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Mutation Research 347 (1995) 55–60

Mutation Research Letters

XPG protein has a structure-specific endonuclease activity

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Received 13 March 1995; accepted 23 March 1995

Abstract

Biochemically active human DNA repair protein, xeroderma pigmentosum G (XPG), was overexpressed in insect cells by a recombinant baculovirus. The recombinant baculovirus produced XPG with a mobility of ~ 185 kDa in a denaturing polyacrylamide gel. Indirect immunofluorescence studies demonstrated that the recombinant full-length XPG protein was expressed predominantly as a nuclear protein. The recombinant XPG protein was purified to apparent homogeneity using Q-sepharose, S-300 size exclusion, and Mono Q column chromatography. XPG protein showed a structure-specific DNA endonuclease activity, and a preferential affinity to single-stranded DNA and RNA compared to double-stranded DNA.

Keywords: DNA repair; Xeroderma pigmentosum group G; Baculovirus expression system; Nuclear protein; Immunofluorescence, flap endonuclease

1. Introduction

The nucleotide excision repair pathway is one of the major components of the DNA repair systems in prokaryotes and eukaryotes (Friedberg, 1985). This pathway removes a wide range of lesions, including UV-induced photo-products, as well as bulky chemical adducts (Sancar and Tang, 1993). Defects in the excision repair system have serious consequences for the organism, such as in the human cancer-prone syndrome xeroderma pigmentosum (XP) (Cleaver and Kraemer, 1989). We recently cloned the ERCC5 gene on the basis of its ability to correct the excision-repair defect in CHO UV135 cells (Mudgett and MacInnes, 1990; MacInnes et al., 1993). Subsequently, it was discovered that ERCC5 complemented the excision repair defect in cells from XPG patients (Scherly et al., 1993; O'Donovan and Wood, 1993; Shiomi et al., 1994). More recently, it was found that XPG protein possesses the single-stranded DNA, a structure-specific endonuclease, and exonuclease activities (O'Donovan et al., 1994a,b; Habraken et al., 1994a,b). These findings prove that XPG protein is a catalytic component that incises the damaged DNA strand during the nucleotide excision repair. To gain insight into the structure/function relationship of XPG, we characterized the recombinant XPG protein that was overexpressed in insect cells by a baculovirus expression system.

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SSDI 0165-7992(95)00019-4

2. Materials and methods

Cell culture

Spodoptera frugiperda cells (Sf9) were kindly provided by Mr. Glenn Godwin at Gibco BRL (Gaithersburg, MD), and grown in Sf-900 II SFM medium (Gibco BRL). Cells were maintained and infected either as monolayer or in suspension on a rotary shaker in a low temperature incubator set at 28° C. Cells were routinely infected with 10 plaque-forming units of recombinant virus per ml of culture at a density of 5×10^5 cells (> 95% viability) per ml.

Construction of recombinant transfer vector and recombinant baculovirus

Linearized wild-type baculovirus (BaculoGold™) and the transfer vector pVL1392 were purchased from Pharmingen (San Diego, CA) and Invitrogen (San Diego, CA), respectively. The recombinant transfer vector pVL1392-XPGMP2 was constructed from the transfer vector pVL1392 and the plasmid pBSK-XPGA, encompassing the entire XPG sequence from −47 to +3561 relative to the ATG start site (Fig. 1). The resulting construct, pVL1392-XPGMP2, was then co-transfected with the linearized wild-type baculovirus DNA (BaculoGold™) into Sf9 cells by the cationic liposome method, as described by the manufacturer's instruction (Invitrogen). Recombinant BaculoGold-XPGMP2 baculovirus was detected by visual inspection and was further screened by PCR with a pair of primers (P3: 5′ AAACCTATAAATATTCCGGA 3′; P4: 5′ TAGTGCTTCATCTCTTTT 3′), which produced a 528-bp fragment. All methods pertaining to the growth, transfection, screening, and manipulation of the Sf9 cells and baculovirus have been described elsewhere (Summers and Smith, 1987; O'Reilly et al., 1992) unless otherwise indicated.

Antibody preparation

A peptide (aa 747–801) of XPG, which was produced in *E. coli*, was used to immunize mice. The serum collected from the immunized mice was further purified on affinity columns, and the antibody was named anti-XPGHLH.

