Domain swapping between FEN-1 and XPG defines regions in XPG that mediate nucleotide excision repair activity and substrate specificity

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ABSTRACT

FEN-1 and XPG are members of the FEN-1 family of structure-specific nucleases, which share a conserved active site. FEN-1 plays a central role in DNA replication, whereas XPG is involved in nucleotide excision repair (NER). Both FEN-1 and XPG are active on flap structures, but only XPG cleaves bubble substrates. The spacer region of XPG is dispensable for nuclease activity on flap substrates but is required for NER activity and for efficient processing of bubble substrates. Here, we inserted the spacer region of XPG between the nuclease domains of FEN-1 to test whether this domain would be sufficient to confer XPG-like substrate specificity and NER activity on a related nuclease. The resulting FEN-1-XPG hybrid protein is active on flap and, albeit at low levels, on bubble substrates. Like FEN-1, the activity of FEN-1-XPG was stimulated by a double-flap substrate containing a 1-nt 3' flap, whereas XPG does not show this substrate preference. Although no NER activity was detected in vitro, the FEN-1-XPG hybrid displays substantial NER activity in vivo. Hence, insertion of the XPG spacer region into FEN-1 results in a hybrid protein with biochemical properties reminiscent of both nucleases, including partial NER activity.

INTRODUCTION

FEN-1 and XPG belong to the FEN-1 family of structure-specific nucleases, the members of which fulfill diverse biological roles and cleave branched DNA structures with conserved polarity at the junction of single-stranded (ss) and duplex (ds) DNA (1). FEN-1 has an essential role in DNA replication in the processing of Okazaki fragments and has additional functions in base excision repair and in other pathways contributing to the maintenance of genome stability (2,3). XPG plays a central role in nucleotide excision repair (NER) where it makes the incision on the 3’ side of the DNA lesion (4–8). XPG also fulfills a structural role in the assembly of NER complexes since its presence, but not its catalytic activity, is required for the 5’ incision by ERCC1-XPF (9–11). Consistent with the contribution of FEN-1 and XPG to different DNA repair and replication pathways, they show similar but distinct substrate specificities in vitro. XPG shows structure-specific properties cleaving artificial DNA structures that contain ss/ds DNA junctions, including bubbles and splayed arm substrates with the same polarity as in the NER reaction (7,12,13). In contrast, FEN-1 only acts on substrates that have free 5’ ss DNA ends and does not process DNA bubble structures or loops that are substrates for XPG (14,15). In addition, the nuclease activity of FEN-1 is greatly increased on a double-flap structure containing a 1-nt 3’ flap adjacent to the 5’ flap compared to the conventional 5’ flap substrate (16,17).
Such double-flap structures might be the real in vivo substrates for FEN-1 during replication (18).

Members of the FEN-1 family of nucleases have a conserved nuclease core composed of the N-terminal (N-) and internal (I-) regions (1). Crystal structures of FEN-1 family proteins from different species revealed that the N- and I- regions form a conserved globular domain containing the active site (19–22). In most FEN-1 family members, the N- and I- regions are separated by <70 amino acids. This region forms a helical arch that is located above the active site and plays a role in DNA binding and catalysis (23). In addition, FEN-1 contains a small surface-exposed hydrophobic wedge and a 1-nt-binding and catalysis (23). In addition, FEN-1 contains a small surface-exposed hydrophobic wedge and a 1-nt-binding pocket, which provides specificity for double-flap structures and appears to contribute to the positioning of the ssDNA 5' flap near the active site (24,25).

XPG differs from the other FEN-1 family members in that the N- and I- regions are separated by a stretch of over 600 amino acids designated ‘spacer region’, which shows no homology to other known proteins or motifs (26). An obvious role for this spacer region would be in conferring the substrate specificity and mediating protein–protein interactions required for NER. Indeed, some parts of the spacer region interact with the XBP, XPD, p62 and p44 subunits of the transcription/repair factor TFIIH (27) and perhaps also with RPA (28). The interaction between TFIIH and XPG is of particular relevance since TFIIH needs to be present at sites of DNA damage for the recruitment of XPG following the initial damage recognition by XPC-HR23B (29–31). To address the role of the XPG spacer region, we have generated several mutants of XPG in a previous study and showed that deletions in the spacer region can result in loss of NER activity and defective interaction with TFIIH (32,33). Furthermore, the spacer region of XPG contributes to the substrate specificity of XPG as it is required for efficient bubble cleavage activity. These results demonstrate that the spacer region is, to a significant degree, responsible for the NER-specific functions of XPG.

