Molecular analysis of the function of Xeroderma pigmentosum group A protein by site-directed mutagenesis

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Molecular Analysis of the Function of Xeroderma Pigmentosum Group A Protein by Site-directed Mutagenesis

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TABLE OF CONTENTS

1. INTRODUCTION

DNA, the carrier of our genetic information, is constantly damaged by different genotoxic agents such as UV-light, carcinogenic compounds or endogenous reactive metabolites. The resulting DNA lesions, if persistent, can induce mutations and ultimately lead to cancer or cell death. To avoid such adverse effects, all living organisms have evolved an intricate network of DNA response pathways, including multiple DNA repair strategies [1-3].

Nucleotide excision repair (NER) is an extremely versatile DNA repair pathway that eliminates bulky base adducts, including UV-light induced photoproducts and DNA adducts generated by carcinogenic chemicals. Inherited defects in this DNA repair mechanism cause xeroderma pigmentosum (XP) in humans, which is a rare syndrome characterized by a >1'000-fold increased risk of sunlight-induced skin cancer. Individuals affected by XP are classified into seven repair-deficient complementation groups designated XP-A through XP-G [3-7].

The NER pathway requires the coordinated activity of approximately 30 different proteins. This mechanism involves a multistep "cut and patch" reaction in which the damaged DNA strand is incised on either side of the lesion and then the base adduct is released as the component of an oligonucleotide fragment. The excised oligomer is replaced by DNA repair synthesis using the intact complementary strand as the template. In the context of mammalian chromosomes, this repair activity is subdivided in two subpathways [2,5]. "Global genome repair" (GGR) removes DNA lesions from the entire genome regardless of whether any specific sequence is transcribed or not. In addition, "transcription-coupled repair" (TCR) pathway removes DNA adducts preferentially from the transcribed strand of active genes.

The core subunits required for damage excision in the GGR pathway include XPC, a human homolog of yeast Rad23B (hHR23B), transcription factor IIH (TFIIH), replication protein A (RPA), XPA, excision repair cross complementing 1 (ERCC1), XPF and XPG [8-11]. The order of arrival of these core NER factors is still debated, but the favored model proposes the assembly of a multi-subunit complex triggered by XPC protein together with hHR23B and centrin-2 [11-13]. After initial recognition of damaged sites by the XPC subunit, the NER pathway is thought to proceed with the sequential recruitment of TFIIH, XPA and RPA. As a last step, the two endonucleases XPG and XPF-ERCC1 are recruited.

The present thesis is concerned with the function of XPA protein, which undergoes direct interactions with many other NER subunits and is absolutely required for both the GGR and TCR pathway [14-16]. The role of XPA during assembly of the NER complex was unknown, but several possible functions have been assigned to this subunit. XPA protein displays a weak binding preference for damaged DNA substrates in biochemical experiments. Therefore, it was first thought that XPA may constitute the initial damage sensor [17-18]. Alternatively, it has been suggested that XPA protein may be incorporated in the NER complex, after recruitment of XPC and TFIIH, to verify the presence of a lesion [9]. Another study led to the hypothesis that XPA may adopt an architectural role, which is not related to DNA damage recognition, by monitoring the correct three-dimensional assembly of the incision complex [19].

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2. WORKING HYPOTHESIS

XPA is a key subunit in both GGR and TCR, but the molecular function of this protein is highly controversial. It has been proposed that XPA protein may constitute a DNA damage sensor, but it was not clear how a single repair factor may acquire a universal affinity for the very wide spectrum of DNA lesions that are processed by the NER pathway.

The main goal of the project was to test the hypothesis that XPA may not be involved in damage “recognition” or damage “verification”. Instead, XPA may have a regulatory role in inducing and monitoring the correct three-dimensional assembly of the incision complex before activation of the two endonucleases that are responsible for double DNA incision.

3. RECENT STATE OF THE FIELD

A. DNA Repair

Although integrity of the genetic information is essential for normal development, viability, longevity and the health of organisms, the cellular DNA is under permanent attack not only from environmental genotoxic agents but also from endogenous metabolic byproducts that alter its chemical structure. To counteract the continuous formation of genetic damage, living organisms are equipped with a network of DNA repair systems. Briefly, placental mammals employ six major DNA repair pathways to cope with mutagenic insults [1]: (i) mismatch repair to correct replication errors, (ii) DNA damage reversal to remove alkyl groups, (iii) non-homologous end joining to repair double-strand breaks, (iv) homologous recombination to rescue corrupted or deleted chromosomal sequences, (v) base excision repair to eliminate modified or incorrect bases, and (vi) nucleotide excision repair (NER) to remove bulky lesions.

B. Nucleotide Excision Repair

The NER system eliminates DNA lesions by promoting the excision of single-stranded oligonucleotides from damaged strands followed by restoration of an intact double helix by DNA repair synthesis and DNA ligation (Fig1). This type of repair reaction has evolved in all three biological kingdoms to excise photoproducts induced by short-wavelength ultraviolet (UV) light [primarily cyclobutane pyrimidine dimers and (6-4) photoproducts] as well as a wide array of bulky DNA adducts generated by electrophilic carcinogens [2-4]. Other known NER substrates include a subset of oxidative lesions [5-7] and protein-DNA crosslinks [8].

The importance of preventing genetic mutations caused by DNA photoproducts and other NER substrates is illustrated by a direct link between defects in the NER pathway and a devastating cancer-prone disorder in humans. In fact, many NER proteins are encoded by genes that, when mutated, give rise to xeroderma pigmentosum (XP), an inherited disease characterized by extreme photosensitivity and a 2000-fold increased incidence of sunlight-induced skin cancer [1, 9]. XP patients also have a higher risk of internal tumors and, in some cases, neurological complications, probably reflecting the essential role of NER in the removal of oxidative DNA damage [5, 10]. Individuals suffering from this recessive disorder

have been assigned to different complementation groups by cell-fusion experiments and the respective NER genes (*XPA* through *XPG*) were named after the complementation group with which they associate.