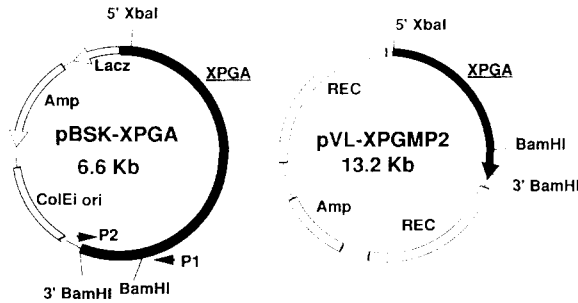

Fig. 1. Construction of the baculovirus transfer vector pVL1392-XPGMP2. The recombinant transfer vector pVL1392-XPGMP2 was constructed from the transfer vector pVL1392 and the plasmid pBSK-XPGA, encompassing the entire XPG sequence from −47 to +3561 relative to the ATG start site. Due to the presence of a *Bam*HI site at +2836 in the XPG cDNA, 2.9 kb *Xba*I-*Bam*HI fragment of XPG gene was first ligated to the *Xba*I-*Bam*HI-digested pVL1392 to create pVL1392-XPGMP1. The rest of the XPG cDNA molecule (+2836 to +3561) was synthesized by polymerase chain reaction (PCR) with a pair of primers (P1: GAGGCCTACCTCAAACCCGTGGTG; P2: CGCGGATC-CGCGTTAGGT TTTCCTTTTTC). The *Bam*HI-digested PCR product was connected to the *Bam*HI-digested pVL1392-XPGMP1 to create the final construct pVL1392-XPGMP2.

SDS-PAGE and Western blot analysis

Sf9 (5×10^5) cells were harvested at 0–72 h post infection. Cells were washed with phosphate buffered saline (PBS, pH 6.2) (O'Reilly et al., 1992), and solubilized in 600 μl SDS sample buffer, boiled for 5 min, passed through a 27 gauge hypodermic needle in a 1 ml size syringe, and 10 μl was electrophoresed in SDS/4–20% gradient acrylamide gels. Proteins were then transferred electrophoretically to nitrocellulose filter papers, incubated with the anti-XPG1322, and detected with alkaline phosphatase and 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/ nitroblue tetrazolium (NBT) (Blake et al., 1984).

Immunofluorescence microscopy

Sf9 cells ($0.5–1 \times 10^4$) were cytofuged to glass slides, fixed in methanol for 10 min at −20° C, permeabilized in 0.1% Triton X-100 for 5 min, incubated with anti-XPG1322 for 1 h (50 ng/ml in 3% BSA/PBS, pH 7.5), and reacted with 1:200 diluted FITC-conjugated secondary antibody

(Sigma, St. Louis, MO) for 1 h. Then cells were analyzed by an epifluorescence microscope (Zeiss), and images were recorded by Kodachrome (ASA 400).

Purification of XPG protein

Wild-type XPG protein from Sf9 cells, expressing the recombinant baculoviruses, was purified using the previously published procedure (Habraken et al., 1994a).

Assays

DNA substrates for flap cleavage were prepared, and the flap-endonuclease assay was performed as previously described (Harrington and Lieber, 1994). Single-stranded DNA endonuclease assay was done as described by Habraken et al. (1994b). Binding of purified XPG protein to single-stranded DNA was determined by the retention of [^{3}H]DNA (prepared from M13 phage) on nitrocellulose filter (Kim et al., 1992). Reaction mixtures contained increasing amounts of purified XPG protein plus 25 ng DNA, and were incubated for 30 min in 40 mM Tris-HCl, pH 7.4, 80 mM NaCl, 1 mM EDTA, 1.5 mM dithiothreitol (DTT) on ice. For competition of DNA binding, reaction mixtures contained 10 ng of XPG protein plus 25 ng DNA and were incubated as described above in the presence of competing nucleic acids.