In the present study, we tested whether the spacer region is sufficient for mediating NER-specific function of XPG or whether additional parts of the protein contribute. For this purpose, we inserted the XPG spacer region between the N- and I- regions of the FEN-1 protein and investigated the biochemical and cell biological properties of this FEN-1-XPG hybrid protein. Our studies reveal that FEN-1-XPG displays specificities on model bubble substrates. DNA cleavage and binding assays were performed using the primer (5'-AATTCGATGTCATCAGAAGCCTGGC) and (5'-AAAGTTAAAAATGCGGCAGG) and (5'-AATTCAAGGCTCGTACAGGTTG) and the plasmid pET28a-FEN-1 as template. The primers incorporated a NotI restriction site 5' of the FEN-1 ORF and a HindIII restriction site 3' of the PCR fragment. Primers (5'-AATTCAACATTTATGCTGAGCGCAGAAAGCAGG) and (5'-AATTCAATGCCTAAATGATAGGTGTGTGTTGGTGTTGCTTTCCCTTAAACTTCC) were used to amplify the FEN-1 sequence from nucleotide 316 to 1143 (fragment II), introducing a 5' Asel restriction site, a His6 tag and the stop codon followed by a KpnI restriction site at the 3' end. pFastBac1-XPG (13) was digested with NotI and Asel, and the 2.2 kb NotI-Asel XPG fragment was ligated together with the Asel-KpnI digested FEN-1 fragment II and the NotI-KpnI digested pFastBac1 vector in a three-point ligation. The NotI-HindIII digested FEN-1 fragment I was ligated into the NotI-HindIII digested pFastBac1 vector.

For transduction of XP-G/CS fibroblasts, FEN-1-XPG (in pFastBac1) and FEN-1 (in pET28a) cDNA constructs were cloned into the pLOX/EWgfp lentiviral vector by replacing the GFP cDNA (32).

**XPG, FEN-1 and FEN-1-XPG hybrid proteins expression and purification**

XPG and FEN-1-XPG were expressed in S99 insect cells and purified as described previously (13), with minor modifications. The majority of XPG and FEN-1-XPG eluted from the nickel beads at 100 mM imidazole. XPG eluted from the HiTrap S column between 400 and 600 mM NaCl and FEN-1-XPG between 350 and 500 mM NaCl. From the HiTrap Q column, XPG eluted between 450 and 600 mM NaCl and FEN-1-XPG between 500 and 650 mM NaCl. Protein concentrations were determined using the Bradford protein assay with BSA as a standard. Concentrations between 0.1 and 0.3 mg/ml of purified proteins were obtained with an overall yield of 0.2-0.5 mg.

**Human FEN-1 cDNA** was amplified by PCR from HeLa RNA first-strand cDNA synthesis (Clontech), equipped with a C-terminal His6 tag and cloned into pET28a using NcoI and XhoI restriction sites. The plasmid was transformed into the Rosetta pLysS strain (Novagen) and grown at 37°C to an OD600nm of 0.6 and induced with 1 mM IPTG for 4–5 h at 37°C. The cells were lysed by sonication in buffer A (500 mM NaCl, 20 mM Heps pH 8, 10% glycerol, 2 mM β-mercaptoethanol, 0.1% NP-40) and batch purified over Ni-NTA Sepharose using buffer A + 2 mM imidazole for loading and eluted with buffer A + 60 mM imidazole. Fractions containing FEN-1 were dialyzed against buffer Z (20 mM Heps pH 8, 10% glycerol, 2 mM DTT, 1 mM EDTA) + 50 mM KCl and purified on a S Sepharose column using a gradient of 50 mM to 1 M KCl. FEN-1 eluted as a sharp peak at around 400 mM KCl.

