Structural basis for the recruitment of ERCC1-XPF to nucleotide excision repair complexes by XPA

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The nucleotide excision repair (NER) pathway corrects DNA damage caused by sunlight, environmental mutagens and certain antitumor agents. This multistep DNA repair reaction operates by the sequential assembly of protein factors at sites of DNA damage. The efficient recognition of DNA damage and its repair are orchestrated by specific protein–protein and protein–DNA interactions within NER complexes. We have investigated an essential protein–protein interaction of the NER pathway, the binding of the XPA protein to the ERCC1 subunit of the repair endonuclease ERCC1-XPF. The structure of ERCC1 in complex with an XPA peptide shows that only a small region of XPA interacts with ERCC1 to form a stable complex exhibiting submicromolar binding affinity. However, this XPA peptide is a potent inhibitor of NER activity in a cell-free assay, blocking the excision of a cisplatin adduct from DNA. The structure of the peptide inhibitor bound to its target site reveals a binding interface that is amenable to the development of small molecule peptidomimetics that could be used to modulate NER repair activities in vivo.

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Introduction

The repair of chemical insults to DNA caused by UV light and other mutagens is essential for coping successfully with the intrinsic reactivity of DNA and preserving genetic information. Inherited diseases resulting in the failure to correct spontaneous or environmentally induced damage are typically associated with genomic instability and a predisposition to various cancers (Friedberg et al., 2005). Contrarily, DNA repair is undesirable when DNA-damaging agents are used for chemotherapy of cancer and other diseases. In these settings, the ability to modulate the DNA repair activities of cells targeted for destruction is a desirable goal (Ding et al., 2006). The nucleotide excision repair (NER) pathway is essential for normal genomic maintenance, removing bulky chemical adducts from DNA that are otherwise mutagenic or would pose lethal blocks to replication.

NER involves over 30 proteins that recognize damaged sites in DNA and excise an oligonucleotide containing the damage (de Laat et al., 1999; Gillet and Schärer, 2006). Following excision of the damaged DNA segment, the resulting gap is filled by templated DNA synthesis and ligase seals the nick to complete the repair. Cell biological and biochemical studies have shown that NER operates by the sequential assembly of protein factors at the sites of DNA damage, rather than through the action of a preformed repressorome (Houtsmuller et al., 1999; Volker et al., 2001). The recruitment of NER factors into protein–DNA ensembles is guided at each step by numerous protein–protein interactions (Araujo et al., 2001), imparting specificity to the recognition and verification of damaged sites. Damage recognition in NER culminates in the incision of DNA 5′ and 3′ to the lesion site by ERCC1-XPF and XPG, respectively, to release a 24–32 nucleotide segment containing the damage (de Laat et al., 1999; Gillet and Schärer, 2006).

For the NER pathway, DNA cleavage by ERCC1-XPF requires physical interaction with XPA, a scaffold protein that interacts with DNA and several repair proteins, including RPA, TFIIH and the ERCC1 subunit of ERCC1-XPF (Li et al., 1994, 1995; Park and Sancar, 1994; Saijo et al., 1996). Although XPA was originally described as a DNA damage-specific sensor or verification protein, recent work suggests that XPA instead recognizes the DNA structural intermediates arising during processing by NER (Jones and Wood, 1993; Camenisch et al., 2006). XPA recruits ERCC1-XPF to NER complexes (Volker et al., 2001), positioning the XPF nuclease domain at the 5′ side of the damage site (Enzlin and Schärer, 2002). ERCC1-XPF has other roles in DNA metabolism outside of NER, notably in interstrand crosslink repair and homologous recombination (Hoy et al., 1985; Niedernhofer et al., 2001). The importance of these additional, NER-independent functions of ERCC1-XPF is underscored by the pronounced sensitivity to crosslinking agents caused by mutations of ERCC1 or XPF in mice and humans (McWhir et al., 1993; Weeda et al., 1997; Niedernhofer et al., 2006).
However, the exact biochemical role(s) of ERCC1-XPF in crosslink repair remain to be discovered.