3. Results and discussion

Expression and purification of recombinant XPG protein in insect cells

To examine the expression of XPG in Sf9 cells infected with baculoviruses encoding full-length XPG protein, we performed a time course study using Western blot analysis (data not shown). Expression of recombinant XPG protein can be detected in whole cell extracts as early as 24 h after infection with BaculoGold-XPG2, and reaches a maximum level of expression between 40 and 60 h post-infection. The reduced level of expressed XPG protein 72 h post-infection appears to be due to cell death, which is indicated by the reduced level of total cellular protein. A polypeptide with mobility of ~ 185 kDa was detected in recombinant virus infected cells on SDS-PAGE (Fig. 2, lane 2). The expressed recombinant XPG protein in Sf9 cells migrates more slowly (~ 185 kDa) in SDS gels than the predicted molecular weight (135 kDa). Similar result was reported by others (O'Donovan et al., 1994a; Habraken et al., 1994a). A combination of ammonium sulfate precipitation, Q-sepharose, S-300 size exclusion, and Mono S column was used to purify XPG protein from insect cells. Mono S fractions of XPG protein was nearly homogeneous as demonstrated by Coomassie staining (Fig. 2, lane 3) and Western analysis (Fig. 2, lane 4).

Intracellular localization of overexpressed XPG protein in insect cells

To examine the intracellular location of XPG protein, indirect immunofluorescence was employed using anti-XPG1322 on intact Sf9 cells

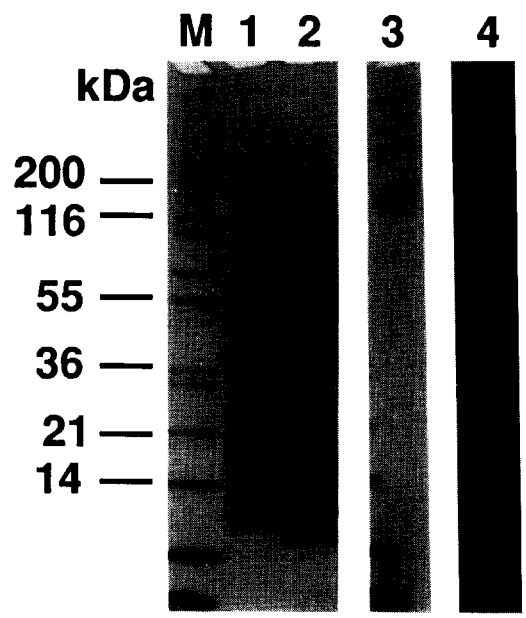

Fig. 2. Overexpression and purification of recombinant XPG protein. Lanes M: molecular weight marker; 1: Sf9 cells infected with the wild type virus; 2: Sf9 cells infected with the BaculoGold-XPGMP2 recombinant virus. Total cell extracts were prepared 48 h post-infection. 3: Coomassie staining of purified XPG (2 μg); 4: Western blot analysis of purified XPG (150 ng) using anti-XPGHLH.

infected with the BaculoGold-XPGMP2 (Fig. 3). No positive immunofluorescence was detected in cells infected with the wild-type virus (Fig. 3A), or when cells expressing XPG were probed with preimmune serum (Fig. 3B). More than 90% of infected Sf9 cells expressed XPG protein, and the XPG-specific immunofluorescence was predominantly localized in the nucleus in insect cells infected with the recombinant baculovirus (Fig. 3C). Putative NLSs in the ERCC5/XPG protein have been proposed (MacInnes et al., 1993; Scherly et al., 1993), and we identified strong candidates for XPG NLS by using the β-galactosidase reporter system (Knauff et al., unpublished observation). The presence of functional NLS signals in the XPG protein provides a molecular basis for our observation of nuclear localization of XPG protein in the nucleus of insect cells.

Structure-specific DNA endonuclease assay

In order to test biochemical activity of the recombinant XPG protein, we subjected the purified XPG for a structure-specific endonuclease

