Published online October 19, 2004

5596–5608 *Nucleic Acids Research, 2004, Vol. 32, No. 18*
doi:10.1093/nar/gkh863

The C-terminal αO helix of human Ogg1 is essential for 8-oxoguanine DNA glycosylase activity: the mitochondrial β-Ogg1 lacks this domain and does not have glycosylase activity

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Received July 28, 2004; Revised and Accepted September 14, 2004

ABSTRACT

The human Ogg1 glycosylase is responsible for repairing 8-oxo-7,8-dihydroguanine (8-oxoG) in both nuclear and mitochondrial DNA. Two distinct Ogg1 isoforms are present; α-Ogg1, which mainly localizes to the nucleus and β-Ogg1, which localizes only to mitochondria. We recently showed that mitochondria from ρ^0 cells, which lack mitochondrial DNA, have similar 8-oxoG DNA glycosylase activity to that of wild-type cells. Here, we show that β-Ogg1 protein levels are ~80% reduced in ρ^0 cells, suggesting β-Ogg1 is not responsible for 8-oxoG incision in mitochondria. Thus, we characterized the biochemical properties of recombinant β-Ogg1. Surprisingly, recombinant β-Ogg1 did not show any significant 8-oxoG DNA glycosylase activity *in vitro*. Since β-Ogg1 lacks the C-terminal αO helix present in α-Ogg1, we generated mutant proteins with various amino acid substitutions in this domain. Of the seven amino acid positions substituted (317–323), we identified Val-317 as a novel critical residue for 8-oxoG binding and incision. Our results suggest that the αO helix is absolutely necessary for 8-oxoG DNA glycosylase activity, and thus its absence may explain why β-Ogg1 does not catalyze 8-oxoG incision *in vitro*. Western blot analysis revealed the presence of significant amounts of α-Ogg1 in human mitochondria. Together with previous localization studies *in vivo*, this suggests that α-Ogg1 protein may provide the 8-oxoG DNA glycosylase activity for the repair of these lesions in human mitochondrial DNA. β-Ogg1 may play a novel role in human mitochondria.

INTRODUCTION

Reactive oxygen species (ROS) generated during normal metabolism can oxidize proteins, lipids and nucleic acids (1,2). Oxidation of DNA is thought to be involved in tumorigenesis, aging and various degenerative disorders (3,4). 8-oxo-7,8-dihydroguanine (8-oxoguanine, 8-oxoG) is perhaps the best-characterized oxidative DNA adduct, and thought to be one of the most common. 8-oxoG pairs with adenine as well as cytosine in DNA (5). If the adenine opposite 8-oxoG in DNA is not removed GC→TA transversions occur during DNA replication (6). The major pathway for the removal of 8-oxoG is base excision repair (7,8). Base excision repair is initiated by a lesion-specific DNA N-glycosylase that recognizes and excises the damaged base. Bacterial Fpg and eukaryotic Ogg1 proteins are the major 8-oxoG DNA glycosylases. Both enzymes possess an associated apurinic/apyrimidinic (AP) lyase activities, such that they remove 8-oxoG and cleave the DNA backbone (9). The biological relevance of the 8-oxoG DNA glycosylase activity is demonstrated by a recent study showing that low Ogg1 activity strongly associates with increased risk of lung cancer (10).

Mitochondria are the primary intracellular source of ROS. Particularly high levels of 8-oxoG have been detected in mitochondrial DNA (mtDNA) (3,11), up to 16 times higher than in nuclear DNA (12). There is a growing body of evidence that accumulation of damage and mutation in mtDNA may contribute to tumorigenesis, aging and age-associated degenerative diseases (13–15). Mitochondria also possess an Ogg1 activity (16–18). Using *OGG1*$^{-/-}$ mice, we recently demonstrated that Ogg1 is the only 8-oxoG DNA glycosylase in mouse liver mitochondria and that liver mtDNA from these animals accumulates 20 times more 8-oxoG than wild type (WT) (19). Similarly, the disruption of *Saccharomyces cerevisiae OGG1* gene causes spontaneous mutations in mtDNA, doubling the rate of petite formation in comparison to WT (20). Thus, Ogg1 appears to be essential for the maintenance of mtDNA integrity in eukaryotes (21).

