCACTGAG-3′), which was biotinylated on the 5′ end, and the bottom strand (5′-CTCAGTGCT CAGTGCTCAGTCAGTGGTAGGCTCGTCGTCGTCGGAAAAAA CATCAGTAGTGGTCTCATCAGTAGGGCGCGGTCGTCGGCTC GC-3′) were synthesized by Operon Biotechnologies (Huntsville, AL). Construction of the duplex substrate and hybridization of the strands was carried out as previously described [19]. The DNA was then irradiated with 245 J/m^2 UVC light.

For protein–DNA binding reactions, purified recombinant native XPA or E2, E3 or E5 mutant XPA proteins (0.3 μg) were mixed with damaged DNA substrate (0.3 μg) in DNA-binding buffer (20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 120 mM NaCl, 0.3% Tween 20) and incubated at 4 °C for 1 h. Streptavidin-coated acrylamide beads (Pierce) were then added to the DNA binding reaction as previously described [19] and incubation was continued at 4 °C for 15 min. The beads/bound DNA and protein were pelleted by centrifugation (2500*g* at 4 °C) and washed four times in DNA binding buffer. The bound proteins were examined by HisProbe-HRP analysis.

Results

Analysis of the recombinant XPA proteins

Examination of the purified native XPA (Fig. 1A, lanes 1 and 2) and E2 mutant XPA (Fig. 1B, lanes 1 and 2) proteins, which eluted from the Ni^{2+}–NTA–agarose and then the DNA–cellulose columns, showed the presence of two bands of approximately 40 and 42 kDa, as ascertained by

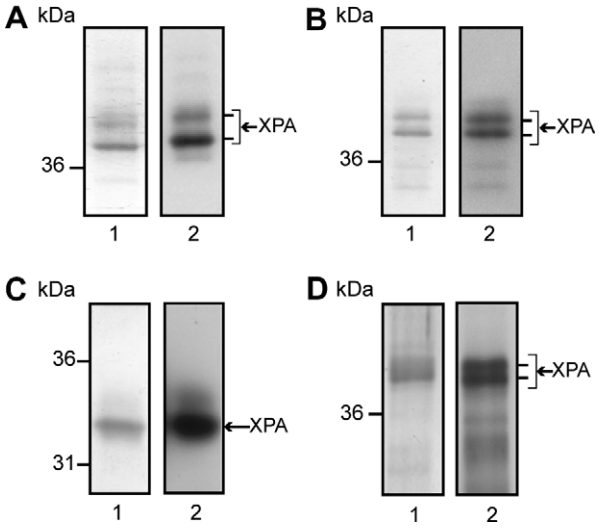

Fig. 1. Electrophoretic analysis of the native and mutant recombinant XPA proteins. The purified XPA proteins were electrophoresed on 12% SDS–PAGE and electroblotted onto nitrocellulose: (A) native, (B) E2 mutant, (C) E5 mutant, and (D) E3 mutant XPA proteins. Lane 1, the membranes were stained with colloidal gold; 2, a duplicate membrane was analyzed by Western blot analysis using HisProbe-HRP. Arrows indicate the location of the XPA protein. The position of molecular weight markers is indicated.

both gold stain and Western blot analysis. The E5 mutant XPA protein eluted as one major band at approximately 33 kDa (Fig. 1C, lanes 1 and 2), which reflects that it is a truncated protein. The E3 mutant protein was purified only on the Ni^{2+}–NTA–agarose column since it was not retained by the DNA cellulose column and came off in the flowthrough. It is possible that the mutation in the zinc finger of this protein affected its affinity for the DNA cellulose. Gold staining and Western blot analysis showed that it eluted as two bands of approximately 40 and 42 kDa (Fig. 1D, lanes 1 and 2).

Influence of the native and mutant XPA proteins on the mechanism of action utilized by endonucleases in XPA cells

For determination of the mechanism of action utilized by endonucleases in XPA cells for locating sites of damage, an assay was utilized that examined endonuclease activity on UVC-irradiated DNA either in the presence or absence of a UVC-irradiated competitor DNA. An endonuclease that acts in a processive manner will scan a DNA molecule and incise at multiple sites of damage before dissociating from it and associating with another DNA molecule [4,5]. The addition of a competitor DNA, which is added after the endonuclease has associated with its substrate DNA, will therefore have little or no effect on the activity of the endonuclease on the substrate DNA. However, if an endonuclease acts in a distributive manner, it will randomly locate and incise sites of damage on both the substrate and competitor DNAs. Its activity on the substrate DNA will decrease after the competitor is added since it will be incising the competitor as well [4,5].

