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Rat 7,8-dihydro-8-oxoguanine DNA glycosylase: substrate specificity, kinetics and cleavage mechanism at an apurinic site

M. J. Prieto Alamo, J. Jurado[1], E. Francastel[1] and F. Laval*

Unité 347 INSERM, 80 Rue du Général Leclerc, 94276 Le Kremlin Bicêtre Cedex, France and [1]Groupe 'Réparation des lésions radio et chimio-induites', LA 147 CNRS, Institut Gustave Roussy, 94805 Villejuif Cedex, France

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ABSTRACT

Reactive oxygen species produce different lesions in DNA. Among them, 7,8-dihydro-8-oxoguanine (8-oxoG) is one of the major oxidative products implicated in mutagenesis. This lesion is removed from damaged DNA by base excision repair, and genes coding for 8-oxoG-DNA glycosylases have been isolated from bacteria, yeast and human cells. We have isolated and characterized the cDNA encoding the rat 8-oxoG-DNA glycosylase (rOGG1). Expression of the cDNA in the *fgp mutY Escherichia coli* double mutant allowed the purification of the untagged rOGG1 protein. It excises 8-oxoG from DNA with a strong preference for duplex DNA containing 8-oxoG:C base pairs. rOGG1 also acts on formamidopyrimidine (FaPy) residues, and the K_m values on 8-oxoG and FaPy residues are 18.8 and 9.7 nM, respectively. When acting on an oligonucleotide containing an 8-oxoG residue, rOGG1 shows a β-lyase activity that nicks DNA 3′ to the lesion. However, rOGG1 acts on a substrate containing an apurinic site by a β–δ elimination reaction and proceeds through a Schiff base intermediate. Expression of rOGG1 in *E.coli fpg mutY* suppresses its spontaneous mutator phenotype.

INTRODUCTION

Reactive oxygen species (ROS), superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) are potent oxidizing agents. They are formed either in the cells by the mitochondrial redox chain, or externally by ionizing radiations, near-ultraviolet light, redox-active drugs and sensitizer dyes. ROS react with DNA to form genotoxic lesions (1), specially 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy) and 7,8-dihydro-8-oxoguanine (8-oxoG) (2). 8-OxoG also arises through the incorporation, during DNA replication, of 8-oxo-dGTP, formed by oxidation of dGTP by ROS (3). 8-OxoG residues are mutagenic, as they give rise to G→T transversions (4). In *Escherichia coli*, the elimination of 8-oxoG residues is mediated by three proteins, Fpg, MutY and MutT (5). Fpg (or MutM protein) excizes FaPy and 8-oxoG residues from DNA and possesses three different activities: it acts as a DNA glycosylase (6), has an AP-nicking activity (7) and excises 5′-deoxyribose phosphate from damaged DNA (8). MutY corrects the 8-oxoG:A mispair by excising the A residue (9), and MutT hydrolyzes 8-oxodGTP, present in the nucleotide pool, to 8-oxodGMP (10).

A human homologue of the *E.coli* MutY (11), and human (12) and rat (13) homologues of the MutT protein have been cloned. Recently, Fpg homologues have been cloned from *Saccharomyces cerevisiae* (OGG1) (14,15), human (hOGG1) (16–20) and mouse (mOGG1) (20,21) cells. These proteins have no sequence similarity with the *E.coli* Fpg protein, but contain a sequence element known as the Helix–hairpin–Helix (H–h–H), Gly/Pro-rich-Asp motif, which is essential for the recognition and the catalysis of the substrate (22).

In this paper, we describe the cloning of the rat OGG1 (rOGG1) gene, the purification and the catalytic properties of the rOGG1 protein that suppresses the mutator phenotype of *fpg mutY E.coli*.

MATERIALS AND METHODS

Bacterial strains and enzymes

The *mutY E.coli* mutant (BH980:CC104 mutY::kan^R),the *fpg mutY* double mutant (BH990:CC104 mutY::kan^R X:tet^R mutM::kan^R) and the purified *E.coli* uracil-DNA glycosylase, Fpg and Nth proteins were obtained from Dr J. Laval (Institut G. Roussy, Villejuif, France).