The human *OGG1* gene has been cloned by several groups [reviewed in (22)]. It consists of eight exons and produces two major distinct transcripts via alternative splicing (23–26). Thus, two isoforms of human Ogg1 proteins have been identified: α-Ogg1 (alternatively Ogg1 type 1a), and β-Ogg1 (type 2a). α-Ogg1 mRNA is transcribed from exons 1 to 7, and β-Ogg1 mRNA from exons 1 to 6 plus exon 8. The α-isoform

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is a 345 amino acids (39 kDa) protein that localizes mainly in nuclei, whereas β-Ogg1 is a 424 amino acids (47 kDa) protein that appears to be localized exclusively in mitochondria (26,27). These studies, along with one report showing an 8-oxoG DNA glycosylase activity for β-Ogg1 using large amounts of recombinant protein (25), led to the idea that β-Ogg1 provides the 8-oxoG DNA glycosylase activity detected in human mitochondrial extracts.

The first 316 amino acids are common between the two isoforms, whereas the C-termini are quite different. The α-Ogg1 protein has been extensively characterized biochemically and structurally. The active site for DNA glycosylase activity requires Lys-249 and Asp-268, both located in the conserved helix–hairpin–helix structural element that is followed by a Gly/Pro-rich loop and a conserved aspartic acid motif (HhH-GPD), which is essential for DNA glycosylase as well as DNA binding activity (28–30). Arg-154 is required for recognition of the base opposite the damage (28,31). Amino acid residues 9–26 comprise a mitochondrial location signal (MLS) (26), though a dominant nuclear localization signal (NLS) is also present in amino acids 335–342 [Figure 1; (32)]. On the other hand, no information regarding substrate specificity, kinetic characteristics or binding activities is available for β-Ogg1.

Human cells that lack mtDNA (ρ^0) are a particularly interesting model for studying the regulation of proteins involved in mtDNA transactions (33,34), nuclear–mitochondrial crosstalk and mitochondrial physiology and biochemistry (35). Although these cells do not have a functional electron transport chain, they still have mitochondria with a transmembrane potential and undergo mitochondrial-dependent apoptosis (36). We have recently demonstrated that ρ^0 cells retain most mtDNA repair activities, including 8-oxoG incision activity, at levels only slightly lower than WT cells (37). Here, we found a dramatic reduction in β-Ogg1 protein levels in the mitochondria of ρ^0 cells. In order to reconcile these observations, we purified a recombinant β-Ogg1 protein to characterize its biochemical properties. However, we failed to detect 8-oxoG DNA glycosylase activity in the recombinant protein. Since the C-terminal region of α- and β-Ogg1 differ substantially and β-Ogg1 lacks an αO helix that has been implicated in activity (38), we investigated whether the amino acid sequence different for the β-isoform could account for its lack of DNA glycosylase activity. For these studies, we generated a series of mutant proteins, with specific amino acid substitutions in α-Ogg1 to identify the amino acid changes that could account for the inability of β-Ogg1 to catalyze 8-oxoG excision. Our results indicate that the C-terminal αO helix is necessary for Ogg1 activity. We also identified a novel amino acid, Val-317, necessary for the DNA binding and glycosylase activities of this protein.

MATERIALS AND METHODS

Chemicals and reagents

Complete EDTA-free protease inhibitor cocktail was purchased from Roche. All other reagents were from Sigma.

Cell lines and growth conditions

ρ^0 143B TK$^-$ human osteosarcoma cells were a generous gift from G. Attardi (California Institute of Technology). Wild type (WT) 143B TK$^-$ cells were acquired from the American Type Cell Collection (ATCC) (ATCC CRL 8303). Both WT and ρ^0 cells were cultured under identical conditions, as described earlier (37) (see Supplementary data).

