**Nucleotide excision repair 3′ endonuclease XPG stimulates the activity of base excision repair enzyme thymine glycol DNA glycosylase**

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**ABSTRACT**

An ionizing radiation-induced DNA lesion, thymine glycol, is removed from DNA by a thymine glycol DNA glycosylase with an apurinic/apyrimidinic (AP) lyase activity encoded by the *Escherichia coli* endonuclease III (*nth*) gene and its homolog in humans. Cells from Cockayne syndrome patients with mutations in the XPG gene show ∼2-fold reduced global repair of thymine glycol. Hence, I decided to investigate the molecular mechanism of the effect of XPG protein observed in vivo on thymine glycol removal by studying the interactions of XPG protein and human endonuclease III (HsNTH) protein in vitro and the effect of XPG protein on the activity of HsNTH protein on a substrate containing thymine glycol. The XPG protein stimulates the binding of HsNTH protein to its substrate and increases its glycosylase/AP lyase activity by a factor of ∼2 through direct interaction between the two proteins. These results provide in vitro evidence for a second function of XPG protein in DNA repair and a mechanistic basis for its stimulatory activity on HsNTH protein.

**INTRODUCTION**

Oxidative DNA damage is possibly the most common form of DNA damage (1–4). It is engendered by normal cellular metabolism and by exposure to oxidants or ionizing radiation (1–4). An ionizing radiation-induced DNA damage, thymine glycol, is mainly repaired by the base excision repair pathway initiated by endonuclease III (Nth) in *Escherichia coli* (5,6). A human homolog of NTH (human endonuclease III; HsNTH) has been cloned recently (7,8) and shown to be, like the *E. coli* enzyme, a thymine glycol DNA glycosylase with an apurinic/apyrimidinic (AP) lyase activity (8).

Cockayne syndrome (CS) is a rare genetic disorder with severe growth defects, neuronal demyelination and mental retardation (9). Cells from CS patients are moderately sensitive to UV-irradiation and defective in the preferential repair of UV-induced cyclobutane dimers in the transcribed strand of active genes; a repair pathway known as transcription-coupled repair (9,10). In addition to the CS-A and CS-B groups, complementation analysis assigned some CS patients to rare xeroderma pigmentosum (XP) groups B, D and G. Surprisingly, XP-G/CS cells also show reduced global repair and lack of transcription-coupled repair of thymine glycol which is removed primarily by base excision repair (11). Mutation analysis revealed that XP-G/CS patients have mutations which result in severely truncated XPG protein while classical XP-G cells have XPG protein with missense mutations which inactivate its function in excision repair (12). These data suggest that XPG protein has a role in the repair of thymine glycol in addition to its role as a structure-specific endonuclease in nucleotide excision repair.

Since XP-G/CS cells have reduced global repair of thymine glycol which is primarily repaired by HsNTH protein, the effect of XPG protein on the activity of HsNTH protein was studied. Surprisingly, the nucleotide excision repair endonuclease XPG protein enhances the DNA binding activity of base excision DNA glycosylase, HsNTH protein, to its substrate thymine glycol through the direct interaction of the two proteins and stimulates the repair activity of HsNTH protein.