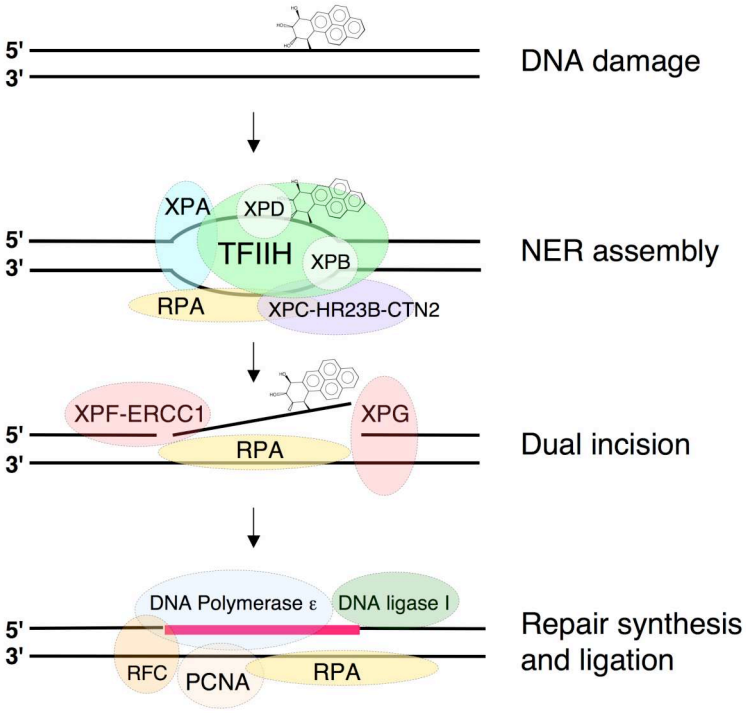

Figure 1: Scheme of the human NER pathway. The damaged strand carries a polycyclic aromatic hydrocarbon adduct. Abbreviations: ERCC1, excision repair cross complementing-1 protein; HR23B, homolog of RAD23B; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A; TFIIH, transcription factor IIH; XPA-XPF, xeroderma pigmentosum complementation group A-F proteins. DNA damage recognition by the action of XPC, XPA, TFIIH and RPA is followed by double DNA incision through the endonucleases XPF-ERCC1 and XPG. The synthesis of repair patches is dependent on RFC, a matchmaker that binds to the excision gap and loads PCNA, which in turn acts as a sliding clamp for DNA polymerases. Finally, the newly synthesized repair patches are sealed by DNA ligase I.

Core subunits of the mammalian nucleotide excision repair

In human cells, the proteins necessary and sufficient for excision activity in the presence of naked substrates *in vitro* without the aid of any other accessory protein include XPC-hHR23B, TFIIH, RPA, XPA, XPG and ERCC1-XPF [11-13]. The order of arrival of these core NER factors is still debated, but a favored model illustrated in

Fig. 1 proposes the assembly of a multi-subunit complex triggered by XPC protein together with one of the mammalian homologs of yeast RAD23 (HR23B) and the calcium-binding protein centrin-2 (CTN2) [14-16]. After initial recognition of damaged sites by the XPC subunit, this pathway is thought to proceed with the sequential recruitment of transcription factor IIH (TFIIH, containing XPB, XPD and other 8 subunits), XPA (a possible homodimer), replication protein A (RPA, 3 subunits), XPG and XPF-ERCC1 (a heterodimer composed of XPF and excision repair cross complementing-1 protein).

Nucleotide excision repair reaction

Irrespective of the order of NER assembly, which will be discussed in more detail below, it has been demonstrated that XPA, XPC, TFIIH and RPA participate in the formation of a stable recognition intermediate [17, 18] characterized by transient unwinding of the duplex substrate (Fig. 1). In conjunction, these four factors generate an open DNA structure that contains "Y-shaped" double to single strand junctions flanking the lesion [18-20]. The endonucleases XPF-ERCC1 and XPG act as "scissors" to cut out DNA damage by cleaving the damaged strand at each of the "Y-shaped" transitions of this open intermediate, thereby releasing injured residues as part of an oligomeric segments of 24-32 nucleotides in length [21]. XPF-ERCC1 makes the 5' incision, whereas XPG is responsible for the 3' incision [22-24]. The DNA scissions are introduced 15-25 nucleotides away from the damaged base on the 5' side but only 3-9 nucleotides away on the 3' side.

To restore duplex integrity, all excision repair reactions depend on the redundancy of the genetic code consisting of two complementary strands. If the nucleotides of one strand are damaged, they are excised and the intact opposing strand is used as a template to direct the synthesis of repair patches. Because the NER pathway generates two separate incisions, it is essential that both scissions occur in the same damaged strand, such that the opposite native sequence is preserved during the excision reaction and later in the pathway can serve as the complementary template for error-free DNA synthesis [11, 25]. However, it is not yet clear what molecular constraints are responsible for the accurate targeting of the incision endonucleases to either side of damaged deoxyribonucleotides.

Assembly of the DNA damage recognition complex

The mechanism of DNA lesion recognition in the NER pathway is a matter of intense debate, raised by the fact than none of the individual core proteins displays a high enough specificity to function as a unique sensor of damaged substrates. In mammals, the NER reaction occurs by the individual recruitment of repair factors to sites of damage, rather than by the action of a preassembled "repairosome". A full excision complex with approximately 20 polypeptide subunits would achieve a mass of >1 MDa, but studies monitoring diffusion rates in the nuclei of intact cells indicate that the NER proteins are present as separate factors and not as part of a large "repairosome" complex. Also, the rapid movement of NER factors to foci of DNA damage is not compatible with the existence of large preassembled complexes and instead favors the individual recruitment of each subunit to lesion sites [26]. One advantage of this stepwise process is that multifunctional proteins may shuttle between repair, transcription, replication, recombination or other nuclear pathways [27]. In addition, the mathematical modeling of various scenarios suggests that an ordered and consecutive assembly of freely diffusing proteins is more efficient than alternative strategies such as the random aggregation of repair factors, or their preassembly into a "repairosome" complex [28].