Molecular cloning and sequencing

A cDNA plasmid expression library established from rat hepatoma cells (H4 cells) (23) was amplified by PCR (Expand Kit, Boehringer Mannheim) using primers corresponding to the 5′ (5′-GTC TGG GCG GGG TCT TTG GGC-3′) and 3′ ends (5′-GGA TGG GGA GAG AGA AGT GGG-3′) of the hOGG1 cDNA (20). The ribosome binding site and *Xba*I and *Bam*HI restriction sites were introduced in amplification steps using the following primers: 5′-TGG ATC TAG AGG GCT GGA GGC TGC TAT CCA AAT G-3′; 5′-TGG ATC TAG AGG GCT GGA

*To whom correspondence should be addressed. Tel: +33 1 49 59 18 53; Fax: +33 1 49 59 19 59; Email: laval@kb.inserm.fr

AGG AGC TAT CCA AAT G-3′; 5′-GGA AAG GAT CCG TGA CAT CTT TTG TCC CCT GGG C-3′.

The 1.1 kb DNA fragment containing the rat OGG1 cDNA was cloned into the *Xba*I–*Bam*HI sites of the pET11a vector (Novagen, USA) and sequenced on both strands using an ABI Prism 310 Genetic Analyzer. The nucleotide sequence is deposited in the GenBank database (accession no. AF029690).

Protein overexpression and purification

The rOGG1 cDNA was subcloned into the polycloning site of the pAlter-Ex2 plasmid (Promega, USA), under the control of the tac promoter. The resulting fragment (1.2 kb) was subcloned into the *Eco*RI–*Hin*dIII sites of the pBluescript plasmid (Stratagene, USA) producing the pBROGG plasmid. BH990 *E.coli* harboring the pBROGG plasmid were grown at 37°C in LB medium until A_{600} = 0.9, then grown for an additional 3 h in the presence of IPTG (1 mM final concentration). The cells were harvested, washed and suspended in 50 ml of buffer A (50 mM HEPES–KOH pH 7.6, 1 mM EDTA, 5 mM β-mercaptoethanol and 5% glycerol) containing 250 mM NaCl, 1 mM PMSF and 0.1% Triton X-100. After addition of lysozyme (0.2 mg/ml final concentration), the suspension was incubated for 10 min at 0°C, then for 15 min at 37°C and 10 min at –70°C. This freezing–thawing cycle was repeated twice. After centrifugation (30 000 *g* for 30 min at 2°C), the supernatant (fraction I, 50 ml) was loaded on a QMA anion exchange column (Water ACELL:50 ml bed volume), washed and equilibrated with buffer A containing 250 mM NaCl. Under these conditions, the 8-oxoG glycosylase activity was not retained and was separated from the bulk of nucleic acids (fraction II = 120 ml). Fraction II was dialyzed against buffer A containing 100 mM NaCl and applied to an SP-Trisacryl-M column (IBF-LKB, 25 ml bed volume) equilibrated with the same buffer. The 8-oxoG glycosylase activity was eluted with a linear gradient of NaCl (100–600 mM in buffer A). The active fractions eluted at 300–350 mM NaCl. They were pooled (fraction III), diluted in buffer A to 50 mM NaCl and loaded on a monoS HR5/5 column (Pharmacia) equilibrated with buffer A containing 50 mM NaCl. The column was eluted with a linear NaCl gradient (50–600 mM) and the active fractions recovered at 230–280 mM NaCl.

Enzyme assays

The 8-oxoG-DNA glycosylase activity was measured using as substrate a 34mer oligonucletide containing an 8-oxoG at position 20 (Table 1), 5′-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, then hybridized to complementary sequences with a cytosine, guanine, thymine or adenine opposite 8-oxoG, by heating at 70°C for 10 min. The assay mixture contained (final volume 100 µl) 70 mM HEPES pH 7.5, 2 mM Na_2–EDTA, 100 mM KCl (buffer A), 100 fmol ^{32}P-oligonucleotide and either Fpg or rOGG1 protein. After 15 min at 37°C, the reaction was stopped by adding formamide-dye loading buffer and heating for 5 min at 95°C, then the products were separated by 20% denaturing PAGE containing 7 M urea. They were quantified using an InstantImager (Packard).