Li et al (1994, 1995) identified residues 59–114 in XPA as the site of interaction with ERCC1, and showed that deletion of three conserved glycines (Gly72, Gly73, Gly74) abrogates the XPA–ERCC1 interaction as well as the ability of the XPA protein to confer UV resistance to XP-A cells. Furthermore, the expression of a truncated protein comprising residues 59–114 of XPA renders cells sensitive to UV light and cisplatin (Rosenberg et al, 2001), suggesting that this region is sufficient to disrupt the interaction of the native XPA protein with ERCC1-XPF. Conversely, it can be inferred from several previous studies that residues 92–119 of ERCC1 are necessary for the interaction with XPA (Li et al, 1994; Sijbers et al, 1996; Gaillard and Wood, 2001).

Following these seminal studies, understanding of the biochemical and structural basis for XPA’s interaction with ERCC1 has not advanced, although more is known about the individual proteins. XPA contains a well-defined central domain (residues 98–219; Figure 1A), although the remainder of the protein including the ERCC1 interaction domain appears to be poorly structured (Buchko et al, 1998, 2001; Ikegami et al, 1998; lakoucheva et al, 2001). Residues 92–119 of ERCC1 fall within the central domain of ERCC1 (Tsodikov et al, 2005) that is structurally homologous to the nuclease domain of the archaeal XPF-like proteins Aeropyrum pernix Mus81/XPF (Newman et al, 2005) and Pyrococcus furiosus Hef (Nishino et al, 2003). AV-shaped groove on the surface of ERCC1 corresponds to the nuclease active site of XPF (Enzlin and Schärer, 2002; Nishino et al, 2003; Newman et al, 2005), except that ERCC1’s groove lacks the catalytic residues of a nuclease active site and is instead populated with basic and aromatic residues (Gaillard and Wood, 2001). We previously showed that the central domain of ERCC1 binds to single-stranded DNA in vitro (Tsodikov et al, 2005), and proposed the V-shaped groove as the DNA-binding site.

These observations prompted us to investigate the structural and functional basis for the interaction of XPA and ERCC1, and its role in recruiting the XPF-ERCC1 endonuclease to sites of NER. Here, we describe the structure of a short peptide motif from XPA in complex with the central domain of ERCC1. We show that this XPA peptide specifically inhibits the NER reaction in vitro, creating an opportunity for structure-based design of NER inhibitors targeting this protein–protein interaction. Point mutations in the corresponding region in XPA abolish NER activity in vitro, underscoring the importance of the XPA–ERCC1 interactions for NER. In addition to providing insights into how protein–protein interactions mediate progression through the NER pathways, our studies will provide a blueprint to develop small molecules that interrupt the interaction between XPA and ERCC1. Such molecules should be valuable for studying the biochemical functions of ERCC1-XPF in NER and other repair pathways including DNA interstrand crosslink repair and homologous recombination by selectively inhibiting NER.

Results

Induced fit of the XPA peptide upon interaction with ERCC1

Previous reports have suggested that the ERCC1-interacting region of XPA (Figure 1A) is unfolded in solution, based on NMR studies and its sensitivity to proteolytic cleavage (Buchko et al, 2001; lakoucheva et al, 2001). To investigate the structure of the XPA ligand, we collected HSQC NMR spectra of an 15N-labeled XPA59–93 peptide alone and in complex with unlabeled central domain of ERCC1 (the ERCC192–214 protein; Figure 1B). In the absence of ERCC1, the resonance signals for XPA cluster in a narrow range of chemical shifts (Figure 1B, inset) that is characteristic of an unstructured polypeptide with poor spectral dispersion. In the complex with ERCC1, a subset of XPA backbone amides become well dispersed and the peaks are broader. These changes are indicative of a well-structured region within the bound XPA peptide. Only a few resonance peaks are markedly perturbed when XPA59–93 is bound to ERCC1, and among these, three glycine residues (assigned as Gly72, Gly73 and Gly74) are strongly perturbed in the complex. In order to overcome the peak broadening observed in NMR spectra of the XPA59–93 peptide at concentrations above 0.1 mM, we sought to identify shorter XPA peptide ligands for ERCC1. A minimal, 14-residue XPA67–80 peptide (described below) was identified by examining a series of overlapping XPA fragments from the region previously shown to interact with ERCC1 (Li et al, 1994).