Initially, two opposing mechanisms have been proposed for the damage recognition step: "XPC first" or "XPA first" [18]. In the "XPC first" model, XPC represents the primary sensor that binds to lesion sites and initiates the NER pathway by recruiting TFIIH and other successive factors [29-31]. This scenario is supported by competition experiments aimed at determining the order in which NER proteins are recruited to the DNA substrate. For example, damaged plasmids preincubated with XPC-HR23B are more rapidly repaired in cell extracts than those previously incubated with XPA and RPA [30], indicating that the NER complex works more efficiently in vitro when XPC is allowed time to bind to DNA before addition of the remaining subunits. In apparent conflict with these reports, other researchers observed that preincubation of damaged DNA with XPA-RPA promotes *in vitro* excision repair more effectively than when the damaged substrate is first incubated with XPC-HR23B [32]. These conflicting models have been reconciled by the notion that XPC, XPA and RPA may act in a cooperative manner to locate the lesions and recruit the TFIIH complex [33]. A conceptual advantage of this concerted action is that three subunits may achieve an increased affinity for damaged DNA by combining the

modest selectivity of each component alone. This cooperative model of damage recognition, although not supported by other evidence, provides a plausible explanation for the efficient *in vitro* excision of cyclobutane dimers, albeit no single subunit displays a significant affinity for this particular UV lesion [31, 34].

An experimental strategy to study NER assembly exploits the nuclear trafficking of core subunits in living cells. Fibroblast monolayers were exposed to UV light through filters with small pores to obtain localized foci of DNA damage. The translocation of XPC and XPA from unirradiated nuclear regions to these damaged foci was monitored by immunological staining after formaldehyde fixation. Volker et al. [14] observed that XPC protein readily accumulates in the DNA repair foci of both wild-type and XP-A cells, but they did not detect any accumulation of XPA protein in the DNA damage foci of XP-C cells. These results have been taken as further evidence for XPC being the first factor that recognizes DNA lesions, whereas XPA is apparently not able to interact with damaged sites in the absence of the XPC subunit [14-15, 19, 30, 34].

The accessory role of UV-DDB

UV-damaged DNA-binding (UV-DDB) protein has been isolated by virtue of its ability to interact preferentially with UV-irradiated DNA fragments [35]. UV-DDB accelerates the excision of UV dimers in human cells [36], but contradictory results have been reported as to how this factor contributes to the NER reaction [18, 37, 38]. UV-DDB is a heterodimer of p127 (DDB1) and p48 (DDB2), with the small subunit being encoded by the *XPE* gene [39-41].

UV-DDB seems to be an initial damage sensor because of its extraordinary preference for UV-irradiated substrates [42, 43]. It binds with high affinity to (6-4) photoproducts and, unlike XPA or XPC, also interacts preferentially with DNA duplexes containing cyclobutane pyrimidine dimers [44]. The binding of UV-DDB to damaged substrates leads to bending of the DNA by an angle of 55° [45], prompting the hypothesis that UV-DDB may recognize photoproducts and distort the DNA around the lesion to promote the subsequent recruitment of XPA or XPC. XP-E cells lacking UV-DDB activity are compromised in the repair of cyclobutane dimers but retain the ability to excise (6-4) photoproducts [46]. Similarly, rodent cells, which fail to express DDB2 protein because of promoter methylation, are inefficient in cyclobutane dimer repair [47]. Finally, DDB2 accumulates at sites of UV-induced

DNA lesions and the recruitment of XPC to DNA repair foci containing exclusively cyclobutane dimers has been reported to be dependent on UV-DDB [38, 48].

An early "hit-and-run" hypothesis suggested that DDB1 possesses damage-specific DNA-binding activity and that DDB2 leaves the complex after mediating the association of its larger partner with the DNA substrate [46]. Other studies indicated that UV-DDB activity is dependent on the presence of both subunits [44, 49]. Yet different results have been obtained by Kulaksiz et al. [37], who tested the binding of each purified UV-DDB subunit to a 50-mer DNA duplex containing a centrally located (6-4) photoproduct. These authors came to the conclusion that the DNA-binding domain maps to the smaller DDB2 subunit. A separate study, performed in intact human fibroblasts, showed that the knockdown of DDB1 does not prevent DDB2 from accumulating at foci of UV damage, thus supporting the view that DDB2 is sufficient for target site binding [50]. A previous report already demonstrated that, upon UV irradiation, DDB1 translocates into the nucleus [51], but this nuclear accumulation is prevented by *DDB2* mutations in XP-E cells [52]. On the other hand, not only XP-E cells lacking DDB2 but also DDB1 knockdown cells are defective in photoproduct excision, indicating that both subunits are required for efficient repair [50].

The consequences of a DDB1 knockdown may be explained by its role as an adaptor that connects a ubiquitin ligase complex to protein targets [53]. Ubiquitin is a 76-amino-acid polypeptide modifier that modulates the function of proteins or marks them for proteasomal degradation. In general, the ubiquitylation reaction is directed by a three-enzyme cascade involving the ubiquitin-activating enzyme E1, a ubiquitin-conjugating enzyme (E2) and a ubiquitin protein ligase (E3). DDB1 forms a molecular adaptor for the Cul4A-Roc1 ubiquitin ligase by mediating the recognition of WD40-repeat proteins, including DDB2 itself [54, 55]. Known substrates of the DDB1-Cul4A-Roc1 machinery include DDB2 [56, 57], XPC [58] and histones [59, 60]. Ubiquitylated DDB2 looses its damaged DNA-binding activity [58] and is rapidly degraded [61], which contradicts its presumed role in the recognition of cyclobutane dimers because the majority of these lesions are still not repaired when most DDB2 is destroyed. Instead, the concurrent ubiquitylation of XPC is reversible and does not lead to protein degradation.

To summarize, DDB2 is considered to be the first factor that recognizes UV damage while its interaction partner, DDB1, mediates the physical handover of the lesions to the next recognition subunit. However, this model fails to explain the efficient removal of cyclobutane dimers in a number of *in vivo* or *in vitro* systems lacking DDB2 [38, 62]. It may be possible that the ubiquitylation initiated by UV-DDB plays another role that is independent of NER activity. One intriguing observation is that skin fibroblasts taken from XP-E patients, or embryonic fibroblasts derived from $DDB2^{-/-}$ knockout mice, are more resistant than wild-type controls to UV-induced cell killing [63, 64]. Further analysis of $DDB2^{-/-}$ cells revealed that this factor is involved in a regulatory circuit that controls the level of p53 in response to DNA damage. These results lend support to an alternative hypothesis whereby the primary role of UV-DDB is to trigger an apoptotic signaling cascade in response to genotoxic stress.