Plasmids and oligonucleotides

α- and β-Ogg1 expression vectors, pUC18-*HOGH1* and pPR171 were gifts from Erling Seeberg (University of Oslo) and Serge Boiteux (CNRS/CEA), respectively. pQE-30Xa vector was purchased from Qiagen. Chaperon plasmid set was purchased from Takara (Kyoto, Japan). Oligonucleotides used in this study as PCR primers and substrates for biochemical assays were purchased from Integrated DNA Technologies (Coralville, IA) and Midland (Midland, TX), respectively (Supplementary Table I).

Construction of expression vectors of His-tagged human Ogg1 proteins

WT human α-*OGG1*-encoding region was amplified from pUC18-*HOG1* by PCR using primers OG1-F-Bam and OG1-A-R-HIN as shown in Supplementary Table I.

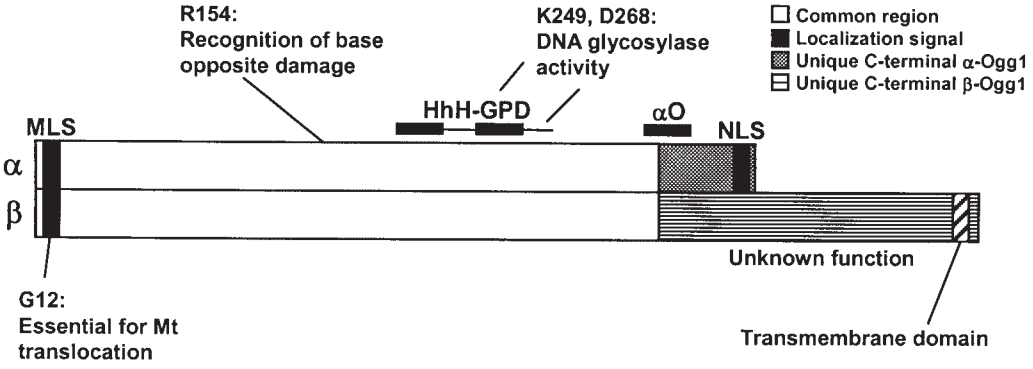

Figure 1. Structure of the two major human Ogg1 isoforms. Schematics of α- (345 amino acids) and β-Ogg1 (424 amino acids) proteins are shown. The first 316 amino acids are common for both isoforms, while the C-termini vary considerably. The mitochondrial localization signal (MLS, position 9–26), nuclear localization signal (NLS, 335–342) and HhH-GPD motif are indicated. The functions of amino acids Gly-12, Arg-154, Lys-249 and Asp-268 are indicated, based on previous functional studies. The function of the β-Ogg1 C-terminus is unknown. A transmembrane domain (position 400–422) in a highly hydrophobic region was predicted by the SOSUI system (32).

β-*OGG1*-coding region was amplified from pPR171 using primers OG1-F-Bam and OG1-B-R-HIN. For construction of α-Ogg1 Stop325, primer Stop325 was used instead of OG1-A-R-HIN. Forward and reverse primers contained BamHI and HindIII restriction sites, respectively, and after digestion with appropriate restriction endonucleases, PCR-amplified fragments were inserted into the BamHI–HindIII site in pQE-30Xa. All mutant proteins were generated from the pQE-30Xa *OGG1*-WT plasmid by QuikChange site-directed mutagenesis kit II XL (Stratagene) with sets of forward and reverse primers according to the manufacture. To construct α-Ogg1 fused with β-Ogg1 C-terminus, α-Ogg1 coding region at positions 1–327 was initially cloned into pQE-30Xa BamHI–SalI site, followed by the cloning of the coding region of position 317–424 of β-Ogg1 into the SalI–HindIII site. The DNA sequence of all of constructs was verified.