The results showed that the number of breaks produced in UVC-irradiated DNA by endonucleases in chromatin-associated proteins from normal cells remained the same when the competitor was added (Fig. 2A), indicative of a processive mechanism of action. Addition of the recombinant XPA protein had no effect on endonuclease activity (data not shown). These results confirm our previous findings [5]. In contrast, the number of breaks produced by the endonucleases in chromatin-associated proteins from XPA cells on UVC-irradiated DNA decreased after the competitor DNA was added (Fig. 2B). Incisions were approximately 50% of those produced when no competitor was present. This is characteristic of endonucleases that act distributively. However, when native recombinant XPA protein was added, the number of incisions created returned to the levels produced when no competitor was present (Fig. 2B). XPA was thus able to switch the mechanism of action of the endonucleases to a processive one, confirming our previous findings [5].

When the E2 mutant XPA protein was added to the assay, the number of incisions produced by the XPA endonucleases on damaged DNA returned to the levels seen in the absence of competitor (Fig. 2C). After 130 min of incubation with the competitor DNA, the number of incisions was 101% of those produced when no competitor was present. Thus in the presence of the E2 mutant XPA protein, the endonucleases in the XPA chromatin-associated proteins were able to act processively.

Addition of the E3 mutant XPA protein to the assay system did not increase the number of incisions produced by XPA endonucleases on UVC-damaged DNA when a competitor was present (Fig. 2D). After 130 min incubation with the competitor DNA, the number of incisions produced were 50% of those observed when no competitor was present. Since the E3 mutant XPA protein had only been purified on a Ni^{2+}–NTA column, the action of the native XPA protein, purified in a similar manner was examined. Upon addition of the native XPA protein, the number of incisions created returned to the levels produced when no competitor was present (Fig. 2E), just as is seen for the native XPA protein that had been purified further on a DNA–cellulose column (Fig. 2B). Therefore, these results were not affected by not including the DNA cellulose column in the purification scheme.

Addition of the E5 mutant XPA protein to the assay system did not lead to an increase in the number of incisions produced by the XPA endonucleases on UVC-irradiated DNA after the competitor was added (Fig. 2F). After 130 min incubation with the competitor, the number of incisions produced was 48% of those produced when no competitor was present. In the absence of the E5 mutant XPA protein, the number of incisions was also 48% of those produced when no competitor was present. Thus the E5 mutant XPA protein was not able to confer processivity on the endonucleases in the XPA chromatin-associated proteins.

Binding of the native and mutant XPA proteins to UVC-irradiated DNA

The native and mutant recombinant XPA proteins were examined for their ability to bind to a 93-bp DNA substrate containing cyclobutane pyrimidine dimers. Western blot analysis showed that the native XPA protein binds to the UVC-irradiated DNA (Fig. 3A, lane 1) and had little or no affinity for either the streptavidin-coated beads alone (Fig. 3A, lanes 2 and 4) or undamaged DNA (Fig. 3A, lane 3).

The purified E2 mutant XPA protein also bound to the UVC-irradiated DNA (Fig. 3B, lane 1) and remained bound after successive washes with no affinity for the beads alone (Fig. 3B, lane 2) or for undamaged DNA (data not shown).

The E3 mutant XPA protein, in contrast, showed no affinity for the damaged DNA (Fig. 3C, lane 1). It had no affinity for the beads alone (Fig. 3C, lane 2) or for undamaged DNA (data not shown). The native XPA protein purified only on the Ni^{2+}–NTA column bound to the damaged DNA (Fig. 3C, lane 3) and had little affinity for the beads alone (Fig. 3C, lane 4) just as did the native XPA protein that had been further purified on the DNA cellulose column (Fig. 3A). Thus whether the XPA protein