The special case of transcription-coupled repair

NER operates through two subpathways that differ in the initial recognition of base damage. In "global genome repair" (GGR), XPC, XPA, TFIIH and RPA bind to damaged sites and induce DNA incision irrespective of whether the target sequence is silent or actively engaged in transcription. However, living organisms have evolved a more efficient "transcription-coupled repair" (TCR) reaction that eliminates DNA lesions from the transcribed strand of active genes [65]. Due to this specialized pathway, DNA excision repair is highly non-uniform in the context of mammalian chromosomes. For example, cyclobutane dimers are removed more rapidly from transcribed genes than from transcriptionally silent regions [66, 67], and DNA lesions are repaired in the template strand of RNA polymerase II-transcribed genes more rapidly than in the non-transcribed coding strand [68]. GGR and TCR play different biological roles: GGR protects from damage-induced mutations that ultimately lead to cancer, while TCR ensures that the genes are efficiently and correctly transcribed, thus protecting from premature aging [69].

The progression of RNA polymerase II along transcribed strands is generally obstructed by DNA modifications [see for example Ref. 70], indicating that, in TCR, the exquisite sensitivity of RNA polymerases to DNA lesions provides a facilitated mechanism of damage recognition. After immobilization at damaged sites, the stalled RNA polymerase II serves as a "bait" for the recruitment of XPA, TFIIH, RPA, as

well as the endonucleases XPG and XPF [71], whereas UV-DDB and XPC are no longer necessary [72]. DNA repair only occurs if the RNA polymerase enzyme, which occludes the site of damage, is temporarily relieved. This poorly understood process involves the Cockayne syndrome (CS) complementation group A and B proteins [73]. The characteristic hallmark underlying the hereditary condition known as CS syndrome is a defect in the recovery of mRNA synthesis after UV irradiation [74] and this TCR deficit causes a clinical phenotype of postnatal growth failure, progressive neurodegeneration and symptoms reminiscent of segmental accelerated aging [75]. CSA is a WD40-repeat protein that acts as a cofactor for Cul4A-containing E3 ubiquitin ligases [55]. CSB, on the other hand, is a member of the SWI2/SNF2 family of DNA-dependent ATPases with chromatin remodeling activity [76, 77].

Several hypotheses have been raised regarding the TCR mechanism. For example, the arrested RNA polymerase II is phosphorylated and subsequently polyubiquitylated by a reaction that involves the CSA and CSB proteins [78, 79] and two interpretations of the role of ubiquitylation have been proposed. One indicates that ubiquitin marks the RNA polymerase II molecule for degradation, leaving the damaged strand accessible for repair [80]. The other hypothesis suggests that a blocked RNA polymerase II molecule does not need to be degraded and that ubiquitylation is a signal for activation of DNA repair or other cellular responses [81]. More recent findings indicate that CSB, TFIIH and XPG cooperate to remodel the RNA polymerase II complex in an ATP-dependent manner. This conformational shift would allow access to the lesion without removal or disruption of the transcription machinery [82].

Bipartite substrate discrimination in the GGR pathway

How does the GGR system, without any help from the transcriptional machinery, detect DNA damage in a versatile manner and, at the same time, avoid futile repair cycles among the 3 billion base pairs of the human genome? A major decision point during the sequential assembly of NER complexes is related to the question of how the system “knows” whether cleavage should occur, which is appropriate only if a lesion is actually present. This fundamental problem of versatile DNA damage recognition is solved by a bipartite discrimination strategy that employs several distinct subunits to detect different characteristic features of damaged DNA.

Hanawalt and Haynes [83] were the first to propose that the need for excision repair is determined by comparing the conformation of damaged DNA to that of the normal Watson-Crick double helix. Elaborating on this concept, Gunz et al. [84] showed that the efficiency of bulky lesion recognition by the human GGR complex correlates with the degree of helical destabilization due to the loss of base pairing properties resulting from the formation of a particular DNA adduct. It was, therefore, expected that the GGR factors responsible for the initial damage recognition step would show an affinity for helical distortions caused by DNA lesions. Simple base mismatches or nucleotide bulges are, however, not processed by the GGR machinery, indicating that the local thermodynamic destabilization of duplex DNA is not sufficient to qualify as a NER substrate [85, 86].

Understanding the strategy used by the NER system to discriminate between normal DNA and damaged sites required the construction of highly defined substrates amenable to molecular manipulations. In fact, the notion of bipartite substrate discrimination originated from *in vitro* excision assays demonstrating that the human GGR complex remains inactive on DNA duplexes containing a "non-distorting" DNA adduct that preserves normal hydrogen bonds between complementary bases [87]. However, such a "non-distorting" DNA adduct in conjunction with local disruption of canonical base pair interactions, caused by mismatches or a DNA bulge, induces strong NER reactions. These experiments revealed that the molecular hallmark leading to GGR activity consists of two distinct elements, i.e., disruption of Watson-Crick base pairing and altered chemistry of the damaged deoxyribonucleotide residue [87, 88]. Neither defective base pairing alone, in the absence of bulky adducts, nor defective chemistry in the absence of helical distortions, is able to elicit an excision response, but the combination of these two substrate alterations results in the assembly of productive excision complexes. Thus, the term of "bipartite recognition" has been introduced to indicate that the GGR factors use at least two principal levels of discrimination to recognize damaged substrates.

Common conformational features of NER substrates

As mentioned before, the NER system is able to process diverse UV lesions, including the more abundant cyclobutane pyrimidine dimers and the less frequent (6-4) photoproducts, as well as wide range of bulky carcinogen-DNA adducts, oxidative lesions, crosslinked purines, protein-DNA crosslinks and other modifications that

share no overt structural similarity [84, 89]. To reach this substrate versatility, the NER proteins are thought to recognize a conformational distortion of the double helix induced by UV photoproducts and other types of DNA damage [90]. An increased flexibility of damaged DNA duplexes, relative to the undamaged double helix, may provide such a generic property of different NER substrates. In fact, base stacking is the predominant energetic force leading to the intrinsic rigidity of DNA [91] but the loss of base stacking, resulting in a flexible hinge, is a common consequence of bulky lesion formation [92, 93].