The structure of XPA in complex with ERCC1

A synthetic XPA67–80 peptide with amino-acid sequence KIIDTGGFILEEEE forms a stable complex with ERCC196–214 that can be purified by gel filtration chromatography. Like full-length XPA protein, the XPA59–93 and the XPA67–80

Figure 1 XPA domain organization and structure of the ERCC1-binding peptide. (A) The ERCC1-binding region of XPA (residues 67–77) is located between the central domain (Zn2+–binding and DNA-binding subdomains; residues 98–219) and an N-terminal region (residues 1–58) that is dispensable for functional complementation of NER in whole-cell extracts from XP-A mutant cells (Miyamoto et al, 1992) and a TFIIH-binding region (Park et al, 1995). (B) 15N HSQC spectrum of 15N-labeled XPA59–93 in complex with unlabeled ERCC1, and in the unbound state (inset). The spectrum of the unbound XPA59–93 (inset) is characteristic of an unfolded peptide. The appearance of new well-dispersed NMR peaks in the XPA spectrum upon addition of ERCC192–214 (shown in the larger spectrum) indicates that a portion of the XPA peptide adopts a defined conformation in complex with ERCC1.
peptides behave similarly and efficiently co-purify with ERCC1, suggesting that XPA\textsubscript{67–80} contains all significant binding determinants. We confirmed that XPA and ERCC1 form a stoichiometric 1:1 complex by estimating the amount of each subunit in the purified complex using an Edman degradation reaction, and by analytical centrifugation of the complex. Equilibrium sedimentation data for the complex (Supplementary Figure 1C) were best fit to the expected masses for a 1:1 complex of XPA\textsubscript{59–93} and ERCC1\textsubscript{92–214} (\(M_w = (19.4 \pm 1.2)\) kDa) and unbound ERCC1\textsubscript{92–214} (\(M_w = (15.0 \pm 1.0)\) kDa). We confirmed that ERCC1\textsubscript{96–214} binds stoichiometrically to the XPA\textsubscript{67–80} peptide with a \(K_D\) of 0.78 μM (Supplementary Figure 2). A structure of the XPA\textsubscript{67–80}–ERCC1\textsubscript{96–214} complex (Figure 2A) was determined by a combination of NMR-derived distance restraints and X-ray diffraction data extending to 4 Å resolution (Table 1 in the Supplementary data and Materials and methods) as described below.

Identifying the ERCC1-binding site in complex with XPA

The binding site for XPA on the surface of ERCC1 (Figure 2B) was identified using two-dimensional HSQC experiments. The spectrum of unliganded \(^{15}\)N-labeled ERCC1\textsubscript{92–214} (blue, Figure 3) showed significant differences from that of the complex with unlabeled XPA\textsubscript{67–80} (red, Figure 3). However, complexes of ERCC1\textsubscript{96–214} with either XPA\textsubscript{67–80} or XPA\textsubscript{59–93} were identical, suggesting that the shorter XPA peptide makes all of the significant binding contacts. The \(^{15}\)N HSQC spectrum of the ERCC1–XPA complex is consistent with a slow-exchange regime, implying a dissociation equilibrium constant below 1 μM for the complex. The XPA-binding site on the ERCC1 central domain was identified using the backbone assignments for ERCC1\textsubscript{96–214} alone and in complex with XPA (see Materials and methods). A comparison of the \(^{15}\)N HSQC spectra for ERCC1 in the presence and absence of XPA reveals that, with only one exception, the most prominent changes in chemical shifts involve a cluster of residues within a V-shaped groove of the ERCC1 central domain (Figures 2B and 3).

The bound XPA peptide fits snugly into the V-shaped groove of ERCC1 (Figure 2) that we previously speculated could be a binding site for single-stranded DNA (Tsodikov et al., 2005). Three consecutive glycines (Gly72, Gly73, Gly74) of the XPA peptide insert into the groove, making a U-turn with close steric complementarity to the binding site. These are the same three conserved glycines previously reported to be essential for the interaction of XPA with ERCC1 and required for the functional complementation of XP-A cells (Li et al., 1994, 1995). A total of 1039 Å\(^2\) of

Figure 2 Structure of the XPA–ERCC1 complex. (A) The XPA\textsubscript{67–80} peptide (orange) is bound to a V-shaped groove of the central domain of ERCC1\textsubscript{96–214} (green). An orthogonal view of the bound XPA peptide (left side) is shown in comparison to the peptide in complex with ERCC1 (right-hand side). (B) The XPA-binding site on the surface of ERCC1 (colored red) was identified by resonance perturbations larger than 0.2 ppm that are indicative of direct interactions with XPA.
accessible surface area from XPA peptide is buried in the complex with ERCC1, accounting for 61% of the solvent accessible surface area of XPA residues 67–77, which are in close proximity to the binding site. The XPA ligand derives many interactions from the core sequence motif (shown in boldface; KIIDTGGGFILEE) of the XPA 67–80 peptide. The side chains of Phe75, Leu77 and Thr71 of XPA are clustered together at the mouth of the V-shaped groove (Figure 2A) where Phe75 stacks against Asn110 of ERCC1, and the Ile76 side chain packs against the aliphatic portion of ERCC1 side chains Arg144 and Leu148. The binding groove in ERCC1 is capped by XPA Leu77.