Expression and purification of human α-Ogg1 proteins

Escherichia coli BK3004 [BL21(DE3) *fpg::Kan*] (gift from Erling Seeberg, University of Oslo) was transformed with appropriate hOgg1 expression vector and grown in 25 ml of Luria–Bertani (LB) broth (1% trypptone, 0.5% yeast extract and 0.5% NaCl) supplemented with 200 μg/ml ampicillin, 25 μg/ml kanamycin and 1% glucose at 37°C for 16 h. The culture was then transferred to 500 ml of LB broth supplemented with 200 μg/ml ampicillin and 25 μg/ml kanamycin, and incubated with vigorous shaking (200 r.p.m.) until OD_{600} reached 0.5. Culture flasks were placed on ice for 5 min to equilibrate the culture temperature, and 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added. Bacteria were further incubated at 15°C for 20 h with vigorous shaking. Cells were collected by centrifugation at 3000 *g* for 10 min, washed once with phosphate-buffered saline (PBS), and the pellet was stored at −80°C until use. Cell pellets were suspended in 10 ml of lysis buffer [20 mM HEPES–NaOH pH 7.4, 300 mM NaCl, Complete EDTA-free protease inhibitor cocktail, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% Triton X-100, 10% glycerol]. After two cycles of freeze–thawing, the cells were sonicated and clarified by centrifugation at 27 000 *g* for 30 min. After addition of 20 mM imidazole to the supernatant, the sample was mixed with 2 ml of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer containing 20 mM imidazole. After gentle mixing for 1 h at 4°C, unbound proteins were washed with 40 ml of lysis buffer containing 20 mM imidazole, and bound proteins were eluted with 10 ml of lysis buffer containing 60, 300 and 500 mM imidazole, sequentially. Most of the His-tagged human Ogg1 eluted in the 300 mM imidazole fraction. The active fraction was dialyzed against 1000 volumes of 20 mM HEPES–NaOH pH 7.4, 50 mM NaCl, 1 mM DTT and 50% glycerol, and stored at −20°C.

Expression and purification of human β-Ogg1 proteins

GST-tagged β-Ogg1 was expressed in BK3004 cells. Pre-cultures were grown in 5 ml of LB medium supplemented with 200 μg/ml ampicillin, 25 μg/ml kanamycin and 1% glucose, transferred to 250 ml of 2× YT medium supplemented with 200 μg/ml ampicillin and 25 μg/ml kanamycin, and incubated at 37°C until OD_{600} reached 0.6. The temperature was then reduced to 15°C, and bacteria were incubated without IPTG-induction for 16 h. The cell pellet, obtained as described above, was stored at −80°C until use. Cell pellets were suspended in 10 ml of B/PERII reagent (Pierce) supplemented with protease inhibitor cocktail, and gently shaken for 10 min. Samples were clarified by centrifugation at 27 000 *g* for 15 min. GST–β-Ogg1 was purified using a GST fusion protein purification kit (Pierce) according to the manufacturer's instructions. The active fraction was dialyzed to remove glutathione; glycerol was added to a final concentration of 10%, and the samples stored at −80°C.

His-tagged β-Ogg1 was expressed in *E.coli* BK3004 cells that were previously transformed with pG-Tf2, which expresses the GroES–GroEL chaperone protein. BK3004 bacteria containing both pG-Tf2 and pQE-OGG1B were pre-cultured in 30 ml of LB medium supplemented with 200 μg/ml ampicillin, 25 μg/ml chloramphenicol and 25 μg/ml kanamycin. The pre-culture was transferred to fresh LB medium (3 l) containing 200 μg/ml ampicillin, 25 μg/ml chloramphenicol, 50 μg/ml kanamycin and 10 ng/ml tetracycline. After reaching an OD_{600} of 0.4, IPTG was added to a final concentration of 0.5 mM, and cultures were incubated for an additional 5 h at 30°C. Cells were spun down at 3000 *g* for 10 min, and washed once with PBS. Cell pellets were kept at −80°C until use. Cell pellets were suspended in Buffer A [20 mM HEPES–NaOH pH 7.4, 30 mM NaCl, 0.2% polyoxyethylene ether W-1 (Lubrol PX), 0.05% Triton X-100 and 10% glycerol] containing Complete EDTA-free protease inhibitor cocktail and 5 mM imidazole, and the cells were disrupted by sonication. For dissociation of the chaperone proteins from the recombinant Ogg1, 10 mM $MgSO_4$ and 10 mM ATP were added to the sample, followed by incubation at 37°C for 10 min. Samples were kept on ice for 5 min and clarified by centrifugation at 20 000 *g* for 45 min at 4°C. Imidazole was added to the supernatant at 5 mM final concentration, the sample was mixed with 2 ml of Ni-NTA agarose equilibrated with buffer A containing 5 mM imidazole, and incubated at 4°C for 1 h with gentle rotation. Unbound proteins were washed out with 40 ml of buffer A containing 5 mM imidazole, and bound proteins were eluted with 20 ml of buffer A containing 20, 60, 300 and 500 mM imidazole, respectively. A single band eluted with 500 mM imidazole. This fraction was then dialyzed with 25 mM HEPES–NaOH pH 7.4, 50 mM NaCl, 20% glycerol and 0.5 mM PMSF. Samples were concentrated using a Microcon YM-30 (Millipore) and analyzed by SDS–PAGE, and the fraction containing Ogg1 was stored at −80°C.