Even the native double-stranded DNA is not a static molecule and the DNA strands are constantly in motion due to thermal oscillations, such that the distance between complementary strands exhibits fast and small variations [94-96]. In the absence of DNA damage, the picosecond-to-nanosecond timescale of these small strand vibrations is probably too short to be recognized by DNA repair factors. However, molecular dynamics simulations predict that the introduction of a single lesion, for example a cyclobutane pyrimidine dimer, provokes longer-lived and larger openings of the double helix relative to undamaged DNA [97]. In the case of pyrimidine dimers, the covalently bonded residues move together in phase, forcing the undamaged bases in the opposite strand to synchronize and give rise to more prominent oscillations compared to native sites. These large fluctuations between complementary strands are expected to appear 25 times more frequently at a cyclobutane dimer position than in undamaged DNA sequences. Also, the amplitude of these oscillations is drastically increased because the strength of interactions between the two complementary strands is weakened. Interestingly, these dynamic changes triggered by base damage generate mainly oscillations of the intact complementary sequence across bulky lesions, as the strand containing base adducts is less flexible than native DNA [97]. Thus, the simulation of macromolecular dynamics lends support to the hypothesis that damage-induced DNA fluctuations may provide a truly universal signal for the recruitment of repair factors.

XPC protein is a sensor of abnormal strand oscillations

To account for its substrate versatility, it has been suggested that XPC protein, one of the key initiators of the GGR pathway, is sensitive to damage-induced deformations of the double helix [31, 34], but the molecular basis of this recognition function remained unknown for a long time. The XPC subunit (125 kDa) is found in

complexes with HR23B, a 58-kDa homolog of the yeast NER protein RAD23 [98], and centrin-2 (CTN2), a 18-kDa calcium-binding protein [16]. XPC protein possesses DNA-binding activity, whereas the HR23B and CTN2 partners exert accessory functions in stabilizing the complex and stimulating its action in DNA repair [34, 99]. XPC protein alone or in conjunction with HR23B binds preferentially to damaged DNA substrates containing, for example, (6-4) photoproducts, acetylaminofluorene adducts or cisplatin crosslinks [31, 34, 100, 101]. Scanning force microscopy studies showed that the binding of XPC protein to damaged double-stranded DNA induces a kink in the nucleic acid backbone [102]. The structural determinants for the recruitment XPC protein have been probed with artificial substrates, thus revealing a general affinity for sites that deviate from the canonical Watson-Crick geometry, including single-stranded loops, mismatched bubbles or single-stranded overhangs [34, 86, 103].

There is ambiguity over the precise amino acid region of XPC protein involved in the complex formation with HR23B. A two-hybrid study reported by Li et al. [104] mapped the HR23B-interacting region of XPC to residues 776 through 801. In contrast, Uchida et al. [105] performed a bidirectional truncation study to map the minimal HR23B-interacting region of XPC between amino acids 496 and 734. A more recent report using a series of XPC fragments expressed in bacteria proposed that there is an additional HR23B-binding site in the N-terminal region of XPC protein [106]. This N-terminal domain is also responsible for an interaction with XPA [106], which may mediate the transition from an initial recognition intermediate (involving XPC and TFIIH) to the formation of an ultimate incision complex that includes the two endonucleases [107]. In fact, XPC protein behaves like a “molecular matchmaker” as it initiates the assembly of a repair complex but leaves the DNA substrate before completion of the incision reaction [15, 108]. The carboxy-terminal tail of XPC protein mediates the association with CTN2 (residues 847-863) and with TFIIH (residues 816-940) [16, 105].

Recently, Maillard et al. [109] discovered a sequence homology between RPA-B, one of the single-stranded DNA-binding domains of human RPA, and an XPC region extending from residue 621 to 730. The observed homology (27% amino acid identity and 73% similarity) includes most of the conserved elements of secondary structure characteristic of the oligonucleotide/oligosaccharide-binding fold (OB-fold) responsible for the tight interaction of RPA with single-stranded DNA. A sequence

similarity of 64% and 66%, respectively, has also been observed between XPC residues 621-730 and two distinct OB-folds of BRCA2.

The single-stranded DNA-binding activity of each OB-fold in RPA or BRCA2 correlates with the presence of two structurally conserved aromatic side chains that mediate stacking interactions with closely spaced DNA bases [110]. The search for functionally analogous aromatics in human XPC protein revealed that Trp690 and Phe733 are critically required for DNA binding and GGR activity [109]. Consistent with the presence of a putative OB-fold motif, XPC protein displays a preference for single-stranded oligonucleotides, implying that it recognizes the local single-stranded character of DNA containing bulky lesions. Surprisingly, XPC exhibits an unfavorable binding to damaged oligonucleotides compared to the more efficient interaction with undamaged single-stranded counterparts [101, 109]. This finding points to an indirect mode of bulky lesion recognition that exploits the local loss of normal duplex properties and the appearance of a single-stranded character in the undamaged complementary sequence opposite to the actual damage.

To summarize, the striking affinity of XPC protein for single-stranded oligonucleotides, in combination with its aversion to interact with damaged single strands, indicates that one of the early recognition steps in the GGR pathway is guided by an association with the native strand of damaged duplexes. This mechanism of action fits with the appearance of large and long-lived oscillation in the native DNA strand across lesion sites, thus predicting that XPC protein operates at sites of bulky lesions by capturing the local and transient formation of a single-stranded conformation in the undamaged complementary sequence. One advantage of this inverted model of substrate discrimination is that the early recognition step is independent of the variable chemistry of lesion sites and, hence, confers the ability of the GGR machinery to detect a very wide array of DNA adducts.

TFIIH is a sensor of defective deoxyribonucleotide chemistry

The TFIIH complex shuttles between sites of transcription by RNA polymerase I or II, and sites of excision repair [27]. TFIIH can be resolved in two main components: the core complex consisting of 6 polypeptides (XPB, TTDA, p62, p52, p44, p34), which assemble in a ring-like structure with a central hole [111, 112] and a protruding CAK (Cdk-activating kinases) complex containing cdk7, cyclin H and MAT1 [113]. This CAK component is dispensable for the NER function. XPD protein, which

appears to play a crucial role in damage recognition, is found in both subcomplexes. Recently, p8 has been identified to be the tenth subunit of TFIIH and, together with XPC-hHR23B, to trigger DNA opening by stimulating XPB ATPase activity [113-115].