**DNA binding and cleavage and in vitro NER assays**

Oligonucleotides described previously (see Table 1 in (13)) were used to generate the splayed arm (oligonucleotide A20 and B19), flap (oligonucleotide A20, B19 and C19) and bubble substrates. DNA cleavage and binding assays were
performed as described previously (13). To construct the double-flap substrate containing a 1-nt 3' flap, oligonucleotide C_{20} that contains one additional A nucleotide at the 3' end was used instead of oligonucleotide C_{19}. Substrates were annealed with a 2-fold excess of the unlabeled oligonucleotide(s). In DNA cleavage assays, the reaction was activated by addition of 0.5 mM MnCl_{2} for XPG and 1 mM MnCl_{2} for FEN-1 and FEN-1-XPG. Cleavage assays were analyzed by denaturing and binding assays by native PAGE. Each gel was quantified using a Typhoon 9400 PhosphorImager (Amersham Biosciences) and Image Quant version 5.2 software from Molecular Dynamics. In all studies, the amount of substrate and product was quantified, and the percentage of substrate cleavage was determined by the product/(substrate + product) ratio. This method allows for correction of any loading errors among lanes. All assays were performed at least in duplicate, and representative gels are shown.

For the in vitro NER assay, a 24-mer oligonucleotide containing a unique GTG sequence (5'-TCTTCTT CTGTGCCACTTCTTCTTCT) was platinated to produce a 1,3-intrastrand GpTpG-crosslink. This oligonucleotide was used in primer extension reaction with ssDNA of pBluescript KS as a template, and purified as described previously (34). The NER assay was performed as described previously (32).

**Cell culture conditions, preparation of whole-cell extracts and transduction with lentiviral recombinants**

For the generation of whole-cell extracts, SV40 transformed fibroblasts XPCS1RO (35) were cultured in Modified Eagle's Medium (MEM) (Gibco) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator. Recombinant lentiviruses containing a unique GTG sequence (5'-TCTTCTT CTGTGCCACTTCTTCTTCT) were produced as described previously (32). Infection was carried out by adding the pseudogenes for FEN-1-XPG and FEN-1 (Promega) according to the manufacturer’s instructions. The number of proliferating cells was measured by recording the absorbance at 490 nm with a 96-well plate reader (Bio-Rad). Viability assays were performed independently four times using three independent sets of transduced cells. For immunodot blots, cells were irradiated with a UVC dose of 20 J/m^{2} and pelleted at different times post-UVC. DNA was isolated using the Puregene method (Genta systems) according to the manufacturer’s instructions. Immunodot blots were performed as described previously (39) using 64M-2 antibodies against 6-4PPs (gift from O. Nikaido).

**Local UV irradiation and immunofluorescence**

Local UV irradiation and immunofluorescence were performed as described previously (32) with the following modifications. The primary antibodies, mouse monoclonal anti-XPG (clone 1G12, gift from J.M. Egly) at 1/1000 dilution and rabbit polyclonal anti-p89 at 1/600, were added for 1 h at room temperature in 1× PBS containing 1% BSA. Detection of 6-4PPs was performed as described previously. Transduced cells were analyzed for the presence/absence of foci, and untransduced cells were used as negative control. Stained cells were observed in a Zeiss Axiovision microscope using the Axiovision software.

**RESULTS**

**Construction and purification of FEN-1-XPG hybrid protein**

In a previous study, we have shown that the deletion of the spacer region in XPG resulted in a defect in NER and in reduced bubble cleavage activity (32). We wanted to test whether the spacer region is sufficient to mediate these XPG-specific functions and reasoned that the insertion of the 600 amino acid spacer region of XPG into the homologous FEN-1 protein would be a suitable experimental approach to test this notion. FEN-1 shares a conserved nuclease domain (N- and I-regions) with XPG, but its N- and I-regions are separated by only about 70 amino acids, defined as a helical clamp structural motif. Based on FEN-1 crystal structures (21,22), we hypothesized that the insertion of XPG spacer into FEN-1 between amino acid residue 104 and 105, in an unstructured region just N-terminal to helix 4, should not dramatically alter the structure of the FEN-1 nuclease active site. We incorporated new HindIII and Asel restriction sites into FEN-1 by PCR and inserted the XPG DNA fragment corresponding to the XPG spacer region from amino acid residue 108 to 732 (Figure 1A), as described in the ‘Materials and methods’ section. We refer to this hybrid protein as FEN-1-XPG.