Table 1. Oligonucleotides used as substrates

5′ AAA TAC ATC GTC TGG G8-oxoGC ATG TTG CAG ATC C 3′
5′ AAA TAC ATC GTC TGG G U C ATG TTG CAG ATC C 3′

To measure the AP-nicking activity, we used a 34mer oligonucleotide containing an apurinic site at position 20 (Table 1), hybridized to a complementary strand with a C opposite the AP-site. It was prepared by incubating a ^{32}P-labeled oligonucleotide containing a single uracil residue in 50 mM HEPES pH 7.8, 100 mM KCl, 1 mM Na_2–EDTA and 5 mM β-mercaptoethanol with 5 ng of uracil-DNA glycosylase for 30 min at 37°C. The substrate was purified by phenol–chloroform extraction, precipitated with ethanol and redissolved in H_2O (0.1 pmol/µl). The AP-nicking activity was measured by incubating 0.3 pmol of this substrate with either the Fpg or the rOGG1 protein in buffer A (final volume 30 µl) for 10 min at 37°C. The samples were analyzed as described above.

The activity on formamidopyrimidine residues was measured by incubating, for 15 min at 37°C, the rOGG1 protein with poly(dG.dC) containing [^{3}H]FaPy residues, prepared as described (7), in buffer A (final volume 100 µl). After ethanol precipitation, the [^{3}H]FaPy residues liberated were characterized by separation by HPLC (24) and quantitated by liquid scintillation counting.

DNA trapping assays

Reactions were performed (final volume 20 µl) in a buffer containing 0.5 mM Na_2PO_4 pH 7.8, 1 mM EDTA, 100 mM $NaBH_4$, 0.3 pmol of ^{32}P-labelled double-stranded apurinic oligonucleotide and either the FPG or rOGG1 protein. After 10 min incubation at 37°C, SDS (0.5% final concentration) was added and the samples heated to 60°C for 10 min. The samples were loaded on 15% polyacrylamide–0.1% SDS gels run at 200 V (25).

Mutation frequency in *E.coli*

BH 980 and BH 990 *E.coli* strains were transfected either with the pBROGG or with the control pBluescript plasmids. About 1000 cells were inoculated in LB broth containing 50 µg/ml kanamycin, 100 µg/ml ampicillin and 25 µg/ml tetracycline, if necessary. Mutations were analyzed by counting rifampicin-resistant colonies on agar plates containing rifampicin (100 µg/ml) and lactose revertant colonies on minimal lactose (0.2%) agar plates.

RESULTS

Cloning of the rat 8-oxoguanine-DNA glycosylase

The rat cDNA obtained by amplification of the cDNA library was sequenced. The cDNA (1.035 kb) contains an open reading frame encoding a 345 amino acid protein, which shows extensive homology with the human and mouse OGG1 proteins. The rat protein (rOGG1) is a 38 686 Da protein, as deduced from the nucleotide sequence, with a calculated isoelectric point of 8.86. It contains the characteristic H–h–H DNA binding domain, and the conserved lysine and aspartic acid residues present in the proposed active site of the hOGG1 protein (22). A putative nuclear localization signal (PAKRKKG) (26) is located in the C-terminal region of the protein (positions 342–348).

Substrate specificity of rOGG1

The rOGG1 protein cleaves a double-stranded 8-oxoG-containing oligonucleotide, releasing a product with a mobility slightly different from that released by the *E.coli* Fpg protein (Fig. 1A). It shows a low activity on a single-stranded substrate, corresponding to <10% of the activity measured on an 8-oxoG:C substrate

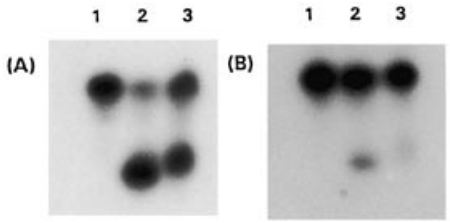

Figure 1. Nicking of 8-oxodG containing oligonucleotide by rOGG1. The ^{32}P-labelled oligonucleotide containing a single 8-oxodG residue opposite C was incubated with the rOGG1 or Fpg proteins for 15 min at 37°C. (**A**) Double-stranded oligonucleotide incubated alone (lane 1) or with 10 ng Fpg protein (lane 2) or 80 ng rOGG1 protein (lane 3). (**B**) Single-stranded oligonucleotide incubated alone (lane 1) or with 0.2 µg Fpg (lane 2) or 1.6 µg rOGG1 (lane 3) protein.