The glycine-rich loop of XPA 67–80 extends far into the groove of ERCC1 where main chain atoms of these XPA residues stack against the side chains of Tyr145 and Tyr152 from ERCC1 (Figure 2A). The main chain amides of these glycines could participate in hydrogen-bonding interactions with the backbone amides of Tyr145 and Tyr152, respectively. The side chain of XPA Asp70 could participate in electrostatic interactions with the side chain His149 of

Figure 3 XPA67–80 binds in a shallow groove of ERCC1. (A) A comparison of the two-dimensional HSQC spectra for 15N-labeled ERCC192–214 in the presence and absence of an unlabeled XPA67–80 peptide. The 15N HSQC spectra reveal significant chemical shift changes for some ERCC1 residues in the absence (blue) or presence (red) of unlabeled XPA67–80. (B) Combined average chemical shift perturbations are calculated as \( \Delta_{\text{ave}} = \sqrt{\left(\Delta_{1H}^2 + \Delta_{15N/5}^2\right)/2} \) for each backbone amide of ERCC1 and shown as a histogram.

Figure 4 The XPA67–80 peptide is an effective inhibitor of NER activity. (A) XPA67–80 inhibits the in vitro NER reaction, whereas the mutant XPA67–80 F75A peptide has no effect. HeLa cell extracts were incubated with a plasmid containing a 1,3-intrastrand cisplatin adduct in the presence of increasing concentrations of either XPA67–80 or XPA67–80 F75A (lane 1, no XPA; lanes 2 and 7, 46 nM XPA peptide; lanes 3 and 8, 460 nM; lanes 4 and 9, 4.6 \( \mu \)M; lanes 5 and 10, 46 \( \mu \)M; lanes 6 and 11, 92 \( \mu \)M). Products were visualized by a fill-in reaction following annealing to an oligonucleotide complementary to the excision product with a 4-nt overhang (Shivji et al., 1999). The marker DNA ladder is labeled LMW DNA ladder. (B) XPA67–80 and XPA67–80 F75A do not affect the intrinsic nuclease activity of ERCC1-XPF. The stem12-loop22 substrate (6.6 nM) was incubated with different concentrations of ERCC1-XPF (lanes 2, 4 and 6: 6.7 nM ERCC1-XPF; lanes 3, 5 and 7: 26.8 nM) and 0.4 mM MnCl2 in the presence of no peptide (lanes 1–3), 92 \( \mu \)M XPA67–80 (lanes 4 and 5), and 92 \( \mu \)M XPA67–80 F75A (lanes 6 and 7). The DNA substrate and the cleavage products are indicated.
ERCC1. It is notable that a solvent-exposed salt bridge between the side chains of Asp129 and Arg156 of ERCC1 (PDB code 2A1; Tsodikov et al., 2005) becomes almost completely buried when XPA is bound.

Phε75 of XPA is completely buried within the ERCC1-binding site (Figure 2A). We tested whether an alanine substitution at this position interferes with binding to 15N-binding site (Figure 2A). We tested whether an alanine completely buried when XPA is bound.

For the former possibility, we tested the effects of XPA et al., 2007). The wild-type, F75A, G73A and G73A/G74A XPA proteins all bound with similar affinity to a three-way junction (Figure 5B), indicating that the mutant proteins are fully proficient in DNA binding and unlikely to be misfolded or otherwise inactive. These results show that single point mutations in XPA can result in a defect in NER.