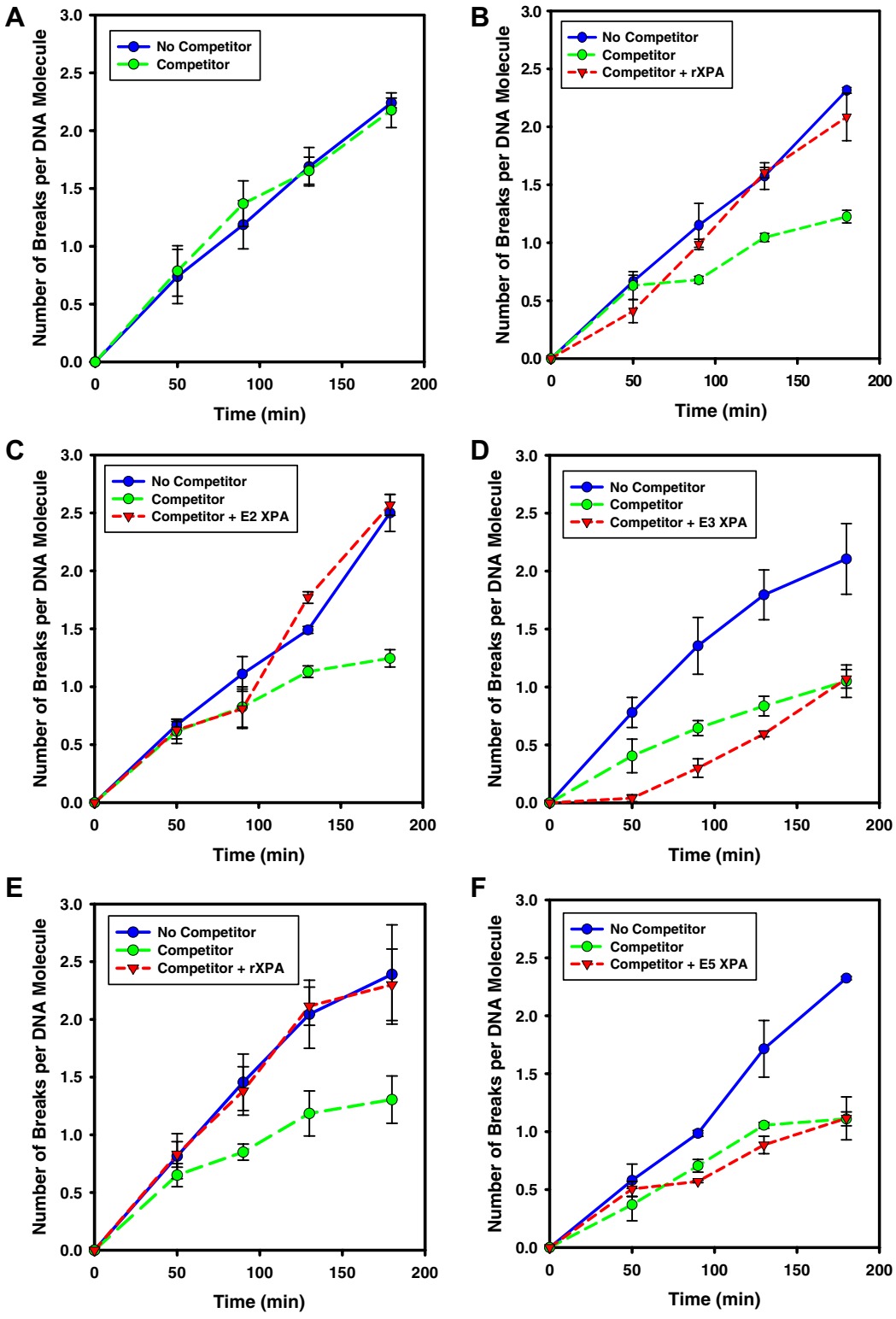

Fig. 2. Influence of the native and mutant XPA proteins on the mechanism of action utilized by NER endonucleases in XPA cells for locating sites of damage. Endonuclease activity was measured on UVC-irradiated DNA in the presence of a similarly damaged competitor DNA. (A) Chromatin-associated proteins from normal cells were incubated with UVC-irradiated DNA for 40 min after which time the UVC-irradiated competitor DNA was added and incubation continued. (B–F) Chromatin-associated proteins from XPA cells plus either native or mutant XPA proteins were incubated with UVC-irradiated DNA for 40 min and then the UVC-irradiated competitor DNA was added and incubation continued. The XPA proteins included in the reactions were: (B) native XPA (rXPA); (C) E2 mutant; (D) E3 mutant purified only on a Ni^{2+}–NTA column; (E) rXPA purified only on a Ni^{2+}–NTA column; and (F) E5 mutant. Endonuclease activity was expressed as the number of breaks per DNA molecule. Vertical lines represent ±SEM for three to four experiments.

