Where Multifunctional DNA Repair Proteins Meet: Mapping the Interaction Domains Between XPG and WRN.

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Abstract

The human genome is constantly subjected to both endogenous and exogenous damage from cellular metabolism, environmental sources of ultraviolet (UV) and ionizing radiation, and mutagenic chemicals, which generate several kinds of lesions throughout cellular DNA. Natural by-products of oxidative phosphorylation in mitochondria in addition to other cellular processes of the cell generate reactive oxygen species, which are estimated to produce 50,000-100,000 oxidative DNA lesions per mammalian cell per day, each of which could potentially cause errors in DNA replication or transcription if not repaired. Such lesions may elicit point mutations or chromosomal rearrangements, single-strand or double-strand breaks in DNA during replication, or trigger a stress response via a signal transduction pathway, which are known precursors to premature aging, cancer and a number of genetic diseases. The rapid recognition and repair of DNA damage is essential for the maintenance of genomic integrity and cellular survival. Multiple DNA repair pathways have evolved within cells to preserve the human genome, though the coordination of these processes may be compromised by a break down in any one of the numerous multifunctional proteins involved in initiating and mediating communication between repair and replication pathways. This study demonstrates a novel interaction between two such repair proteins, XPG and WRN, that are both associated with inherited genetic disorders when mutated. The interaction of XPG and WRN was mapped to the C-terminal region of XPG and two regions within the C-terminus of WRN. Understanding these proteins which link necessary DNA repair processes will ultimately contribute to our understanding and prevention of cancer and premature aging.

Objectives

- Determine the XPG domain responsible for the interaction with WRN *in vitro*, using Far Western analysis
- Determine the WRN domains which complement the interacting XPG domains by the same approach

Introduction

A

[Photograph by D. Atherton. Reproduced from Lehmann, A. R. (1995) Trends Bio-chem. Sci. 20, p. 402-405]

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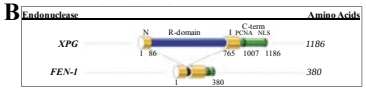


Figure 1 | **A.** Cockayne syndrome sufferers have multiple systemic disorders due to a defect in the ability of cells to repair DNA that is being transcribed. Cockayne syndrome has been linked to abnormalities in XPG protein.

B. Schematic representation of the 1186-amino acid XPG protein and a comparative structure of another such endonuclease, FEN-1, where sequence homology is conserved between the catalytic sub-domains N and I of XPG and FEN-1. R- and C-terminus domains remain unique to XPG and impart XPG with additional functional capabilities.

•XPG protein is a structure-specific endonuclease required for the repair of UV-damaged DNA, which is attested by the existence of dramatic inherited genetic disorders that result by its mutation.

•Point mutations in XPG cause the disease xeroderma pigmentosum (XP), wherein essential repair pathways to remove UV-induced DNA damage are disrupted, resulting in clinical features of extreme sun sensitivity, marked thickening of skin and a high incidence of skin malignancies.

•More severe are truncation mutations, in which patients have a combined disease of XP with the developmental disorder Cockayne syndrome (CS) and display profound postnatal neurological and developmental dysfunction, premature aging and severe wasting that become evident and lethal within the first few years of life.

XPG and WRN Proteins Interact

A

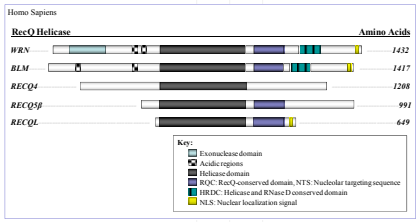


Figure 2 | **A.** Schematic representation of the highly conserved, five-member RecQ family of DNA helicases for *H. Sapiens*, to which WRN protein belongs. RecQ helicases are critical in the maintenance of genome integrity as implicated by heritable cancer predisposition disorders that result by defects in BLM, RECQ4, and WRN. Amino acid designations of each are shown on the right and each respective domain within the proteins correspond to the colors explained in the key below. WRN protein uniquely imparts an exonuclease domain in the amino-(N)-terminal region. **B.** Nonfunctional WRN protein leads to Werner's syndrome (WS), a premature aging disorder with increased cancer incidence.

B

WS Patient Age 15 yrs | Same Patient Age 48 yrs

•Combination approaches of proteomic analysis and directed searching have identified WRN protein as a novel XPG binding partner.