The XPA peptide inhibits NER in mammalian cell extracts

The direct interaction of XPAΔ7−80 peptide with the ERCC1-binding pocket raised the possibility that this peptide might specifically interfere with the recruitment of the ERCC1-XPF nuclease into the NER reaction pathway. We investigated the effect of XPAΔ7−80 and the mutant XPAΔ7−80 peptide on the dual incision of a DNA lesion during NER in cell-free extracts. A plasmid containing a single site-specific 1,3-cisplatin intrasstrand crosslink was incubated with HeLa cell-free extract in the presence of increasing concentrations of XPA peptide (Shivji et al., 1999). In the absence of XPA peptide, the characteristic NER excision products of 28–33 nucleotides containing the lesion were evident (Figure 4A, lane 1). Increasing concentrations of XPAΔ7−80 interfered with excision of the oligonucleotide, and complete inhibition was achieved at a concentration of XPA peptide in the low micromolar range (Figure 4A, lanes 2–6). In contrast, the addition of XPAΔ7−80 did not affect NER activity at concentrations up to 92 μM, the maximum concentration tested (Figure 4A, lanes 7–11).

The XPA peptide might inhibit NER activity in vitro by directly interfering with the endonuclease activity of ERCC1-XPF, instead of blocking its interaction with XPA. To account for the former possibility, we tested the effects of XPA peptides on the DNA incision reaction catalyzed by purified ERCC1-XPF using stem-loop DNA substrate (de Laat et al., 1998). ERCC1-XPF efficiently cleaves on the 5′ side of the loop and the XPA peptide has no effect on this activity (Figure 4B) even at a concentration (92 μM) that completely abolishes NER activity (Figure 4A). We conclude that the inhibitory effect of XPAΔ7−80 on the NER reaction results from disrupting the interaction of ERCC1 with XPA, an essential protein-protein interaction for the dual incision of DNA by the NER pathway.

Mutations in the ERCC1-binding epitope of XPA abolish NER

The specificity of inhibition of NER by XPAΔ7−80 suggested that mutations of single residues such as F75 might diminish the NER activity of the XPA protein. We generated mutant XPA proteins containing an F75A mutation and ΔG73 single and ΔG73/ΔG74 double deletion, and compared their activities to that of the wild-type XPA protein. The ability of the XPA proteins to mediate NER activity was tested by incubating a plasmid containing a 1,3-cisplatin intrasstrand crosslink with a cell-free extract generated from XPA-deficient cells supplemented with purified wild-type or mutant XPA protein (Shivji et al., 1999). Addition of wild-type XPA protein to this mixture resulted in robust NER activity, as evidenced by formation of the characteristic excision products of 24–32 nts in length (Figure 5A, lanes 1–2). By contrast, no NER activity was observed following addition of the F75A or G73A/G74A mutants, while the G73A single deletion mutant displayed marginal NER activity.

To test if these XPA mutations only affected binding to ERCC1, we also compared the DNA-binding activities of wild-type and mutant XPA proteins. We investigated the binding of wild-type and mutant XPA to a DNA three-way junction, representing a high-affinity target for XPA in band-shift assays (Missura et al., 2001). The wild-type, F75A, G73A and G73A/G74A XPA proteins all bound with similar affinity to a three-way junction (Figure 5B), indicating that the mutant proteins are fully proficient in DNA binding and unlikely to be misfolded or otherwise inactive. These results show that single point mutations in XPA can result in a defect in NER.

Figure 5  Mutation of the ERCC1-binding epitope of XPA abolishes NER but not DNA-binding activity. (A) XP-A (XP2OS) cell extracts were incubated with a plasmid containing a 1,3-intrasstrand cisplatin adduct in the presence of wild-type XPA (XP-A WT) or mutant XPA proteins (XP-A-F75A, XPA-G73A or XPA-G73A/G74A). The reaction products were visualized by a fill-in reaction after annealing the oligonucleotides were separated on an 8% native polyacrylamide gel. The reaction products generated with different concentrations of XPA are shown: 0 (lane 1), 4 nM (lanes 2, 7, 13, 17), 10 nM (lanes 3, 8, 13, 18), 25 nM (lanes 4, 9, 14, 19), 60 nM (lanes 5, 10, 15, 20) and 150 nM (lanes 6, 11, 16, 21).
activity by weakening the interaction between ERCC1 and XPA. Due to the highly cooperative nature of NER (Moggs et al., 1996), other NER functions and interactions may be disrupted as a result of blocking the recruitment of XPF–ERCC1.