**MATERIALS AND METHODS**

**Cloning of HsNTH cDNA and purification of recombinant proteins**

HsNTH cDNA was obtained by RT–PCR using mRNA prepared from GM00969C (normal human cell line) as a template and 5′-ATGGATCCGATGTGTAGTCCG-3′ and 5′-GGCGAATTCTCAGAGACCTGGGC-3′ as primers. HsNTH cDNA was cloned into BamHI and EcoRI sites of pRSET(b) from Invitrogen (CA). Recombinant (His)6-HsNTH protein was overexpressed in BL21 (DE3) cells and purified by phosphocellulose and Ni-NTA columns (7). Cell lysate from 2 l of BL21 (DE3) cells harboring pRSET-HsNTH was applied to 25 ml phosphocellulose P11
column (Whatman, UK) equilibrated with buffer A (25 mM HEPES–KOH, pH 7.4, 1 mM EDTA, 10 mM β-mercaptoethanol and 10% glycerol) containing 0.1 M KCl. Bound proteins were eluted with a linear gradient of KCl from 0.1 to 1.5 M in buffer A. The proteins eluted at 0.7–0.8 M KCl were pooled and dialyzed against buffer A containing 0.5 M KCl and 1 mM imidazole. After extensive washing of the column with buffer A containing 0.5 M KCl and 1 mM imidazole, bound (His)_6-HsNTH protein was eluted with buffer A containing 0.5 M KCl and 0.15 M imidazole. Protein peak fractions were pooled and dialyzed against storage buffer (25 mM HEPES–KOH, pH 7.9, 100 mM KCl, 12 mM MgCl_2, 0.5 mM KCl, 0.5 mM MgCl_2, 0.2 mM EDTA, 11% glycerol and 1% NP-40) for 30 min on ice. To study the effect of XPG protein on the binding activity of HsNTH protein, (His)_6-HsNTH (210 ng) was incubated with immobilized DNA (50 fmol) in the presence of XPG protein for 30 min on ice. After extensive washing of the immobilized DNA with pull-down buffer, the bound (His)_6-HsNTH protein was eluted by 1% SDS and separated on 10% SDS–PAGE. (His)_6-HsNTH protein was analyzed by western blotting with Anti-Xpress Antibody (Invitrogen, CA).

DNA binding assay

Biotinylated 140 bp substrate DNA containing a single thymine glycol at position 70 was immobilized on streptavidin-magnetic beads (DYNAL, NY). (His)_6-HsNTH protein (210 ng) was incubated with different amounts of the immobilized DNA in 25 µl of pull-down buffer (10 mM HEPES–KOH, pH 7.9, 60 mM KCl, 5 mM MgCl_2, 0.2 mM EDTA, 1% glycerol and 1% NP-40) for 30 min on ice. To study the effect of XPG protein on the binding activity of HsNTH protein, (His)_6-HsNTH (210 ng) was incubated with immobilized DNA (50 fmol) in the presence of XPG protein for 30 min on ice. After extensive washing of the immobilized DNA with pull-down buffer, the bound (His)_6-HsNTH protein was eluted by 1% SDS and separated on 10% SDS–PAGE. (His)_6-HsNTH protein was analyzed by western blotting with Anti-Xpress Antibody.

RESULTS

Stimulation of HsNTH repair activity by XPG

In order to understand the molecular mechanism of the global repair of thymine glycol, the effect of XPG protein on HsNTH activity was studied. 5'-end-labeled substrate of 139 bp containing a single thymine glycol residue at position 70 in one strand was incubated with HsNTH protein in the presence or absence of XPG protein. HsNTH protein generates a 69 nucleotide length fragment from this substrate by the combined action of DNA glycosylase and AP lyase activity. XPG protein stimulates the activity of HsNTH protein by a factor of 2 (Fig. 1A–C). Since the global repair of thymine glycol in XP-G/CS cells is reduced by a factor of ~2 (11), the 2-fold stimulation of HsNTH activity by XPG protein in vitro is in reasonable agreement with the in vivo data. Structure-specific endonuclease XPG protein itself does not generate a nick on thymine glycol containing DNA (Fig. 1A, lane 10). Furthermore, an XPG nuclease active site mutant, XPG (D812A) (17), shows the same stimulatory effect on the activity of HsNTH (data not shown) demonstrating that the endonuclease activity of XPG protein is dispensable for this effect. This stimulatory effect is specific for HsNTH protein as XPG protein does not stimulate the repair activity of Nth from E.coli (Fig. 1C).