In transcription, DNA unwinding by TFIIH allows the nascent RNA molecules to escape from the promoter region and progress towards the elongation phase [116]. In the GGR process, TFIIH is presumably recruited to GGR sites by XPC protein through interactions with the XPB and p62 subunits [117]. TFIIH then separates the two strands around the lesion, until an approximately 30-nucleotide "bubble" is formed. This unwinding activity generates an open intermediate characterized by double-stranded to single-stranded transitions on either side of the lesion, thus providing the substrates for double DNA incision by structure-specific endonucleases [17, 20].

Central to the local unwinding process are the two DNA helicases, XPD and XPB. The XPD subunit functions primarily in DNA repair because its helicase activity, which has 5' to 3' polarity, is required for NER but is dispensable for transcription [118]. It is thought that XPD has a more structural role in transcription by acting as a bridge between the core TFIIH ring and the CAK protrusion [111]. XPB unwinds double-stranded DNA with opposite 3' to 5' polarity and this activity is required for both transcription and the NER process [119]. Since the bases are buried inside the double helix, the two DNA strands have to be separated for the efficient localization of base lesions. The initial interaction of XPC protein with the undamaged strand may facilitate the subsequent loading of XPD onto the damaged strand, such that this DNA helicase constitutes the first subunit that comes in direct contact with the offending residue. Such a role of XPD in the detection of DNA damage is suggested by an analogy with the bacterial DNA helicase UvrB, a recognition subunit of the prokaryotic NER pathway. Whereas XPD and UvrB do not share an overall sequence identity, both proteins display conserved helicase motifs that provide a common scaffold for structural comparisons [120]. Thus, a model for the human XPD protein has been established based on its similarities to the bacterial UvrB subunit, for which high-resolution crystal structures are available [121-123]. This model suggests that XPD, like UvrB, deploys a b-hairpin domain that, once inserted between the strands of the duplex, is able to sense the presence of DNA lesions [120, 124].

Using a yeast ortholog of XPD, the Rad3 helicase of *Saccharomyces cerevisiae*, it has previously been demonstrated that this molecular engine is arrested by DNA lesions located on the strand along which the enzyme is translocating. Like XPD, Rad3 protein requires single-stranded regions to initiate unwinding and this loading strand must be free of damage to promote full ATPase and DNA helicase activity. If, however, the loading strand contains DNA lesions, both enzymatic activities cease when Rad3 protein encounters the chemically altered residue. Furthermore, the presence of base damage induces the formation of stable Rad3 protein-DNA complexes, indicating that Rad3 protein becomes sequestered on DNA at lesion sites [125, 127]. In contrast, lesions in the opposite complementary strand have no effect on this tracking mechanism [125].

As described before, the hypothesis that XPD and the yeast homolog Rad3 may participate directly in the detection of DNA damage is supported by a site-directed crosslinking study revealing that XPD is located in close proximity to bulky lesions within the excision complex [126]. It seems intuitive to propose that inhibition of the 5' to 3' helicase activity has been adopted to localize damaged deoxyribonucleotides during the NER process.

XPA displays an affinity for distorted DNA structures

When migrating in denaturing gels, XPA protein forms several bands with an apparent mass of 40-45 kDa. The discrepancy with the calculated molecular mass (31 kDa) has been ascribed to the presence of disordered regions in the polypeptide fold. Also, the presence of multiple electrophoretic bands is thought to reflect distinct polypeptide conformations and this molecular flexibility of XPA has been related to its function in accommodating a disparate variety of bulky lesions [128, 129]. In fact, internal motions may alter the nucleic acid interaction surface to fit different kinds of damaged DNA substrates [130]. The retention time of recombinant human XPA protein in gel filtration experiments suggests the formation of homodimers in solution [131], but *in vivo* studies indicate that the majority of XPA molecules diffuse rapidly in monomeric form within the nuclear compartment [132].

XPA associates with several other NER subunits and specific interaction domains have been identified by deletions studies (Fig. 2). The N-terminal portion (residues 1-58) and a central region (residues 153-176) contain sequences for binding to RPA. An association of XPA with the large 70-kDa subunit of RPA (RPA70) is essential for

the NER function [133]. The C-terminal region (residues 226-273) binds to TFIIH [134]. XPA also forms complexes with the ERCC1-XPF heterodimer, and amino acid sequences involved in the interaction with this endonuclease are the polyglutamic acid cluster (residues 78-84) as well as a nearby tetrapeptide consisting of residues 72-75 [135-137].

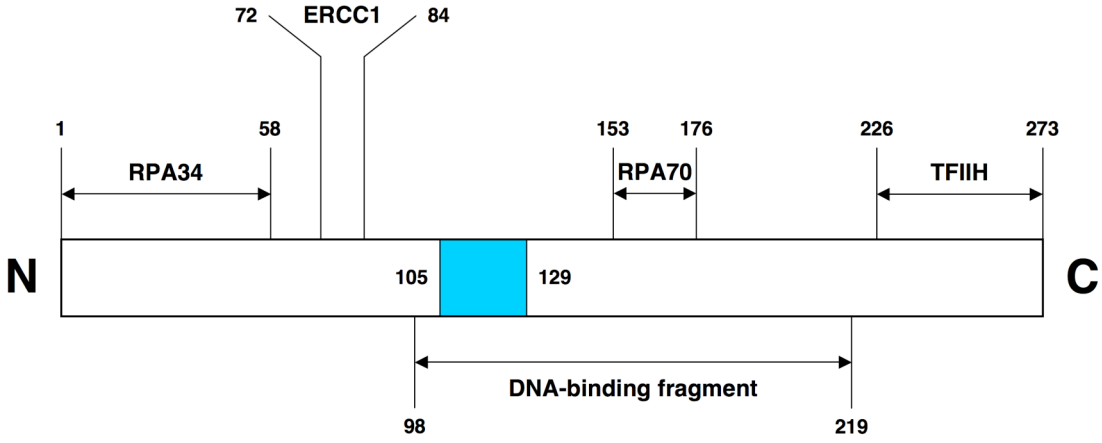

Figure 2: Domain structure of human XPA protein. Blue, zinc finger domain. The minimal DNA-binding fragment is described in Refs. 141-143. Domains for the interaction with the 34- and 70-kDa subunits of RPA as well with ERCC1 and TFIIH have been identified in Refs. 133-137.