XPG and FEN-1-XPG protein with a C-terminal his tag were expressed in Sf9 insect cells and purified as described previously (13) (Figure 1B). The FEN-1-XPG protein of 1005 amino acids was soluble and presented similar elution profiles to XPG during protein purification, indicating that the hybrid protein is properly folded. Since the spacer region of XPG is very acidic (pI 4.5), its insertion into FEN-1 yielded a hybrid protein with...
significantly lower pI of 4.9 compared to FEN-1 (pI of 8.8). As observed for XPG, the FEN-1-XPG hybrid protein of 112 kDa migrated above its molecular weight on SDS-PAGE (Figure 1B), providing further evidence that the low pI of the spacer region is responsible for the abnormal migration behavior of XPG (32).

FEN-1-XPG exhibits endonuclease activity on flap and bubble substrates

We first wanted to examine whether the insertion of the 600 amino acids spacer of XPG into FEN-1 protein has any effect on the proper folding of the FEN-1 nuclease domain. We performed nuclease assays with increasing concentrations of purified XPG, FEN-1-XPG and FEN-1 on a flap substrate, which is processed by both XPG and FEN-1 (12,40). FEN-1-XPG showed comparable activity to XPG on the flap substrate in the presence of either Mg$^{2+}$ or Mn$^{2+}$ (Figure 2A, compare top and middle panel, Figure 2C, left panel and data not shown). This result confirms that the insertion of the 600 amino acid spacer region into FEN-1 does not interfere with the proper folding of the nuclease domain. However, the hybrid protein showed somewhat reduced nuclease activity compared to FEN-1 (Figure 2A, compare middle and bottom panel; Figure 2C, left panel). It has been previously shown that the flexible loop between the nuclease domains in FEN-1 assumes an ordered conformation upon substrate binding, which might be important for efficient cleavage by properly positioning the substrate (22,41). It is likely that the insertion of the spacer region in the flexible loop interferes with a structural rearrangement, resulting in reduced activity of FEN-1-XPG compared to FEN-1.

XPG exhibits nuclease activity on bubble substrates, while FEN-1 is unable to cleave such substrates (14,15,40). Since the XPG spacer region was shown to be essential for
efficient bubble cleavage activity (32), we tested whether FEN-1-XPG displayed bubble cleavage activity. As expected, XPG efficiently cleaved the bubble substrate, whereas FEN-1 did not (Figure 2B, top and bottom panel, Figure 2C, right panel). FEN-1-XPG showed significant activity on the bubble compared to FEN-1 (Figure 2B, compare middle and bottom panels), albeit at much lower levels than XPG (Figure 2B, top panel). FEN-1-XPG cleavage activity reached a plateau at 10% conversion (Figure 2C, right panel), and longer incubation times did not lead to significantly higher activity (data not shown).

The failure of FEN-1 and the low activity of FEN-1-XPG to cleave the bubble substrate were not caused by a reduction in DNA substrate binding. Electrophoretic mobility shift assays revealed no significant differences in the binding efficiencies of XPG, FEN-1-XPG and FEN-1 to a bubble substrate in the presence of a 50-fold excess of cold duplex competitor DNA, demonstrating specific binding to ss/dsDNA junctions (Figure 3).