XPG protein binds to HsNTH protein in vitro

To gain insight into the molecular mechanism of the stimulation of HsNTH activity by XPG protein, I looked for a specific interaction between XPG protein and HsNTH protein. An HsNTH protein column was prepared with (His)_6-HsNTH and Ni-NTA resin, and purified XPG protein was applied to the column. The XPG protein was retained on the HsNTH column, but not on the control column.
Single thymine glycol was biotinylated and immobilized on HsNTH protein to its substrate was studied. DNA containing a interaction, the effect of XPG protein on the binding activity of HsNTH protein, the immobilized DNA was washed extensively streptavidin-magnetic beads. After incubation with XPG protein and HsNTH protein, the immobilized DNA was washed extensively with buffer, and then the amount of HsNTH protein bound to DNA was analyzed by western blotting. In the absence of XPG protein, the HsNTH protein, as expected, binds to thymine glycol containing DNA in a substrate concentration-dependent manner (Fig. 3A). This binding is enhanced by the XPG protein (Fig. 3B). In either the presence or absence of XPG protein, the HsNTH protein did not bind to DNA containing a (6–4) photoproduct. (B) HsNTH protein binds to the MBP-XPG column. An aliquot of 1.4 µg of (His)6-HsNTH protein was applied to an MBP-XPG or MBP-XPA column. After washing with binding buffer, bound proteins were eluted and analyzed on 10% SDS–PAGE. XPG protein was analyzed by western blotting with anti-XPG monoclonal antibody. Lane 4, (His)6-HsNTH protein (1.4 µg); lane 5, MBP-XPG column; lane 6, MBP-XPA column. Nearly 100% of the (His)6-HsNTH protein was retained on an MBP-XPG column. About 5% of the input (His)6-HsNTH protein was retained non-specifically both on the MBP-XPA column and on an amylose control column (data not shown).

Figure 2. Specific interaction between HsNTH protein and XPG protein. (A) XPG protein binds to (His)6-HsNTH protein column. 600 ng of XPG protein was applied to a (His)6-HsNTH column or a control column, Ni-NTA resin. After washing with binding buffer, bound proteins were eluted and separated on 10% SDS–PAGE. XPG protein was analyzed by western blotting with anti-XPG monoclonal antibody. Lane 1, XPG protein (360 ng, 60% of input XPG protein); lane 2, (His)6-HsNTH column; lane 3, Ni-NTA column (control). Nearly 100% of the XPG protein was retained on the (His)6-HsNTH column. The bands below the XPG protein are a mixture of degraded XPG proteins and minor contaminating proteins in the XPG protein preparation which are detected by the anti-XPG antibody. (B) HsNTH protein binds to the MBP-XPG column. An aliquot of 1.4 µg of (His)6-HsNTH protein was applied to an MBP-XPG or MBP-XPA column. After washing with binding buffer, bound proteins were eluted and analyzed on 10% SDS–PAGE. (His)6-HsNTH protein was analyzed by Anti-Xpress Antibody. Lane 1, XPG protein (360 ng, 60% of input XPG protein); lane 2, (His)6-HsNTH column; lane 3, Ni-NTA column (control). Nearly 100% of the (His)6-HsNTH protein was retained on the (His)6-HsNTH column. The bands below the XPG protein are a mixture of degraded XPG proteins and minor contaminating proteins in the XPG protein preparation which are detected by the Anti-Xpress Antibody.}

Figure 1. XPG protein stimulates HsNTH activity on thymine glycol containing DNA. (A) 5′-end-labeled 139 bp DNA (3 fmol) containing a single thymine glycol at position 70 was incubated with 30 ng of HsNTH protein alone (lanes 2–5) or 30 ng of HsNTH protein in the presence of 30 ng of XPG protein (lanes 5–7). Aliquots were taken from the reaction mixture at indicated time points and analyzed on an 8% polyacrylamide sequencing gel. C, control DNA. The incision rates by HsNTH alone (open diamond) and HsNTH and XPG (closed circle) were quantified. XPG stimulated HsNTH activity 1.5-fold at 5 min, 1.7-fold at 10 min and 1.5-fold at 20 min in this particular experiment. Substrate DNA was incubated with HsNTH protein (30 ng, lane 9) and XPG protein (30 ng, lane 10) at 30°C for 10 min. (B) Substrate DNA was incubated with HsNTH protein alone (30 ng, lane 2) and also in the presence of XPG protein (30 ng, lane 3; 60 ng, lane 4) at 30°C for 10 min. The results of four independent experiments were quantified and the degree of stimulation of HsNTH activity by XPG protein is shown. The error bars represent standard deviation. (C) XPG protein does not stimulate Nth activity. Substrate DNA was incubated with 10 ng of Nth protein (lane 2) or 30 ng of HsNTH protein alone (lane 5) and also in the presence of XPG protein (30 ng, lanes 3 and 6; 60 ng, lanes 4 and 7) at 30°C for 10 min. The incision rates by HsNTH protein with XPG protein (black bars) and Nth protein with XPG protein (gray bars) were quantified and the degrees of stimulation are shown. (Fig. 2A). In order to confirm this interaction, an XPG protein column was prepared with MBP-XPG protein and amylose resin and then HsNTH was applied to this column. HsNTH protein was retained on the MBP-XPG column, but not on the MBP-XPA column used as a control (Fig. 2B). These data demonstrate a specific interaction between XPG protein and HsNTH protein even in the absence of DNA, although I cannot exclude the possibility that minor contaminants in either XPG or HsNTH protein preparations are mediating the observed interactions.