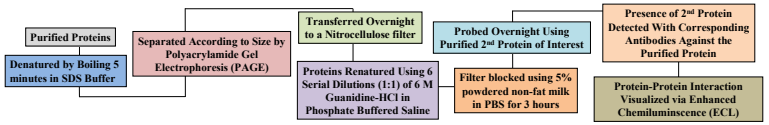
•Further studies have established that XPG increases WRN helicase activity by increasing the rate of DNA unwinding, and that XPG possesses intrinsic annealing activity requiring both the R- and C-terminal domains that is cooperative with WRN.

•The structural interaction with WRN suggests a novel replication-associated role for XPG.

•We now examine which domains of XPG are responsible for coordinating these novel structural roles, as well as the domains of WRN that enable this interaction in order to learn more about these repair proteins.

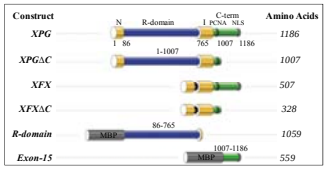
Materials and Methods

Far Western analysis was used to examine protein –protein interaction among XPG and WRN deletion constructs according to the procedure below:



WRN Interacts with the C-terminal Domain of XPG, *in vitro*

A



B

WRN Binding
+
–
+
–
–
+

C

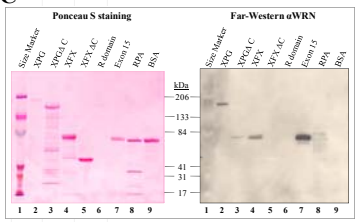
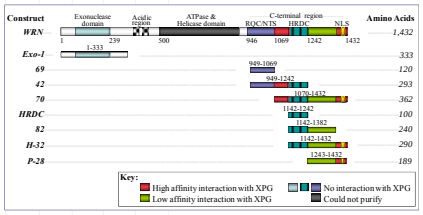


Figure 3 | **A.** Series depiction of domain constructs applied for the interaction mapping with WRN. Protein constructs R-domain and Exon-15 are distinct from other constructs in that they are recombinant fusion peptides generated by fusing the N-terminal domains of the XPG construct with the C-terminus of a 42-3 kDa (380 amino acid) maltose- binding protein (MBP). This technique aided in the affinity purification process of the constructs. These constructs were kindly provided by Miaw-Sheue Tsai of the EMB Core at LBNL. The *numbers* above each construct represent amino acid positions in within full length XPG. **B.** Indication of the ability of each peptide to bind to WRN as detected by Far Western analysis. Deletion constructs XFX and Exon-15 maintain the interaction with WRN that is viewed with full length XPG protein. All other constructs fail to uphold WRN binding. **C.** Separated by 4-12% Tris-glycine SDS-PAGE and stained with Ponceau S Solution for temporary protein visualization, the purified constructs and control proteins RPA (+ control), BSA (– control) are shown on the nitrocellulose filter. To the right, protein-protein interaction between WRN and XPG deletion constructs are signaled by bands at designated migration areas on the filter following Western analysis using WRN antibody (AB200-Abcam).

•XPG constructs missing the C-terminal domain fail to interact with WRN, thus the C-terminus is found to be necessary and sufficient for WRN binding.

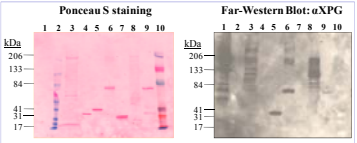
Recombinant WRN Deletion Constructs Used for Interaction Mapping with XPG

Figure 4 | The 160 kDa WRN protein, *top* of the diagram, essentially consists of four domains: a central conserved helicase domain found in all RecQ helicases and a unique N-terminal exonuclease domain that are flanked by divergent N- and C- terminal regions. *Below* WRN are shown the eight member series of recombinant truncated WRN variants used to more finely map the domains responsible for interaction with the C-terminus of XPG. Most of the WRN-interacting proteins have been shown to bind to multiple regions on WRN, thus all eight constructs were very critical in carrying out the reciprocal Far Western analysis. The sub-fractions enable isolation of the RQC, HRDC, NLS C-terminal domains, in addition to the regions between these complexes. Inability to produce a soluble and purified helicase domain construct precludes the ability to identify all interaction sites for XPG on WRN. All full-length WRN fractions and WRN domain constructs were kindly provided by the lab of Steve Yannone (Life Sciences Division, LBNL, Berkeley, CA). Protein constructs were appended with an N-terminal His6 tag, cloned into a vector and recombinantly overexpressed in bacterial or insect-cells. Cells were resuspended in Tris-HCl buffer (pH 7.5 and affinity-purified from sonicated cell lysate by conventional nickel-nitrilotriacetic acid agarose resin column chromatography. Subsequent column chromatography methods allowed purification of the constructs to near homogeneity.