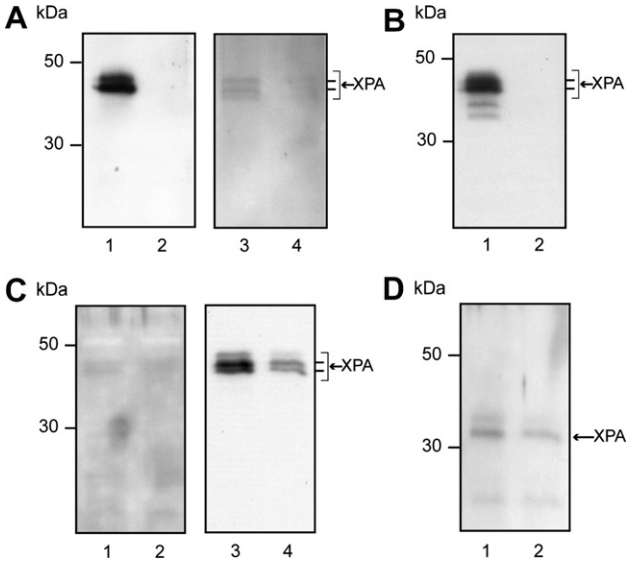

Fig. 3. Binding of native and mutant XPA proteins to UVC-irradiated DNA. A biotinylated 93 bp DNA substrate was irradiated with UVC light and reacted with native or mutant XPA protein. Streptavidin-coated acrylamide beads were added to the reaction and the bound proteins eluted and analyzed by Western blot. Binding of: (A) native XPA protein to: damaged DNA (lane 1), beads alone (lanes 2 and 4), undamaged DNA (lane 3), (B) E2 mutant XPA protein to damaged DNA (lane 1), beads alone (lane 2), (C) E3 mutant XPA protein to damaged DNA (lane 1) or beads alone (lane 2); native XPA protein purified only on a Ni^{2+}–NTA column to damaged DNA (lane 3) or beads alone (lane 4), (D) E5 mutant XPA protein to: damaged DNA (lane 1), beads alone (lane 2).

had been purified through the DNA cellulose column or not did not affect the results of the binding assay.

The purified E5 mutant XPA protein had little binding affinity for the UVC-irradiated DNA (Fig. 3E, lane 1). Binding was only slightly above background binding to the beads alone (Fig. 3E, lane 2) or to undamaged DNA (data not shown).

Discussion

We have proposed that the XPA protein has a new and additional role in NER, of a processivity factor needed to confer a processive mechanism of action on the endonucleases, XPF and XPG, involved in producing incisions at sites of DNA damage [4]. We have additionally shown that these endonucleases from XPA cells are unable to incise damaged DNA by a processive mechanism of action and instead utilize a distributive mechanism of action, which is of particular importance when DNA is assembled into nucleosomes [4,5]. The current studies now show that the DNA binding domain of XPA is needed in order for it to act as a processivity factor and to correct the defect in the ability of these endonucleases from XPA cells to incise damaged DNA by a processive mechanism of action.

Two specific domains of XPA were examined for their importance in the ability of XPA to act as a processivity factor. These were the N-terminal domain, which is needed for incision activity of the endonucleases involved in NER [20,21], and the central DNA binding domain which is needed for binding to damaged DNA (Fig. 4) [22–24]. Within the N-terminal domain of XPA is a region encoded by exon 2 that associates with ERCC1 and is essential for NER activity (Fig. 4) [21,25]. The 12-bp deletion created in this region has been shown to inhibit the ability of XPA to interact with ERCC1 and to increase repair activity in XPA cells or extracts [21]. However, this mutant XPA protein was able to bind to UV-damaged DNA [21]. Thus this region of XPA is necessary for incision activity in NER but not for binding to damaged DNA [21].

In the present study, production of this same E2 mutation in XPA similarly did not affect its ability to bind to UVC-irradiated DNA. In addition, the E2 mutant XPA protein was able to restore the ability of the NER endonucleases from XPA cells to incise damaged DNA by a processive mechanism of action. These results demonstrate that a mutation in *XPA* in exon 2 that disrupts binding of XPA to ERCC1 neither affected the ability of XPA to bind to damaged DNA nor to act as a processivity factor.

Within the central, DNA binding domain of the XPA protein (M98-F219) is the zinc-binding subdomain and a loop-rich subdomain (Fig. 4) [22–24,26]. The zinc subdomain contains a zinc finger motif: Cys_{105}-X_2-Cys_{108}-X_{17}-Cys_{126}-X_2-Cys_{129} [22–24,26]. Replacement of any one of