FEN-1 and FEN-1-XPG but not XPG show a preference for double-flap substrates

FEN-1 was shown to exhibit higher nuclease activity on a double-flap substrate with a 1-nt 3' flap overhang in addition to the 5' flap due to higher substrate-binding activity (16,17,42). Structural studies revealed a specific 1-nt-binding pocket in the N-region explaining this preference for the 3' flap (24). We compared the cleavage activity of XPG, FEN-1-XPG and FEN-1 on a double-flap substrate with a 1-nt 3' flap. XPG showed equal activity on the single- and double-flap substrates (Figure 4A, compare lanes 2-7 and 9-14), whereas FEN-1 had higher nuclease activity on the double flap substrate (Figure 4C, compare lanes 2-7 and 9-14). Like FEN-1, the cleavage activity of FEN-1-XPG was strongly stimulated by the presence of the 1-nt 3' flap (Figure 4B, compare lanes 2-7 and 9-14). Although the overall activity of FEN-1-XPG was lower than that of FEN-1, the degree of stimulation of a double-flap compared to a single-flap substrate was similar for both proteins (Figure 4C). This suggests that the insertion of the spacer region of XPG into the helical loop of FEN-1 has no influence on the structure of the 3' 1-nt flap binding pocket. Consistent with this observed preference for cleaving double-flap substrates, FEN-1 and FEN-1-XPG, but not XPG, showed increased binding activity for the double-flap compared to the single-flap substrate (Figure 5).

FEN-1-XPG has partial NER activity in vivo

The spacer region of XPG is necessary to mediate protein–protein interactions required for NER (32). We wondered whether the spacer region would be sufficient for NER activity characteristic of XPG when imbedded in a different structure-specific nuclease. We first tested whether the FEN-1-XPG hybrid protein could perform the incision 3' to the lesion in an in vitro NER assay. We monitored the excision of a 24–32 mer oligonucleotide from a plasmid containing a site-specific 1,3 cisplatin adduct using an extract from a repair-deficient XP-G/CS fibroblast cell line supplemented with recombinant proteins. As expected, the formation of NER excision products was observed upon addition of purified XPG. In contrast, no excision products were detected in the reactions containing FEN-1-XPG or FEN-1 (Figure 6), even at elevated protein concentrations (data not shown). These results suggest that the spacer region of XPG embedded in FEN-1 is not sufficient to support NER incision activity under the conditions used in our in vitro assays.

We then tested whether the FEN-1-XPG protein was able to correct the UV sensitivity of XPG-deficient cells. We had previously observed that the amount of UV resistance conferred by mutant XPG proteins to XP-G/CS cells was a more sensitive measure of NER activity than the in vitro NER assays (32). Primary XP-G/CS fibroblasts (XP20BE) were, therefore, transduced with lentiviral recombinants containing XPG, FEN-1 or FEN-1-XPG. Transduced cells were irradiated with increasing UV doses (0–6 J/m²), and cell viability was measured using a colorimetric assay at 72 h post treatment. Untransduced XP-G/CS cells lacking a functional XPG protein showed a strong sensitivity to UV irradiation (Figure 7A). Similar UV sensitivity was observed when cells were transduced with a lentivirus containing the GFP gene (data not shown). In contrast, when XP-G/CS cells were transduced with XPG, they became resistant to UV irradiation, and more than 80% of these cells survived a UV dose of 6 J/m² (Figure 7A). Surprisingly, we observed an intermediate resistance to UV of transductants expressing FEN-1-XPG (Figure 7A). Although no NER activity in vitro was observed for FEN-1-XPG, it seems that these cells still retain some repair activity in vivo and are able to confer some UV resistance. This is not due to an unanticipated activity of FEN-1 because control transductants...
expressing only FEN-1 were as highly sensitive to UV as untransduced XP-G/CS cells (Figure 7A).