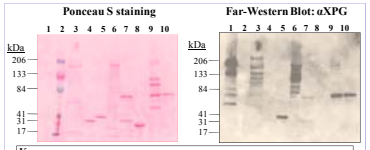
XPG Interacts with the C-terminal domain of WRN

A



Key:
1. XPG
2. Protein Ladder
3. WRN
4. Exo-1
5. H-32
6. BSA- New England BioLabs (– Control)
7. GST (– Control)
8. CSB (+ Control)
9. RPA (+ Control)
10. Molecular Weight Marker

B



Key:
1. XPG
2. Molecular Weight Marker
3. WRN
4. Exo-1
5. H-32
6. CSB (+ Control)
7. RPA (+ Control)
8. GST (– Control)
9. High Range Standard (Containing BSA)
10. BSA- Standard 1:10 (– Control)

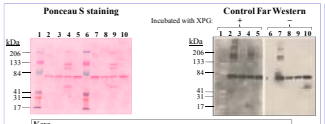
Figure 5 | **A.** Designations for the full length WRN, constructs Exo-1 and H-32 along with the five control proteins that were resolved by 4-12% Tris-glycine SDS-PAGE are provided by the key. XPG was run on the gel itself in lane one to verify the efficiency of the Western detection using XPG antibody (8H7 AB-1; NeoMarkers); definitive interactions should show between a protein and its antibody. Shown *left* is the Ponceau S staining of the nitrocellulose and to its *right*, protein-protein interaction bands are viewed between XPG and WRN, H-32, the positive control proteins XPG, CSB, and RPA, but also the negative control protein BSA after Western detection. These results are confounded by the interaction band with BSA that suggests a non-specific interaction due to error in the methodology. **B.** A subsequent Far Western analysis was performed in similar fashion with the same proteins, except a second source of BSA (Standard 1:10) in addition to an SDS-PAGE high range protein standard, which contains a BSA molecular weight determinant. The Far-Western blot shown on the right, replicates the results produced in the preliminary analysis even among the two new negative control sources of BSA, prompting further investigation.

•Preliminary results show that only WRN deletion constructs that contain the C-terminus domain can mediate an interaction with XPG.

•Strong, unexpected immunoreactive bands among 3 different sources of negative control BSA suggest a non-XPG dependent interaction with Western antibodies.

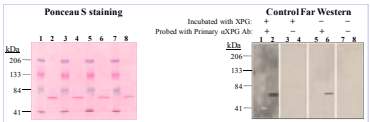
BSA Interacts With Primary XPG Antibody

A



Key:
1. Molecular Weight Marker
2. NEB BSA
3. BSA Standard 1:10
4. Bio-Rad High Range Standard
5. Sigma BSA
6. Molecular Weight Marker
7. NEB BSA
8. BSA Standard 1:10
9. Bio-Rad High Range Standard
10. Sigma BSA

B



Key:
1. Molecular Weight Marker
2. BSA Standard 1:10
3. Molecular Weight Marker
4. BSA Standard 1:10
5. Molecular Weight Marker
6. BSA Standard 1:10
7. Molecular Weight Marker
8. BSA Standard 1:10

Figure 6 | A control Far Western experiment was designed to examine the possibility of non-XPG based interactions as the cause for the immunoreactive response received in the Far Western blots shown in Figures 5 A and B. **A.** Four distinct sources of BSA were loaded twice in a sequence specified by the key onto a 7% gel with 4% stack, resolved by SDS-PAGE, transferred to a nitrocellulose filter and blocked prior to being cut in half. One half of the membrane underwent overnight incubation with XPG protein, and the other half was incubated without XPG protein. Western analysis was conducted on both halves using XPG antibody.

•Equivalently positive bands appeared for all four sources of BSA, thereby dismissing the possibility of source dependent interaction.

•Immunoreactive bands were found with equal intensity on the half of the filter that had no exposure to XPG protein (right half of Western blot) to the half that had received XPG protein probing (left half of Western blot), thus targeting one of the antibodies as the cause for the cross-reactivity.