The DNA-binding activity of XPA is characterized by some selectivity for UV- or chemical carcinogen-damaged duplexes [138]. Compared to UV-DDB and XPC, however, the affinity of XPA for damaged duplexes is orders of magnitude lower. For example, Jones and Wood [139] estimated the binding constant of XPA for (6-4) photoproducts in double-stranded DNA to be ~$3 \cdot 10^6$ M, whereas the reaction constant for binding of UV-DDB to the same substrate is > 5'000-fold greater. Attempts to produce footprints of XPA protein on damaged DNA by nuclease protection or other techniques have failed. Nevertheless, a DNA damage "verification" function has been proposed for XPA because its affinity for nucleic acid substrates is increased in conjunction with RPA [133, 140-142] or ERCC1 [143]. These larger complexes bind to damaged DNA more avidly than each protein alone, indicating that the different subunits may cooperate to promote substrate recognition. XPA also interacts with XPC protein, but it is not yet clear whether this association stabilizes the recognition complex [33] or whether it results in the displacement of XPC from damaged DNA [107].

XPA protein also displays an affinity for distorted DNA structures carrying mismatches, loops or bubbles, even if no actual DNA lesion has been introduced into the substrate [141]. It has a particularly strong preference for distorted DNA molecules, such as three- or four-way DNA junctions, which share the architectural feature of presenting two double strands emerging from a central bend [141].

A nucleic acid interaction domain has been identified by nuclear magnetic resonance (NMR) spectroscopy [144, 145]. This solution structure analysis revealed that the central region of 122 amino acids (residues 98-219) is composed of an acidic subdomain (residues 105-129) containing a zinc finger, and a C-terminal subdomain (residues 138-209) that forms a positively charged cleft on the protein surface. Subsequent chemical shift perturbation experiments conducted in the presence of either DNA or a short RPA peptide sequence led to the unexpected finding that the zinc finger domain is not involved in DNA binding but, instead, is required for the interaction with RPA. Conversely, the cationic cleft has the appropriate curvature and size to accommodate DNA [144, 146], thus prompting us to perform a mutational screen to determine the functional role of each basic residues in the presumed DNA-binding site [147, 148]

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148. Camenisch U., Dip R., Vitanescu M., Naegeli H. Xeroderma pigmentosum complementation group A protein is driven to excision repair sites by electrostatic potential of distorted DNA. DNA Repair *(accepted July 2007)*

4. PUBLICATIONS

A. Recognition of helical kinks by xeroderma pigmentosum group A protein triggers DNA excision repair

Ulrike Camenisch, Ramiro Dip, Sylvie Briand Schumacher, Benjamin Schuler & Hanspeter Naegeli

Nature Structural and Molecular Biology, 2006, Volume 13, Number 3, 278-284.

B. Xeroderma pigmentosum complementation group A protein is driven to nucleotide excision repair sites by the electrostatic potential of distorted DNA

Ulrike Camenisch, Ramiro Dip, Mirela Vitanescu & Hanspeter Naegeli

Accepted for publication in DNA Repair, July 2007

C. XPA gene, its product and biological roles

Ulrike Camenisch & Hanspeter Naegeli

In press as a chapter in the book "Molecular Mechanisms of Xeroderma pigmentosum". Landes Bioscience, Austin.

D. Mechanisms of DNA damage recognition and strand discrimination in human nucleotide excision repair

Ramiro Dip, Ulrike Camenisch, Hanspeter Naegeli

DNA Repair, 2004, Volume 3, 1409-1423

5. DISCUSSION AND CONCLUSION

Xeroderma pigmentosum group A (XPA) protein is absolutely required for both the global genome and transcription-coupled pathways of the mammalian nucleotide excision repair (NER) system. It has been postulated that XPA protein plays a role during the recognition or verification of DNA damage, but it was unclear how a single repair factor might acquire an affinity for all DNA lesions that are processed by the versatile NER system [1, 2]. Therefore, the goal of the present thesis was to analyze the role of XPA in regulating substrate recognition and DNA cleavage during the NER process.

A putative DNA-binding cleft of XPA has been identified by NMR spectroscopy combined to chemical shift perturbation experiments [3, 4]. On the bases of this structural information, we performed a systematic mutational analysis, ultimately demonstrating that a cluster of positively charged residues on the surface of the DNA-binding cleft is indeed required for the efficient association of XPA with target DNA. In particular, the subsequent mapping of this region by electrophoretic mobility shift, site-directed photocrosslinking and host-cell reactivation assays demonstrated that two neighboring basic residues (Lys179 and Lys141), on the N-terminal side of the DNA-binding cleft, form a dual hotspot for recognition of the nucleic acid substrate [5, 6].

The critical residues Lys141 and Lys179 have been converted to negative moieties by mutating these amino acids to glutamic acid. The resulting Lys179Glu/Lys141Glu tandem substitution confers a stronger DNA repair defect than any other combination of double mutants throughout the DNA-binding surface [5]. Like the respective single mutants, this Lys179Glu/Lys141Glu double mutant fails to interact with linear DNA fragments but is still able to bind to 3- or 4-way DNA junction molecules used as a surrogate for kinked substrates arising during the NER process. Surprisingly, the Lys179Glu/Lys141Glu tandem mutant binds to four-way DNA junctions with exactly the same affinity as wild-type XPA, although it generates nucleoprotein products that migrate faster in native gels than the control complexes generated by wild-type protein. Photocrosslinking experiments revealed that the subtle molecular defect underlying the formation of such abnormal complexes resides in the inability of the Lys179Glu/Lys141Glu double mutant to undergo close contacts with the kinked

junction region of the tested DNA structures. Unlike wild-type XPA, the aberrant nucleoprotein complexes formed by the tandem mutant are unable to recruit the XPF-ERCC1 endonuclease [5]. In combination, these results lend support to the hypothesis that the assembly of a productive incision intermediate, which includes XPF-ERCC1, is dependent on the proper association of XPA protein with the bending angle induced by site-specific kinks in the DNA substrate. It has been shown that binding of the damage recognition factor XPC-hHR23B induces a bend in the DNA, which might be a substrate for XPA [7]. In view of these findings, we propose that XPA serves to recognize a DNA kink resulting from the inherently increased DNA flexibility at lesion sites (Fig. 1). Such a local kink is likely to be further stabilized in NER complexes as sharp DNA bends are often introduced when multi-protein machines assemble on DNA [8]. The notion that a site-specific kink may be formed during bulky lesion recognition is supported by the paradigm of NER in prokaryotic model organisms [9].