**XPA competes with single-stranded DNA for binding to ERCC1**

Because XPA binds in the groove on ERCC1 (Figure 2) that was previously implicated in DNA-binding activity (Tsodikov et al., 2005), we directly tested whether or not XPA competes with single-stranded DNA for binding to ERCC1. DNA-binding activity was measured by monitoring fluorescence anisotropy, using single-stranded 40-mer oligonucleotide labeled on the 5' end with 6-carboxyfluorescein. The XPA$_{67-80}$ peptide does not detectably bind to DNA (not shown), although it does compete with DNA for binding to ERCC1 (Supplementary Figure 3). This result confirms that the DNA-binding site on ERCC1’s central domain overlaps with the XPA-binding site. The $K_d$ for binding of XPA$_{67-80}$ is in the micromolar range, but quenching of the fluorescent probe by high concentrations of XPA precluded an accurate measurement of the binding constant. We previously reported an equilibrium-binding constant of 1.5 µM for DNA binding to the central domain of ERCC1 (Tsodikov et al., 2005). By fitting the XPA competition titration data to a competitive binding model (Equation 9 in the Supplementary data), we obtain the estimated binding constant of $K_d = (540 \pm 280)$ nM for the XPA–ERCC1 complex. This result agrees well with the affinity determined directly for this interaction (Supplementary Figure 2). Thus, XPA binds to the central domain of ERCC1 with approximately three-fold higher affinity than single-stranded DNA.

**Discussion**

The removal of bulky and helix-distorting DNA lesions by the NER pathway requires the coordinated assembly of a large multiprotein complex (Houtsmuller et al., 1999; Volker et al., 2001) that exposes the damaged DNA strand and excises an oligonucleotide containing the lesion (Gillet and Schärer, 2006). We have investigated one of the essential protein–protein interactions in this pathway. The specific interaction of XPA with ERCC1 is responsible for recruitment of the ERCC1-XPF nuclease to the DNA repair complex (Li et al., 1994). Our structural studies have defined the XPA ligand as a TGGGF1 sequence motif that inserts into a pocket of the central domain of ERCC1 (Figure 2). It was previously shown that deletion of the GGG triplet within this motif abolishes the interaction of XPA with ERCC1 (Li et al., 1995). These glycines insert deep into the ERCC1-binding site and are likely to make hydrogen-bonding interactions using main chain atoms (Figure 2). The binding site of ERCC1 is mainly a nonpolar surface that is punctuated by several large aromatic side chains (Phe145, Phe152) and a buried salt bridge between Arg156 and Asp129. We show that single point mutations in XPA (F75A or G73A) effectively abolish NER activity in vitro, underscoring the high specificity of the binding interaction between ERCC1 and XPA.

The XPA peptide ligand is unstructured in solution (Figure 1B). It is therefore remarkable that a short peptide segment binds to ERCC1 with submicromolar affinity, given the associated entropic penalty for binding. This peptide–protein interaction is sufficient to block NER activity in cell-free extracts (Figure 4A), raising the possibility that peptido-mimetic ligands could be developed to specifically block NER activity in vivo. Although the competing XPA peptide prevents the double incision of lesioned DNA during NER, the peptide does not interfere with the cleavage of a model DNA substrate by purified ERCC1–XPF (Figure 4B). These results show that the XPA peptide does not block the nuclease activity of XPF-ERCC1 and is instead likely to interfere with the recruitment of the nuclease into the NER protein complex. Mutations in the ERCC1-binding domain of XPA similarly abolish NER activity without affecting the intrinsic DNA-binding activity of XPA. Intriguingly, the central domain of ERCC1 binds to single-stranded DNA in vitro (Tsodikov et al., 2005) and this DNA-binding activity is blocked by XPA (Supplementary Figure 3). Although these competing activities at first appear contradictory, our cleavage assays employed full-length ERCC1 protein in complex with XPF, whereas the in vitro binding studies used the central domain only of ERCC1. Since ERCC1-XPF has multiple DNA-binding sites (Newman et al., 2005; Tripsianes et al., 2005; Tsodikov et al., 2005; Nishino et al., 2005a,b), it is likely that some of the other DNA-binding surfaces of the XPF–ERCC1 heterodimer can compensate for the interference by the XPA peptide. With respect to the overall NER reaction, it is conceivable that DNA and the XPA protein alternatively bind to the same site on ERCC1 during different steps of the repair process. In this regard, a molecular handoff of ERCC1 from XPA to one strand of the unwound DNA substrate could be envisioned as one of multiple, individually weak interactions that drive the progression through the NER pathway in a concerted fashion (Stauffer and Chazin, 2004; Gillet and Schärer, 2006).