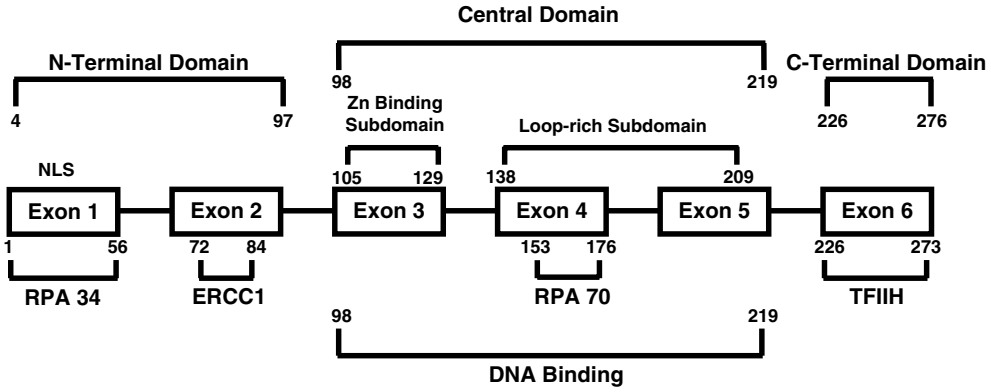

Fig. 4. A map of the *XPA* gene and the domains of the XPA protein each encodes. Numbers refer to amino acid numbers for the XPA protein (adapted from Ref. [30].

the cysteines by a serine leads to disruption of the zinc finger structure and NER [20,27]. In the current study, when the Cys_{105} was mutated to a serine (E3 mutation), the resultant mutant protein was unable to selectively bind UV-irradiated DNA and could not restore the ability of the NER endonucleases from XPA cells to incise damaged DNA by a processive mechanism of action. Therefore, it appears that the zinc-finger subdomain of XPA is important for the ability of XPA to act as a processivity factor.

The major DNA-binding region in XPA is located within the loop-rich subdomain of the DNA binding domain (Fig. 4) [22–24]. It consists of a sheet–helix–loop region and a helix–turn–helix region, between which is a large basic cleft that plays an important role in DNA binding [23,24]. The mutation made in exon 5 (E5 mutation) converts arginine 207, which contributes to the positive charge in this cleft, to a termination. Two different XPA patients, with severe symptoms of XP, have this mutation (XP12RO and XP25RO) [28–30]. In the current study, the E5 mutant XPA protein was unable to bind UV-irradiated DNA and it could not restore the ability of the NER endonucleases from XPA cells to incise damaged DNA by a processive mechanism of action. Thus it appears that this subdomain of XPA is important for its ability to act as a processivity factor. These studies indicate that the ability of XPA to bind to damaged DNA is important for its ability to function as a processivity factor.

A processive mechanism of action increases the efficiency of interaction of a protein with its substrate and accelerates the rate of target location [6–9]. A protein can either act processively itself or processivity can be mediated by another protein. A number of proteins act as processivity factors conferring a processive mechanism of action on specific proteins. These include UL42, PCNA, and RF-C [31,32]. A number of such processivity factors have a well-developed groove or cleft that binds to or has a loose topological association with DNA and allows the protein to translocate along the DNA with its associated protein in a processive manner [7,32].

NMR studies indicate that the basic cleft in the loop-rich subdomain of the XPA binding domain could accommodate the phosphodiester backbone of double-stranded DNA [23,24].

Thus it is possible that the structure of XPA with its basic cleft could allow it to track along the DNA, searching for sites of damage in conjunction with XPC-hHR23B and RPA. A mutation in this cleft and the formation of a truncated protein, such as the one occuring in the E5 mutant, could thus potentially affect the ability of XPA to track along the DNA in its proposed role as a processivity factor.

We have additionally hypothesized that the ability of XPA to confer a processive mechanism of action on XPF and XPG is of particular importance when the DNA is present in nucleosomes [6]. We have shown that when these endonucleases loose their ability to act processively, as that which occurs in XPA cells, there is a deficiency in their ability to incise damaged nucleosomal DNA [4]. Mortelmans et al. [33] and Kano and Fujiwara [34] have similarly shown that endonucleases in XPA cells are able to incise UVC-damaged naked DNA but not damaged nucleosomal DNA. The XPA cell lines used in all these studies had mutations in the DNA binding domain in XPA [30]. These studies, combined with the present results, suggest that the DNA binding domain of XPA is needed for the action of XPA as a processivity factor and for its ability to enable XPF and XPG to incise damaged nucleosomal DNA.

Of particular interest is the finding that XPA patients with some of the most severe clinical symptoms of the disorder have mutations in the *XPA* gene in exons 3, 4, and 5, which code for the DNA binding domain of XPA [22,30]. Thus, it may be possible to hypothesize that the severity of the clinical manifestations of some XPA patients may be related to a deficiency in the ability of the XPA protein to bind to damaged DNA and to act as a processivity factor, which in turn enables the NER endonucleases to incise damaged nucleosomal DNA.

Acknowledgment

This work was supported by Grant R01 ES011298 to M.W.L. from the NIH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.125.

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