B. A second control experiment was designed in order to isolate which antibody was causing the cross-reactivity with BSA. One source of BSA was loaded in an every-other lane sequence onto a 7% gel with 4% stack and subjected to SDS-PAGE. Following the 3 hour block, the nitrocellulose filter was cut in half, and as before, only one half of the filter received treatment with XPG. This half appears on the left of the Control Far Western diagram. Furthermore, each half of the filter was cut in half again, thereby forming 4 quarters, 2 of which that had received treatment with XPG. Only one quarter from each half of the filter was allowed to incubate with primary XPG antibody, in order to isolate the possible cross-reactivity of the primary verses secondary (anti-mouse IgG/horse radish peroxidase) antibodies. These quarters appear as the 1st and 3rd quarters of the nitrocellulose filter shown in the diagram.

•Positive interactions are viewed only on the quarters of the nitrocellulose filter that received primary XPG antibody treatment regardless of exposure to XPG protein, which determined the primary antibody as the source of the cross-reactivity.

XPG Interacts with Two Independent Regions of the WRN C-terminus

A

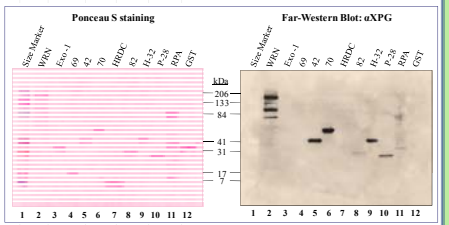
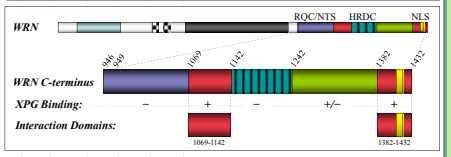


Figure 7 | Preliminary results (Figure 5 A and B) prompted further investigation of the interacting sub-domains of the WRN C-terminus with XPG using 6 additional C-terminal deletion constructs to the already tested H-32 (Figure 4). **A.** Full length WRN, Exo-1 and the total 7 C-terminal sub-constructs, as well as positive control protein RPA, and negative control protein GST, were resolved by 4-20% Tris-glycine SDS-PAGE in the order designated by the construct map in Figure 4. The proteins were blotted to nitrocellulose, blocked and incubated with purified XPG protein prior to Western analysis using XPG antibody, resulting in the blot which appears on the right-hand side of the diagram. Positive interactions are viewed for full length WRN, constructs 42, 70, H-32, P-28, and weakly pronounced for 82.

•Interaction behaviors among the 3 WRN C-terminal derivatives 69, 42, and HRDC together isolate one of the domains of interaction. Constructs 69 and HRDC do not sustain interactions with XPG, however sub-domain 42, which bridges the 73 residue gap between these two constructs, upholds XPG binding activity.

•Interaction behaviors among deletion constructs 82, H-32 and P-28 supports the existence of a 2nd binding site for XPG. 82 stops 50 residues short of the C-terminus and displays a weak XPG interaction, and H-32, which is essentially the same construct with the additional 50 C-terminal residues displays a much stronger interaction. The interaction with H-32 is confirmed by that of P-28.

B



B. Schematic representation of the C-terminal domain of WRN and the regions of the C-terminus that enable the interaction with XPG. Ability of XPG to bind to each C-terminal domain are designated by (+) interaction, (–) no interaction, and (+/–) weak interaction.

•Based on the aforementioned analysis, a 73 residue interaction site (amino acids 1069-1142), resides between the RQC and HRDC domains on WRN.

•A second, 50 residue interaction site (1382-1432) exists at the very C-terminal end of WRN. This interaction region may extend beyond just the final 50 residues.

Conclusions

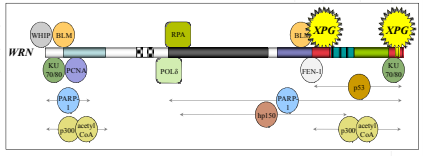


Figure 8 | **Select documented protein binding partners for WRN and novel interacting domains with XPG.** WRN domains are again designated by the same colors used in Figure 4. Protein binding partners indicated by rectangular symbols interact with WRN, though the precise location of the interaction domain within the helicase has not been identified. Binding proteins indicated by round symbols are positioned at approximate, identified binding sites (additional binding sites may exist). Horizontal arrows indicate the limits of binding domains where the site of interaction is broad ranging or mapped less accurately. XPG is shown at its novel binding domains within the WRN C-terminus based on the results of this study.

- The XPG C-terminus is necessary and sufficient for the interaction with WRN protein.
- A 73 amino acid region between the RQC and HRDC domains and a 50 amino acid region at the very C-terminal end of WRN protein sustain XPG binding. These interaction sites appear to function independently of one another, though the interaction is more pronounced when both sites are present.