Homologs of the XPA and ERCC1 proteins are found only in eukaryotic organisms, despite the presence of homodimeric XPF-like endonucleases in the Archaea (Nishino et al., 2003). The ERCC1 residues constituting the XPA-binding site are poorly conserved in *Saccharomyces cerevisiae* (Rad10 protein) and *S. pombe* (Swi10 protein), and the XPA homologs (*S. cerevisiae* Rad14; *S. pombe* Rhp14) are highly divergent from mammalian XPA. Indeed, a different interaction site has recently been reported for Rad14 and Rad10/Rad1, the respective yeast homologs of XPA and ERCC1–XPF (Guzder et al., 2006). These observations suggest that XPA and ERCC1 may have coevolved to interact specifically with each other in higher eukaryotes, perhaps in response to the added complexity and distinct functional organization of the eukaryotic NER pathway. Correspondingly, a BLAST search does not identify the TGGGF1 motif of the XPA ligand in any other mammalian protein.

In conclusion, we have established that only a short peptide segment of XPA is sufficient to form a stable and specific 1:1 complex with ERCC1. The interactions of three consecutive glycines (Gly72, Gly73, Gly74) and several flanking residues of XPA complement a V-shaped, hydrophobic groove in the central domain of ERCC1. This protein–protein interaction is essential for NER activity, and the XPA peptide is an effective inhibitor of NER activity in a cell-free reaction. This work paves the way for development of specific NER inhibitors targeting the surface of ERCC1 involved in XPA binding. ERCC1 has served as a molecular marker for clinical
resistance to cisplatin-based chemotherapy (Reed, 2006), raising the possibility of using ERCC1 antagonists as sensitizing agents for tumors resistant to this and other DNA-damaging agents in the treatment of cancer.

Materials and methods

**Peptide and DNA**
The XPA214–80 peptide corresponding to residues 67–80 of the XPA protein and the mutant peptide XPA214–80 were synthesized by solid phase methods, then HPLC-purified by the Molecular Biology Core Facility at Tufts University (Boston, MA). A 40-mer DNA oligonucleotide 5′-CCGGTGGCCACCGCTCGCCGCTG-3′ with a 5′ 6FAM label (Integrated DNA Technologies) was gel-purified by conventional techniques.

**Protein expression and purification**
The central domain of ERCC1 (constructs ERCC192–214 or ERCC196–214) with an N-terminal His tag was expressed and purified as previously described (Tsodikov et al, 2005). Fragments of the XPA protein (XPA1–273, XPA28–273, XPA59–219, XPA93–93) were cloned into pET19b-pps, in which an N-terminal (His)10 tag is purified as previously described (Tsodikov et al, 2005). The three-way junction DNA substrate described previously (Shivji et al, 2003) was gel-purified by conventional techniques.

**Analytical ultracentrifugation**
Sedimentation equilibrium experiments with ERCC192–214 and the complex ERCC192–214/XPA93–93 were performed using Beckman XL-A Analytical Ultracentrifuge. In both cases, proteins were at concentrations of 0.3–0.5 mg/ml in NMR Buffer (20 mM Tris buffer pH 7.2, 50 mM NaCl, 2 mM -mercaptoethanol and 0.1 mM EDTA). Sedimentation equilibrium data were analyzed as described in the Supplementary data.

**Crystallization of XPA–ERCC1 complex, data collection and analysis**
Protein crystallization and X-ray data collection are described in the Supplementary data. A complete and redundant X-ray data set was collected and processed using HKL2000 (Otwinowski and Minor, 1997). The crystals belong to space group I4 132 with one complex ERCC196–214 or XPA59–93 per asymmetric unit. The structure was solved by molecular replacement (MR) methods using the program PHASER (McCoy et al, 2005) and the crystallographic model of the ERCC1 central domain (Tsodikov et al, 2005; PDB code 2A11) in which the residues C-terminal to residue 214 were deleted. A difference (Fo – Fc) electron density map calculated with phases from the MR solution revealed the bound XPA peptide. The XPA peptide was built into the difference density using distance restraint information from NMR experiments and the structure of the complex was then refined as described below, with strong geometric restraints imposed on the ERCC1 subunit due to the low-resolution diffraction data and the absence of intramolecular distance information for ERCC1. All experimental XPA–ERCC1 distance restraints were accommodated without violations using the structure of unbound ERCC1, suggesting that ERCC1 does not undergo significant conformational changes upon binding to XPA.