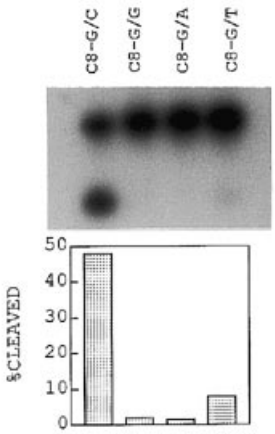

Figure 2. Substrate specificity of rOGG1. The rOGG1 protein (80 ng) was incubated with 8-oxoG:C, 8-oxoG:G, 8-oxoG:A or O-oxoG:T duplexes for 15 min at 37°C, and the reaction products analyzed and quantified as described in Materials and Methods.

(Fig. 1B). rOGG1 cleaves efficiently an oligonucleotide containig 8-oxoG:C, but when the complementary strand contains a G or an A opposite the 8-oxoG residue, there is no significant activity. A slight enzymatic activity is detected when there is a T opposite the lesion (Fig. 2). The activity of rOGG1 protein on poly(dG.dC) containing FaPy residues was measured. This substrate is cleaved efficiently by the rat protein: the K_m values are 18.8 and 9.7 nM, using as substrates the 8-oxoG:C oligonucleotide or the Fapy substrate, respectively.

Mechanism of action of the rOGG1 protein

The *E.coli* Fpg protein excises the 8-oxoG residues yielding an abasic site and then nicks the DNA at AP site through a β–δ elimination reaction. The δ-elimination can be partially suppressed in the presence of β-mercaptoethanol. Incubation of the 8-oxoG:C oligonucleotide with the rOGG1 protein yielded a product migrating as the β-elimination product formed by the Fpg protein (Fig. 3, lanes 1–4), suggesting that the rOGG1 protein acts as a glycosylase and that this step is followed by incision of the oligonucleotide via a β-elimination mechanism. When the rOGG1 protein acts on an oligonucleotide containing an apurinic

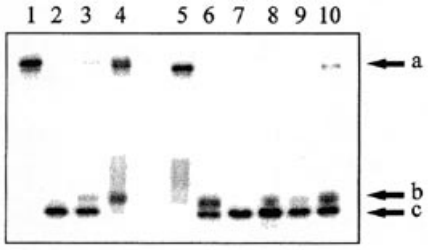

Figure 3. Comparison of the products generated by the rOGG1 and the Fpg proteins. Lanes 1–4, the 8-oxoG:C oligonucleotide was incubated alone (lane 1), or with 10 ng Fpg protein in the absence (lane 2) or presence (lane 3) of 15 mM MSH, or with 80 ng rOGG1 protein (lane 4). Lanes 5–10, the oligonucleotide containing an apurinic site was incubated alone (lane 5), or with 0.2 M NaOH for 15 min at 37°C (lane 6), with 10 ng Fpg protein in the absence (lane 7) or presence (lane 8) of 15 mM MSH, with 80 ng rOGG1 protein without (lane 9) or with (lane 10) 15 mM MSH. Samples were analyzed as described in Materials and Methods. a, position of the unmodified 34mer duplex; b and c, position of the β- and δ-elimination products, respectively.

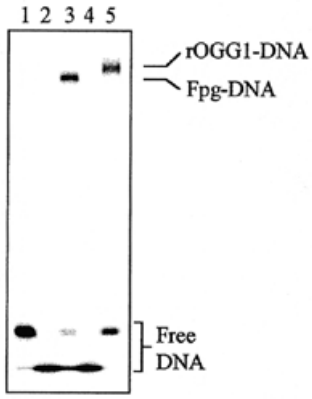

Figure 4. Borohydride trapping analysis of rOGG1 and Fpg proteins. The ^{32}P-labeled oligonucleotide containing an apurinic site was incubated without protein (lane 1), with the Fpg protein in the presence of 0.1 M NaCl (lane 2) or 0.1 M $NaBH_4$ (lane 3), or with the rOGG1 protein in the presence of 0.1 M NaCl (lane 4) or 0.1 M $NaBH_4$ (lane 5). The oligonucleotide was incubated with either protein for 10 min at 37°C, then the samples were analyzed as described in Materials and Methods.

site, the mechanism is different: a β–δ-elimination reaction occurs, as observed in the case of the Fpg protein (Fig. 3, lanes 5–10). Since rOGG1 generates a δ-elimination product in the absence of β-mercaptoethanol (Fig. 3, lane 9), this shows that our preparation is not contaminated by the *E.coli* Nth protein that would have generated a β-elimination product.