To more directly assay DNA repair, we monitored the disappearance of 6-4PPs over time by immunodot blot analysis. XPG, FEN-1-XPG or FEN-1 transductants were exposed to a UV dose of 20 J/m², and genomic DNA was isolated at various times thereafter. The DNA samples were then probed with monoclonal antibodies against the 6-4PPs (43,44). The disappearance of the signals for 6-4PPs over time indicates proper removal of these lesions by NER. As expected for NER-deficient cells, the signals for 6-4PPs persisted up to 48 h post-UV in untransduced XP-G/CS cells (Figure 7B, lane 1, and data not shown). In contrast, these damages were rapidly repaired in XP-G/CS cells expressing XPG (Figure 7B, lane 2). Only background levels of the signal were observed 2–4 h post-UV in XP-G/CS cells expressing XPG (Figure 7B, lane 2), indicating that the presence of functional XPG protein renders these cells NER proficient. In transductants expressing FEN-1-XPG, the 6-4PPs signal levels started to decrease at 8 h post-UV and almost disappeared after 24 h (Figure 7B, lane 3). The disappearance of the 6-4PP signal in these transductants suggests that they possess a significantly increased repair activity compared to untransduced XP-G/CS cells. In contrast, no repair of the 6-4PPs was observed in XP-G/CS cells transduced with FEN-1 (Figure 7B, lane 4). These results are fully consistent with the in vivo survival curves (Figure 7A). Together, they suggest that the presence of the XPG spacer region in the FEN-1 backbone modifies FEN-1 nuclease activity to permit the cleavage of UV lesions with significant efficiency, thereby conferring intermediate UV resistance to the FEN-1-XPG transductants.

Figure 4. Endonuclease activity of XPG, FEN-1 and FEN-1-XPG on a single- and double-flap substrate. The endonuclease activity of XPG (A), FEN-1-XPG (B) and FEN-1 (C) was assessed on a single 5’ flap substrate (lanes 1–7) or on a double-flap substrate with an additional 1-nt 3’ flap (lanes 8–14). 2.5 nM of substrate was incubated for 40 min at 30°C in the presence of 0.5 mM MnCl₂ for XPG or 1 mM MnCl₂ for FEN-1-XPG and FEN-1. The different protein concentrations for XPG, FEN1-XPG and FEN-1 are indicated (in nM) on the top of each gel. The position of the 5’³P label on the substrate is indicated by the asterisk. The length of the products was determined by comparison with authentic standards and is indicated. The percentage of substrate cleavage versus protein concentration is presented graphically (right panels) for each protein. Graphs show the quantification of two independent experiments. Standard deviations are indicated by error bars.

The FEN-1-XPG hybrid protein is not visible at sites of UV damage

The kinetics of the NER reaction and the assembly and disassembly of the proteins involved at sites of UV damage can be monitored by local irradiation of individual cells and staining of cells at various time points after that using antibodies against UV-damaged DNA or NER proteins (29,45). We have previously used this technique to show that an intact spacer region in XPG is required for the efficient recruitment of the protein to the sites of UV damage (32). Here, we asked whether the presence of the XPG spacer region in the FEN-1 protein would be sufficient to allow the recruitment of the hybrid
protein to sites of UV damage. XP-G/CS transductants expressing XPG or FEN-1-XPG were irradiated with 150 J/m² through an 8 μm pore filter. Cells were then fixed 30 min and 24 h post-UV, and the XPG and XPB proteins as well as the 6-4PPs were detected with appropriate antibodies. As shown previously (32), untransduced XP-G/CS cells contain no detectable XPG protein, but the XPB subunit of TFIIH colocalizes with 6-4PPs 30 min post-UV independently of XPG (Figure 8, 30 min post-UV, Untransduced), and these XPB/6-4PP foci persist for over 24 h (Figure 8, 24 h post-UV, XPB/6-4PPs). Hence, XPG is not needed for the earliest damage recognition events but it is required for damage resolution. In XP-G/CS transductants expressing wild-type XPG, this protein was present in nuclear foci at the UV lesions and colocalized with the XPB and 6-4PPs 30 min post-UV (Figure 8, 30 min post-UV, XPG). By 24 h post-UV, foci for XPG, XPB and 6-4PPs have all disappeared, indicating that repair had been completed (Figure 8, 24 h post-UV, XPG).

