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Xeroderma pigmentosum complementation group C protein (XPC) serves as a general sensor of damaged DNA

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ABSTRACT

The Xeroderma pigmentosum complementation group C protein (XPC) serves as the primary initiating factor in the global genome nucleotide excision repair pathway (GG-NER). Recent reports suggest XPC also stimulates repair of oxidative lesions by base excision repair. However, whether XPC distinguishes among various types of DNA lesions remains unclear. Although the DNA binding properties of XPC have been studied by several groups, there is a lack of consensus over whether XPC discriminates between DNA damaged by lesions associated with NER activity versus those that are not. In this study we report a high-throughput fluorescence anisotropy assay used to measure the DNA binding affinity of XPC for a panel of DNA substrates containing a range of chemical lesions in a common sequence. Our results demonstrate that while XPC displays a preference for binding damaged DNA, the identity of the lesion has little effect on the binding affinity of XPC. Moreover, XPC was equally capable of binding to DNA substrates containing lesions not repaired by GG-NER. Our results suggest XPC may act as a general sensor of damaged DNA that is capable of recognizing DNA containing lesions not repaired by NER.

1. Introduction

Exposure to chemical carcinogens and environmental radiation results in DNA damage that, left unresolved, threatens genomic integrity. In order to guard against genetic instability cells have developed an arsenal of biochemical pathways to identify and repair damage as well as coordinate cellular functions such as gene transcription, DNA replication, and cell cycle progression. Critical to the function of these biochemical pathways is the ability to identify the presence of many different types of DNA damage against a high background of unmodified, nearly isomorphous nucleotides (reviewed in [1,2]).

Nucleotide excision repair (NER) is a versatile repair pathway that removes bulky covalent lesions from DNA. The hallmark of NER is the ability to repair a wide variety of chemically distinct DNA lesions without the need for lesion-specific damage recognition factors [3]. Mutations in any of the seven genes encoding for the NER proteins in humans results in Xeroderma pigmentosum (XP), a spectrum of clinical disorders resulting in predisposition to the development of skin cancer, neurodegeneration, and accelerated aging [4]. NER occurs in four steps requiring the assembly and remodeling of protein–protein and protein–DNA complexes [5]. NER is initiated when the presence of a lesion is sensed in a specific DNA locus. The surrounding dsDNA is then unwound and NER proteins assemble on the NER bubble. Dual incision of the damaged strand by nucleases releases an oligonucleotide containing the lesion, and gap filling by a replicative DNA polymerase fills the resulting gap using the undamaged strand as a template, thereby restoring the original DNA sequence [5,6].

NER is divided into two sub-pathways, transcription coupled NER (TC-NER) and global genome NER (GG-NER) (reviewed in [7]). TC-NER is initiated upon stalling of RNA pol II after encountering a DNA lesion and is therefore limited to actively transcribed DNA strands [8]. In contrast, GG-NER removes lesions throughout the entire genome irrespective of the transcriptional activity of the damage locus. XPC, a 940-residue DNA binding protein, is the primary GG-NER initiating protein in humans [9,10]. In cells, XPC forms a complex with the HR23B [11,12] and Centrin2 proteins [13]. XPC–HR23B has previously been shown to be necessary and sufficient to support NER activity in vitro [14–16].

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Previous experiments have shown that XPC–HR23B binds both single stranded (ss) and double stranded (ds) DNA with affinity in the nanomolar range [17,18], and slight preference for binding dsDNA containing bulky nucleotide modifications [15]. Initial recognition of damage repaired by GG-NER is believed to occur via XPC sensing destabilization of dsDNA that is induced by the presence of the lesion [19–21]. The crystal structure of the *Saccharomyces cerevisiae* homolog, Rad4, bound to a duplex DNA substrate containing a cyclobutane pyrimidine dimer (CPD) has been determined. It suggests an indirect read-out model for DNA damage sensing in which there is no direct contact with the lesion. In the crystal structure Rad4 makes extensive contact to the unmodified nucleotides opposite the lesion but CPD lesion itself was disordered and not observed in the structure [20].

In the current model of GG-NER, XPC continuously scans the genome for damage by transiently binding the DNA [9]. Binding to a damage site results in immobilization of XPC and signals recruitment of the basal transcription factor TFIIH, which unwinds the duplex surrounding the lesion to initiate the NER cascade [6,22–24]. Although XPC is primarily active in the GG-NER pathway, recent reports suggest that it may also play a role in the base excision repair (BER) pathway (reviewed in [25]). Cells from XP-C patients display increased sensitivity to oxidative stress and reduced rates of repair by BER [26]. Additionally, XPC has been found to physically interact with several DNA glycosylases, including OGG1 and SMUG1 [27,28].

Although the DNA binding activity of XPC has been the subject of multiple studies, there is a lack of consensus regarding the lesion discrimination properties of XPC. XPC–HR23B is difficult to produce and poor yields of recombinant protein have limited investigations to one or a small number of lesion-containing DNA substrates [15,17,29]. Moreover, experimental conditions vary considerably among previous studies limiting the ability to directly compare XPC–HR23B binding affinities determined for DNA substrates containing various types of lesions [15,17,18,21,30]. To address these uncertainties, we have performed a systematic study of human XPC–HR23B binding to different DNA substrates with a range of different lesions typically associated with DNA excision repair pathways. To minimize the utilization of the protein and maximize accuracy and reproducibility we developed a high-throughput fluorescence anisotropy assay to rapidly measure the DNA binding affinity of XPC–HR23B. Our results suggest that XPC–HR23B serves as a general sensor of DNA helix instability, and that it may play a role in sensing the presence of DNA lesions in DNA repair pathways other than NER.