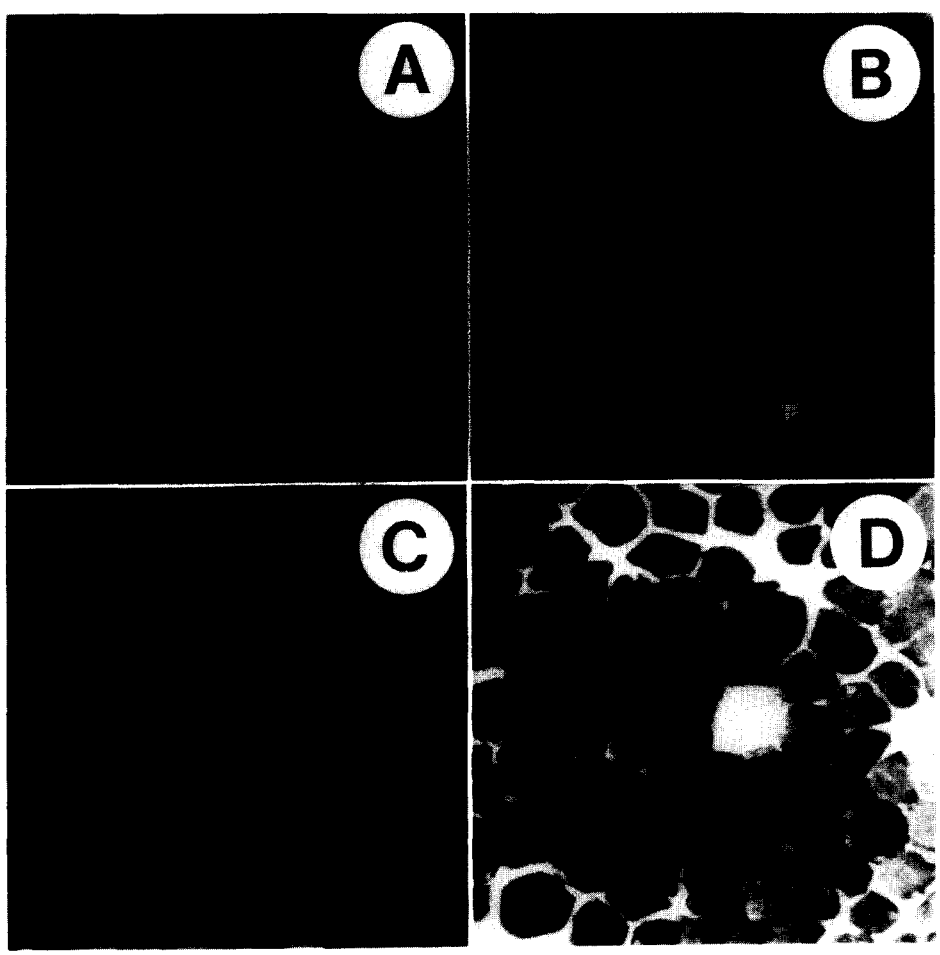

Fig. 3. Indirect immunofluorescence localization of the recombinant XPG protein in Sf9 cells. (A) Sf9 cells infected with the wild-type virus. (B) Sf9 cells infected with the recombinant BaculoGold-XPGMP2 and reacted with a preimmune serum. (C) Sf9 cells infected with the recombinant BaculoGold-XPGMP2 and reacted with anti-XPGHLH. (D) Giemsa-stained Sf9 cells infected with the recombinant BaculoGold-XPGMP2.

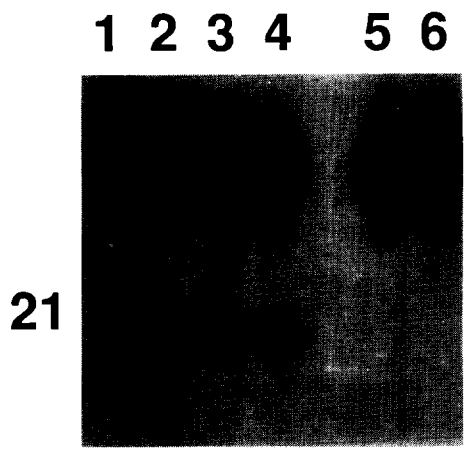

Fig. 4. Flap-endonuclease activity of XPG proteins. Lanes: 1: 0.5 ng; 2: 1 ng; 3: 5 ng; 4: 10 ng; 5: 10 ng of XPG and 5 μg of anti-XPGHLH, 6: 10 ng of heat inactivated XPG. Flap substrate was prepared as described (Harrington and Lieber, 1994). The flap strand (HJ42) was 5′ end-labeled and annealed to the F_{br} (HJ41) and F_{adj} (HJ43) strands. Following incubation of flap substrate with varying amounts of purified XPG and with or without anti-XPGHLH, the reaction product was run on a 12% denaturing polyacrylamide gel. Molecular weight marker is shown in nucleotides.

activity. Purified XPG was able to cut the flap substrate in a concentration-dependent manner (Fig. 4, lanes 1–4). The flap-endonuclease activity was abolished by the preincubation of reaction mixtures with anti-XPGHLH antibody (Fig. 4, lane 5) and heat inactivation of XPG (Fig. 4, lane 6). We also observed the single-stranded DNA endonuclease activity with purified XPG (data not shown).