In the hybrid transductants, FEN-1-XPG protein was not detectable in foci that contained XPB and 6-4PPs 30 min post-UV (Figure 8, 30 min post-UV, FEN-1-XPG). However, the XPB/6-4PP foci had disappeared by 24 h post-UV, indicating that NER had been completed in these cells (Figure 8, 24 h post-UV, FEN-1-XPG). We had previously observed similar results for certain XPG mutants with deletions in the spacer region (32). This behavior of XP-G/CS cells expressing the FEN1-XPG hybrid protein is consistent with the intermediate survival and repair kinetics of these cells. As a possible mechanistic explanation, the FEN-1-XPG protein may be recruited less stably or with slower kinetics to NER repair complexes.

**DISCUSSION**

In this study, we have generated a hybrid human protein containing the nuclease domains of FEN-1 and the spacer region of the XPG protein to ask if the spacer region of XPG is sufficient to confer NER-specific functions to a different nuclease scaffold.

**Biochemical activities of the FEN-1-XPG protein**

The FEN-1-XPG protein displayed biochemical activities that are characteristic of both XPG and FEN-1. Namely, FEN1-XPG was able to cleave bubble substrates, which...
are not processed by FEN-1. At the same time, FEN-1
-XPG showed a preference for double-flap over single-flap
substrates, similar to FEN-1, while the activity of XPG
is equal for single or double flaps. We found that the
preference for double flaps was a result of increased
binding, consistent with the presence of a defined
1-nt-binding pocket in FEN-1 revealed by structural
studies (24). Our studies suggest that this binding pocket
acts independently of the events surrounding catalysis.
The basis for the preferential cleavage of bubbles by XPG
and FEN-1-XPG over FEN-1 is less straightforward.
Our binding studies revealed that XPG and FEN-1-XPG
bind bubble substrates with comparable affinities to FEN-
1. Similarly, we have previously shown that the change in
substrate specificity in XPG proteins with deletion in the
spacer region is not due to initial binding events (32).
We attempted to express the spacer region separately, but
were unable to obtain any soluble protein of that region
using a variety of conditions in the bacterial or
baculovirus expression systems (data not shown).
Furthermore, we were unable to detect this XPG fragment
in living cells by immunofluorescence after transduction of
a lentiviral construct expressing the spacer region into
XPG/CS cells (data not shown). The predicted high degree
of disorder in that region is likely responsible for our
failure to analyze its properties directly. Although one
study has suggested a role for the spacer region in
substrate binding and recognition based on the inhibition
of the DNA-binding activity of XPG using a monoclonal
antibody directed against the spacer region (46), we
believe that a prominent role for the spacer region in
the catalytic step is most consistent with other available data.
For example, it has previously been found that the flexible
loop between the nuclease domains in FEN-1 undergoes a
rearrangement to facilitate catalysis during the incision
reaction (22,41). The fact that FEN-1-XPG, in which this
flexible loop has been disrupted by the introduction of the
XPG spacer region, shows reduced cleavage activity of
flap substrates compared to FEN-1 supports an important
role of this loop in catalysis. Our studies suggest that this
rearrangement occurs subsequent to initial binding and
does not influence the binding affinity. This mechanism of
initial binding and subsequent rearrangement to a
catalytically active conformation seems to be shared by
XPG, as we have previously demonstrated that it displays
distinct requirement for binding and catalysis (13).
The low level of bubble cleavage activity of FEN-1-
XPG indicates that additional regions of XPG contribute
to efficient cleavage activity. Consistent with this, XPG
with a deletion in the spacer region showed reduced, but
substantial bubble cleavage activity (32). It is, therefore,
possible that the N- and I- regions of XPG make

Figure 7. UV damage repair of XP-G/CS transductants expressing the
FEN-1-XPG hybrid. (A) UV survival curves of XP-G/CS cells
expressing XPG, FEN-1 and FEN-1-XPG proteins. XP-G/CS fibro-
blasts (XP20BE) were transduced with the indicated cDNAs in
lentiviral recombinants and irradiated with the indicated doses of
UVC light. Their survival was monitored 72 h later by a colorimetric
assay. Each point represents the average of four independent
experiments. Error bars indicate the standard errors of the means.
(B) Levels of unrepaired UV DNA damage in XP-G/CS cells
transduced with XPG, FEN-1-XPG and FEN-1 cDNA. Cells were
irradiated with a UV dose of 20 J/m², and the subsequent retention of
6-4PPs was analyzed by immunodot blotting with antibodies against
6-4PPs. NI, non-irradiated.