**XPG protein enhances substrate DNA-binding activity of HsNTH protein**

To further examine the significance of this protein–protein interaction, the effect of XPG protein on the binding activity of HsNTH protein to its substrate was studied. DNA containing a single thymine glycol was biotinylated and immobilized on streptavidin-magnetic beads. After incubation with XPG protein and HsNTH protein, the immobilized DNA was washed extensively with buffer, and then the amount of HsNTH protein bound to DNA was analyzed by western blotting. In the absence of XPG protein, the HsNTH protein, as expected, binds to thymine glycol containing DNA in a substrate concentration-dependent manner (Fig. 3A). This binding is enhanced by the XPG protein (Fig. 3B). In either the presence or absence of XPG protein, the HsNTH protein did not bind to DNA containing a (6–4) photoproduct. (B) HsNTH protein binds to the MBP-XPG column. An aliquot of 1.4 µg of (His)6-HsNTH protein was applied to an MBP-XPG or MBP-XPA column. After washing with binding buffer, bound proteins were eluted and analyzed on 10% SDS–PAGE. XPG protein was analyzed by western blotting with anti-XPG monoclonal antibody. Lane 4, (His)6-HsNTH protein (1.4 µg); lane 5, MBP-XPG column; lane 6, MBP-XPA column. Nearly 100% of the (His)6-HsNTH protein was retained on the MBP-XPG column. About 5% of the input (His)6-HsNTH protein was retained non-specifically both on the MBP-XPA column and on an amylose control column (data not shown).

**DISCUSSION**

In summary, I have discovered that the XPG structure-specific endonuclease enhances the binding activity of HsNTH, thymine glycol DNA glycosylase, to DNA containing thymine glycol and that this XPG protein-mediated enhanced binding stimulates the repair activity of HsNTH protein in vitro. This is the first in vitro evidence that one of the nucleotide excision proteins has a role in
Interestingly, XPG protein appears to have at least four functions in DNA repair: (i) a structure-specific endonuclease in nucleotide excision repair (13,14,24); (ii) stabilization of a preincision complex of excision nuclease consisting of XPA, RPA, XPC and TFIIF on damaged DNA (25); (iii) stimulation of the binding activity of HsNTH to thymine glycol containing DNA (this study); and (iv) a specific role in transcription-coupled repair of any type of DNA damage (11,12). The molecular mechanism of transcription-coupled repair is still unclear. XP-G/CS cells show a reduced global repair of thymine glycol but are also defective in transcription-coupled repair of thymine glycol as well as cyclobutane thymine dimer (11,12). I propose the following model for the unique role of XPG protein in base excision repair of transcribed DNA. Thymine glycol in the template DNA inhibits transcription elongation by RNA polymerase II forming a bubble structure containing a thymine glycol in single strand DNA, a stalled RNA polymerase II and a short transcript (T.Bessho and A.Sancar, unpublished observations; 26). HsNTH protein is unable to repair the thymine glycol in this ternary complex because (i) DNA glycosylases show a strong preference for double-stranded DNA as a substrate (5,6) and (ii) RNA–DNA hybrids inhibit DNA glycosylase activity (27). In my model, RNA polymerase II is displaced to eliminate the bubble structure, and at the same time, HsNTH protein is recruited to the thymine glycol lesion for initiation of repair. One possible function of XPG protein in transcription-coupled repair of thymine glycol along with CSB protein and TFIIF might be the recruitment of HsNTH protein to the site of damage in a ternary complex of DNA–RNA–RNA polymerase II. It is interesting that there exists an XP patient apparently without CS symptoms, XP3BR (28). Cells from this patient are sensitive to \( \gamma \)-ray irradiation indicating reduced global repair of thymine glycol. In fact, both global repair and transcription-coupled repair of thymine glycol are reduced in XP3BR cells (S.A.Leadon, personal communications). Further work is required to dissect the functions of XPG protein in global repair of thymine glycol and transcription-coupled repair.

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