To determine whether the rOGG1 activity requires the formation of a Schiff base, trapping assays in the presence of borohydrate were performed. The results show the formation of a complex between the labelled oligonucleotide containing an AP-site and either the Fpg or the rOGG1 protein (Fig. 4). The two complexes migrate at different rates , as expected from the molecular weights of the respective proteins.

Suppression of the *E.coli* mutator phenotype

The *fpg mutY* BH990 double mutant has a high spontaneous mutation rate that can be measured by the appearance of

rifampicin-resistant and lactose revertants colonies, in comparison with the *mutY* BH980 mutant. To measure the ability of rOGG1 to suppress the mutator phenotype of *fpg mutY E.coli*, the double mutant was transformed with the pBROGG plasmid. Controls were performed by transforming BH980 and BH990 *E.coli* with the pBluescript plasmid. Expression of rOGG1 reduces the number of spontaneous rifampicin-resistant and lactose revertants mutation frequencies in BH990, ~20- and 60-fold, respectively (Table 2).

Table 2. Antimutator effect of rOGG1 cDNA in *fpg, mutY E.coli*

	Mutation frequency Rif^R ($\times 10^8$)	Lac^+ ($\times 10^8$)
BH980/pBluescript	4.8 ± 1.6	5.5 ± 1.3
BH990/pBluescript	213.0 ± 72	949.4 ± 95.9
BH990/pBROGG	10.6 ± 3.7	15.4 ± 4.3

The mutation frequencies were calculated from 10 independent cultures.

DISCUSSION

We have isolated the rat OGG1 cDNA coding for a protein of 345 amino acids that has been overproduced in a *fpg E.coli* mutant in order to prevent any contamination by the bacterial Fpg protein. The protein that we have partially purified was not His-tagged in order to avoid possible interferences when investigating its mechanism of action. The rOGG1 protein shares a high sequence homology with the human and mouse OGG1 proteins and contains the proposed active site motif of the base excision repair glycosylases/lyases (22). It acts on an oligonucleotide containing an 8-oxoG residue as an 8-oxoguanine-DNA glycosylase and an AP-lyase via a β-elimination reaction, and shows a strong preference for duplex DNA containing 8-oxoG:C base pairs. However, when the rOGG1 protein acts on an oligonucleotide containing an AP site, it acts through a β–δ-elimination reaction. This mechanism is different from that of the human enzyme which cleaves an abasic site containing DNA by a β-elimination mechanism similar to that observed for the *E.coli* Nth protein (27). rOGG1 acts as the *E.coli* Fpg protein, forming an enzyme–DNA Schiff base intermediate that is reduced by sodium borohydride to yield a covalent complex. rOGG1 releases also FaPy residues from a FaPy poly(dG.dC) substrate, as reported for the hOGG1 protein (27).

A mitochondrial endonuclease that recognizes oxidative damage has been isolated from rat liver mitochondria (28). This protein removes 8-oxoG residues from DNA and incises preferentially 8-oxoG:C base pairs. However this protein does not recognize FaPy residues and is therefore probably different from the rOGG1 protein.

8-OxoG residues are implicated in cancer and aging (29). They are handled in *E.coli* by three proteins, Fpg, MutY and MutM, the 'GO system', and the mouse and human counterparts of these proteins have been cloned. The rat homologue of MutT, which hydrolyzes 8-oxoGTP from the nucleotide pool, has been cloned and characterized (13). The rOGG1 protein is therefore the second activity identified that is implicated in the elaborate system of defenses of organisms against oxidative damage.

The rOGG1 cDNA will allow to modulate the expression of this gene in cells *in vitro* and to determine the contribution of the protein to the cellular resistance to the toxic and mutagenic effects of oxidative DNA damage.

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