**NMR experiments and determination of the structure of XPA–ERCC1 complex**
All NMR data were acquired in the NMR Buffer described above. The protein concentrations were 0.25 mM for free ERCC196–214 or ERCC192–214 (which behaved similarly in all experiments), 0.25 mM for ERCC192–214 in complex with a synthetic XPA28–80 peptide and 0.1 mM for ERCC192–214 complex with XPA93–93 fragment. Higher protein concentrations resulted in line broadening and lower quality NMR spectra. Backbone assignments of the free ERCC192–214 and ERCC196–214/XPA93–93 complex were performed using a standard set of triple-resonance experiments: HNCA/HN(CO)CA, HN(CA)CO and HNCO/HN(CA)CO.

Structural information for the ERCC1–XPA complex was obtained with a differentially labeled sample in which ERCC1 was [15N]-labeled and perdeuterated and the synthetic XPA fragment was unlabeled (D, N-ERCC1/U-XPA) (Walters et al, 1997, 2001). The assignment of the XPA peptide in this sample was performed using homonuclear 2D NOESY and 2D TOCSY experiments acquired in H2O and D2O buffers. The total of 92 intramolecular distance constraints for the XPA peptide were derived from the 2D NOESY experiment acquired in H2O with 100 ms mixing time. Intermolecular distance restraints were derived from a [15N] dispersed NOE-HSQC experiment acquired on the D, N-ERCC1/U-XPA sample using 200 ms mixing time. A total of 23 intermolecular distance restraints between the amide protons of ERCC1 and the protons of XPA were derived from this experiment. The structure of the ERCC1–XPA complex was calculated using simulated annealing protocol in XPLOR-NIH (Schwieters et al, 2003). The total energy term used in the calculation incorporated all of the NMR-derived distance restraints as well as the 4 Å X-ray data. Ten lowest energy structures out of 100 calculated were deposited in the PDB with accession code 2JNW. The solvent accessible surface areas were calculated for the lowest energy structure using Surface Racer 4.0 (Tsodikov et al, 2002) with the solvent probe radius of 1.4 Å.

**Competitive binding equilibrium titrations**
Fluorescence anisotropy measurements were performed as previously reported (Tsodikov et al, 2005). The equilibrium titrations and their analysis are described in detail in the supplementary data.

**Construction and expression of mutant XPA proteins**
Site-directed mutagenesis using the QuikChange kit (Stratagene) introduced point mutations in the expression vector pET15b-XPA was performed. pET15b-XPA served as template and oligonucleotide primers used to generate the mutations contained the desired mutation, and a marker restriction site for selection. The following primers were used (restriction site are underlined and indicated, modified nucleotides are shown in italics):

XPA-F75A: GACACAGGAGGAGGCGCCATCTTAGAAGGAGGAAG
(Xbal)

XPA-A74: GACACAGGAGGATCATCTAGAAGGAGGAAG
(Xbal)

XPA-A73/74: GTAATGTACAGAGATTATCTAGAAGGAGGAAG
(Xbal)

XPA-A73/74: GTAATGTACAGAGATTATCTAGAAGGAGGAAG
(Xbal)

Positive clones were fully sequenced to rule out the introduction of additional mutations. Mutant XPA proteins were expressed in Escherichia coli BL21(DE3) lyS cells and purified by chromatography on nickel-NTA, gel filtration and heparin chromatography.

**Nuclease assay**
ERCC1-XFP was purified and nuclease assays using a stem-loop substrate were carried out as described previously (Enzlin and Schärer, 2002) (see Supplementary data for details).

**DNA-binding assays**
The three-way junction DNA substrate described previously (substrate 7 in Table 1 of Hohl et al, 2003) was 5′-32P-end labeled and incubated at 1 mM concentration with various amounts of XPA in EMSA buffer (25 mM HepesKOH pH 8.0, 30 mM KCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.1 mg/ml BSA) at a reaction volume of 15 μl. After equilibration at room temperature for 30 min, the samples were loaded on a 5% (37.5:1) native polyacrylamide gel containing 0.5 × TBE and electrophoresed at 90 V for 2 h. Gels were dried and the radioactive bands visualized by autoradiography.

**In vitro NER assay**
This assay was performed using an established protocol (Shivji et al, 1999), which is described in detail in the Supplementary data.

**Supplementary data**
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).
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