Figure 8. Recruitment of FEN-1-XPG hybrid to local UV sites of damage in XP-G/CS cells. Cells were irradiated at 150 J/m² through a filter with
8 µm pores and fixed 30 min or 24 h after irradiation. Cells were then
immunolabelled with antibodies against XPG (green), against the XPB subunit
of TFIIH (red) and against 6-4PPs (green). Merged images indicate the overlay of 6-4PPs and XPB staining. Scale bars, 10 µm.
advantageous to employ multiple weak interactions

assembly of the factors involved at the site of damage

NER is a highly dynamic process that acts by sequential

the NER pathway

Multiple weak interaction sites facilitate progression through

the NER pathway

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current detection of the lesions induced and NER proteins

recruited to them by immunohistochemistry (29,45,47).

We used this method to evaluate regions in XPG that

might contribute to the proper recruitment of the protein
to sites of NER. XPG is recruited within 30 min following

irradiation, and it depends on the prior recruitment of

XPC/HR23B and TFIIH. Twenty-four hours after irra-

diation, NER proteins are no longer visible in foci,

indicating that repair has been completed (32,33).

Mutations in the spacer region of XPG can affect

recruitment in two ways; in one case, XPG is recruited

to UV-damaged sites with normal kinetics, but is then

unable to properly execute its function in NER due to

unstable association with the preincision complex (33).

In the second case, the recruitment of XPG to UV-
damaged sites cannot be directly observed. However, since

the damage is ultimately repaired, albeit with slower

kinetics than in the wild-type situation, it can be inferred

that XPG was at some point present at the damaged site

(32). The fact that defects in the interaction with TFIIH

caused by different mutations in XPG can influence the

recruitment of XPG as well as the subsequent processing

of NER substrates is a testimony to the complexity of the

interaction between the two proteins.

Several years ago, it was suggested that sequences

within and downstream of the I-region are also involved in

mediating the interaction with TFIIH and hence con-

tribute to the recruitment of XPG to sites of NER (27).

Indeed, in ongoing work, we have observed that replacing

the C-terminus of FEN-1 in FEN-1-XPG with the

C-terminus of XPG yielded a protein that displayed

wild-type levels of recruitment to sites of UV damage

(data not shown). While the first generation design of this

hybrid protein containing the FEN-1 nuclease core and

spacer and C-terminal regions of XPG appeared not to

result in a fully functional protein and only restored UV

resistance to XP-G/CS cells at levels comparable to the

FEN-1-XPG protein, it provides evidence that two

independent regions in XPG are responsible for the

recruitment of the protein to NER preincision complexes

by interaction with TFIIH (27,29,30).

**Multiple weak interaction sites facilitate progression through

the NER pathway**

NER is a highly dynamic process that acts by sequential

assembly of the factors involved at the site of damage

(4,31,48). To ensure progression through the pathway, it

is advantageous to employ multiple weak interactions

between the proteins and DNA intermediates involved

rather than strong specific interactions such as those

employed by transcription factors binding to their DNA

substrates (49). The transient nature of many of the

interactions involved could serve as a basis for the NER

machinery to assemble dynamically and pass on substrates

from one subcomplex to the next. The modular structure

of the XPG protein and the modular nature of its

interaction with TFIIH and DNA fit perfectly within

that paradigm. At least two regions of XPG, the spacer

region and the C-terminus, interact with the p44, p62,

XPD and XPB subunits of TFIIH (27). Recent studies

have shown that the first 108 amino acids of the p62

subunit of TFIIH make up a pleckstrin homology and

phosphotyrosine binding (PH/PTB) domain that specifi-

cally interacts with XPG (50). This PH/PTB domain is not

required for transcription or the integrity of TFIIH and

NMR studies showed that it forms a defined 3D structure,

providing a first glimpse of a modular domain involved

in the interaction between TFIIH and XPG. An important

future challenge will be to further determine the molecular

basis of the interaction of various modules of TFIIH

and XPG.

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