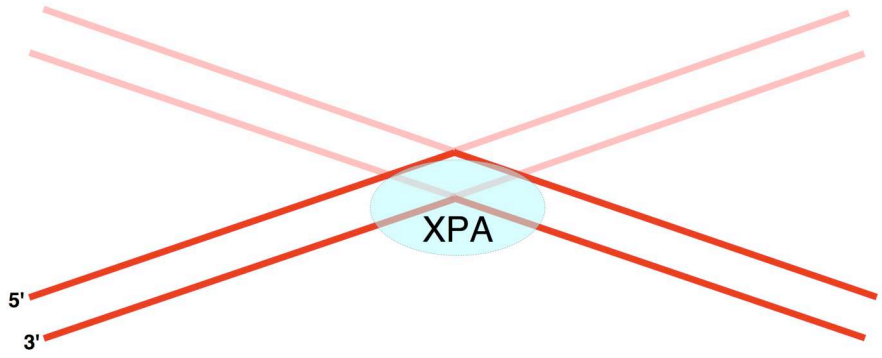

Figure 1: Recognition of DNA kinks by XPA protein. Four-way junctions have been used as model substrates to mimic sharply bent helical backbones.

Furthermore, the requirement for positively charged side chains in the DNA-binding surface, and the preference for kinked DNA, indicates that XPA may represent a molecular sensor of abnormal electrostatic potentials in the nucleic acid substrate. The case of UV endonuclease V, for which detailed crystallographic data is available, illustrates that a cluster of basic amino acids may participate in damage recognition through electrostatic interactions with the unique backbone deformation induced by a DNA kink [10]. This sensor mechanism detects the higher density of

negative charges arising from the closer spacing of phosphate moieties at narrow DNA bends.

Based on these and other recent results from our lab [11] we propose a bipartite substrate discrimination mechanism, in which the NER factors use at least two principal levels of discrimination to recognize damaged substrates, i.e., disruption of Watson-Crick base pairing and altered chemistry of the damaged deoxyribonucleotide residue [12, 13]. In this model, XPC and XPA constitute the sensors of abnormal DNA conformations, whereas TFIIH functions as a tracking enzyme that locates the chemically damaged residues. A salient feature of this bipartite model of substrate discrimination is that the early sensors of DNA damage avoid direct contacts with bulky lesions and, instead, recognize deformations of the double helix. Numerous studies have shown that the efficiency of bulky lesion excision depends on the extent of base pair destabilization in the immediate vicinity to the damaged nucleotide [12, 14-17]. Dynamic simulations reveal that a characteristic property of damaged DNA is the presence of abnormal oscillations between the complementary strands of the double helix [18]. An increased bendability has been identified as another common property of damaged substrates containing bulky lesions [19]. Thus, DNA damage recognition begins when XPC and XPA probe the thermodynamic stability of the double helix and detect abnormal dynamic fluctuations. XPC protein then attracts TFIIH and loads the ring-like helicase domain of TFIIH onto the damaged strand. Driven by the 5' to 3' helicase activity of XPD protein, TFIIH moves up along the damaged strand in search of the lesion [13]. The activity of TFIIH promotes partial unwinding by 20-25 base pairs, thereby separating the duplex. This tracking activity serves to probe the chemical composition of the target strand and to determine the precise location of the adducted nucleotides. Damage recognition is completed when XPD encounters the adduct and becomes sequestered on the damaged strand, thereby marking the location of the genetic insult. This bipartite mechanism for lesion recognition not only results in the ability to detect a wide range of different DNA lesions but, at the same time, also protects undamaged DNA, including the complementary template strand across lesion sites, from inadvertent incisions.

Currently, we are investigating the molecular mechanism by which the sequential recruitment of these core factors is regulated such that the NER complex is formed in a damage- and strand-specific manner. In particular, we are analyzing the role of protein modifiers such as ubiquitin during the NER process and we are tagging the

core factors with fluorescent proteins to monitor their dynamic assembly and disassembly in repair foci of living cells.

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6. STATUTORY REQUIREMENTS

Exams

Laboratory animal science	24.10.2005
Cell biology	26.10.2005
Molecular biology	26.10.2005
Immunology	17.11.2004
Epidemiology and biostatistics	04.11.2004

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03.03.2006

Courses

Epidemiology and Biostatistics. Ph.D. Course. Department of Clinical Veterinary Medicine, Vetsuisse Faculty, University of Berne, August 2004

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Immunology Ph.D. Course. Institute of Veterinary Virology, Vetsuisse Faculty, University of Berne. August/September 2004.

Functional Cell Biology, Ph.D. Course. Institute of Animal Pathology. Vetsuisse Faculty, University of Berne. April/May 2004.

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7. CURRICULUM VITAE

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Meeting Award

2004	Meeting Fellowship Award of the German DNA Repair Network

Talks

2006	Dynamic interplay of DNA damage recognition subunits during the sequential nucleotide excision repair process. 16. Vetpharm Symposium, Hannover, Germany, September 28-29, 2006.
2006	Recognition of helical kinks by xeroderma pigmentosum group A protein during nucleotide excision repair. DNA repair: form molecular mechanism to human disease, Noordwijkerhout, Netherlands, April, 2-7, 2006
2005	Control of nucleotide excision repair by xeroderma pigmentosum group A protein. 15. Vetpharm Symposium, Vienna, Austria, September 29-30, 2005
2004	Molecular analysis of the xeroderma pigmentosum group A protein function by site-directed mutagenesis. 8th Meeting of the German DNA Repair Network, Ulm; Germany, September 28-October 1, 2004.

Posters

2006	Control of nucleotide excision repair by XPA. DNA repair: form molecular mechanism to human disease, Noordwijkerhout, Netherlands, April, 2-7, 2006
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