2. Materials and methods

2.1. Chemical synthesis and construction of damaged DNA substrates

DNA substrate sequences are shown in Fig. 1. Unmodified oligonucleotides and 3′-fluorescein tagged oligonucleotides were purchased from Sigma. Chemically modified 12-mer oligonucleotides ($^{5'}$GCTAGC**G***AGTCC$^{3'}$) were either purchased or synthesized. 8-Oxo-deoxyguanisine (8-oxo-dG) modified oligonucleotide was purchased from Midland Certified Reagent Co., tetrahydrofuran (THF) modified oligonucleotide was purchased from IDT. 12-mer oligonucleotides containing the 1,N^2-ε-guanine [31], 7-(2-oxoheptyl)-ε-guanine [32], 8-hydroxy-1,N^2-ε-guanine [33], 1,N^2-propano-guanine [34], M_1G [34], and methyl-formamidopyrimidine (MeFapy) [35], and modified 11-mer oligonucleotide ($^{5'}$GGCAG**A***TGGTG$^{3'}$) containing the 7,12-dimethylbenz(a)anthracene-adenine (DMBA) [36] lesions were available from previous studies. 60 base pair (bp) and 42 bp substrates were constructed by ligation as described previously [37,38] with the exception that fluorescein was incorporated on the damaged strand which allowed use of fluorescence instead of ^{32}P radioactivity as the probe. 36-mers were synthesized using a previously published sequence [17]. Duplexes were prepared by annealing equimolar amounts of complementary oligonucleotides in TNE buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA), resolved by 8% native PAGE, and eluted in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA).

2.2. Expression of XPC–HR23B complex

Full-length XPC and HR23B were amplified by PCR using indicated oligonucleotides (Supplemental Table S1) that incorporated a tobacco etch virus protease (TEV) cleavable N-terminal 6×His tag in XPC, and a human rhinovirus 3C protease (HRV3C) cleavable N-terminal 6×His tag in HR23B, and sub-cloned into the two multiple cloning sites in pFastBac Dual vector, respectively. The pFastBacDual/XPC–HR23B plasmid was verified by sequencing and transformed to *Escherichia coli* DH10Bac to generate recombinant bacmid and subsequent baculoviruses in Sf9 insect cells. Suspension cultures of Sf9 cells were infected with a high-titer baculovirus encoding XPC and HR23B at a multiplicity of infection of 2, and cells were harvested 70 h post infection.

2.3. Purification of XPC–HR23B complex

Sf9 insect cells were lysed in ice-cold Buffer T1 (50 mM Tris, pH 8.0, 0.5 M NaCl, 10% glycerol, 5 mM Imidazole, 1× Roche Protease Inhibitor cocktail, 1 mM PMSF), 6×His-tagged proteins isolated by TALON chromatography and eluted in Buffer T1 containing 0.3 M Imidazole. Pooled fractions were diluted with Buffer D1 (50 mM Tris, pH 8.0, 10% glycerol) to 0.3 M NaCl and incubated with a combination of HRV3C and TEV proteases overnight at 4 °C. Cleaved XPC–HR23B complex was then immobilized on a 5 mL HiTrap Heparin column (GE Healthsciences) equilibrated in buffer H1 (50 mM Tris, pH 8.0, 0.3 M NaCl, 10% glycerol) and eluted using a linear gradient of buffer H2 (50 mM Tris, pH 8.0, 1.0 M NaCl, 10% glycerol). Heparin-purified XPC–HR23B was re-passed over a TALON column to remove any residual protease or 6×His tag contaminants. Pooled protein was then resolved on an HR10/30 S200 size exclusion column (GE Healthsciences) and dialyzed against buffer S1 (50 mM Tris, pH 8.0, 0.5 M NaCl, 50% glycerol, 1 mM EDTA, 1 mM DTT) overnight at 4 °C. Protein concentration was determined by Bradford assay using the Bio-Rad Protein Assay Dye Reagent and confirmed by measuring UV absorbance at $\lambda = 280$ nm. Dialyzed protein was divided into small aliquots, flash-frozen in liquid nitrogen, and stored at −80 °C.

2.4. High-throughput DNA binding assay

XPC–HR23B and DNA substrates were diluted in binding buffer (25 mM HEPES, pH 7.8, 0.2 M KCl, 5% glycerol, 1 mM DTT). A Bravo automated liquid handling robot (Agilent Technologies) was used to perform a three-fifths serial dilution of XPC–HR23B in Corning #3676 low-volume 384-well microtiter plates and subsequently add the DNA substrates to each well. The plate was covered from light and incubated at room temperature with gentle agitation for 5 min prior to measuring fluorescence anisotropy to ensure samples were homogenized and had reached equilibrium. Fluorescence anisotropy was measured using an EnVision 2100 plate reader (PerkinElmer), plate reader settings were optimized to maximize sensitivity and allow the lowest possible concentration of fluorescein probe (2 nM). Binding measurements were performed in triplicate for each DNA substrate. Apparent dissociation constants (K_d) were determined for each individual titration