By using a nitrocellulose filter binding assay, we observed an efficient binding of XPG to single-stranded DNA (Fig. 5A). In the presence of anti-XPGHLH antibody, XPG binding to single-stranded DNA was abolished (Fig. 5A). XPG binding to single-stranded DNA was best competed with unlabeled single-stranded DNA and to the some extent with total cytoplasmic RNA from HeLa cells (Fig. 5B). Double-stranded DNA was less effective as a competitor for nucleic acid binding. Since XPG protein functions as a single-stranded DNA endonuclease, it was not suprising to see the effective competition by cold circular single-stranded DNA in excess. However, relatively effective binding of RNA to XPG protein was also observed. It may suggest that XPG protein may recognize a localized structure which assumes single-strand conformations after DNA damage. This conformation may have the structural features that are similar between circular single-stranded DNA and RNA.

Nucleotide excision repair requires complex macromolecular interactions. To define these molecular interactions, it is essential to acquire

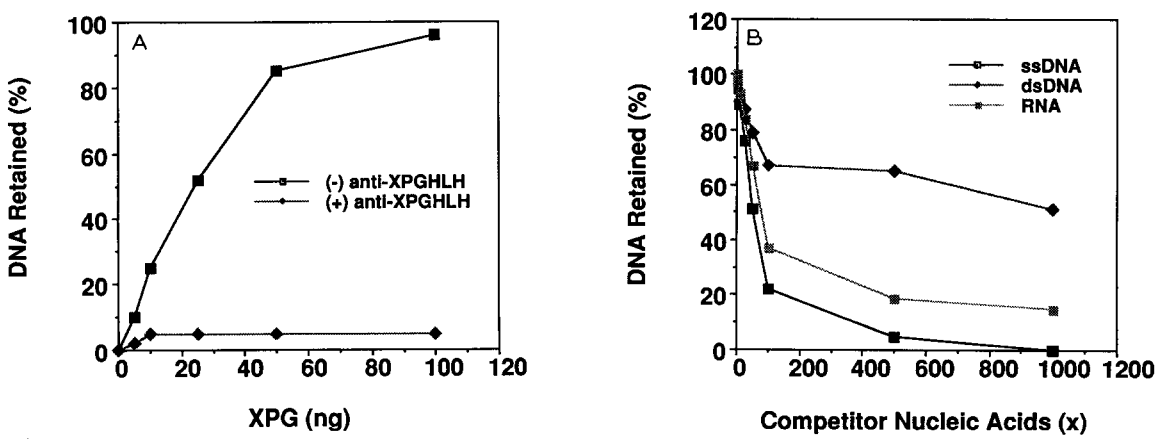

Fig. 5. DNA binding activity of purified XPG protein. (A) Single-stranded DNA binding activity. Binding of purified XPG to single-stranded DNA was determined by the retention of [^{3}H]DNA on nitrocellulose filters. Preincubation of the XPG protein with the anti-XPGHLH (2.5 μg) inhibited single-stranded DNA binding. (B) Competition of XPG protein/single-stranded DNA binding by different nucleic acids. As competitors, total cytoplasmic RNA from HeLa cells (RNA), single-stranded (ssDNA), and double-stranded (dsDNA) M13 DNA were used.

functionally active components. Overexpression of biochemically active XPG protein will not only enable us to study the structure/function relationships of XPG protein, but it will also provide us means to reconstitute nucleotide excision repair in vitro.

Acknowledgements

The authors would like to thank Mr. Glenn Godwin for providing valuable suggestions on baculovirus expression techniques. This study was supported by the Office of Health and Environmental Research of the Department of Energy.

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Communicated by M.D. Shelby