Oligonucleotide labeling for protein activity assays

The 5′-end of each top strand (Supplementary Table I) was labeled with [γ-^{32}P]ATP (3000 Ci/mmol, ICN) using T4 polynucleotide kinase (New England Bio Labs). Unincorporated [γ-^{32}P]ATP was removed using MicroSpin G-25 columns (Amersham), and the ^{32}P-labeled oligonucleotides were annealed with complementary strands (bottom strand in Supplementary Table I).

DNA incision assays

For 8-oxoG DNA glycosylase assay with recombinant proteins, a 30mer oligonucleotide containing an 8-oxoG/C pair was used. ^{32}P-labeled oligonucleotide substrates (10 nM) were incubated with appropriate amounts of purified proteins in 10 μl of reaction buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl,

2 mM EDTA, 100 ng/ml BSA) at 37°C. Reactions were terminated by adding 10 µl of stop buffer (95% formamide, bromophenol blue, xylene cyanol). The samples were incubated at 90°C for 3 min, and immediately placed on ice. 8-oxoG DNA glycosylase assay with human mitochondrial extracts was performed as previously described (37) (see Supplementary data). Samples were electrophoresed on 20% polyacrylamide/7 M urea gel in 1× TBE (89 mM Tris-borate and 2 mM EDTA). Radioactivity was detected by Phosphorimager and analyzed by ImageQuant software.

Electrophoresis mobility shift assay (EMSA)

^{32}P-labeled oligonucleotide substrates (10 nM) were incubated with appropriate amounts of purified proteins in 10 µl of reaction buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 100 ng/ml BSA) on ice for 30 min. Samples were directly loaded on a 5% polyacrylamide gel containing 5% glycerol in 1× TBE, and gel was run at 200 V constant for 2 h, at 4°C. Radioactivity was analyzed as above.

Sodium borohydride-mediated DNA–protein cross-links (DNA trapping assay)

To detect a DNA glycosylase–oligonucleotide complex, 100 mM $NaBH_4$ was added to the same reaction buffer as for the DNA incision assay. After 30 min incubation at 37°C, reactions were boiled in SDS–PAGE sample buffer and the products were separated by SDS–PAGE.

Isolation of mitochondria

Mitochondria from wt and ρ^0 143B TK$^-$ cells were isolated by differential centrifugation with Percoll gradient, as previously described (37) (see Supplementary data). Purified mitochondria (5 mg/ml in protease-free buffer) were immediately treated with 50 µg/ml proteinase K on ice for 10 min. Reaction was stopped by addition of 2 mM PMSF and sample incubated on ice for 10 min. Mitochondria were washed in appropriate buffer at least five times and stored at −80°C until use.

Western blot

Extracts were mixed with SDS–PAGE sample buffer and heated at 95°C for 5 min. Samples were fractionated by SDS–PAGE (8–16% gradient gel). Proteins were blotted to a PVDF membrane (Invitrogen), and western blot was done by conventional technique using mtOGG1 (Novus Biological), human Ogg1 (Novus Biological), Lamin B1 (Santa Cruz), Cytochrome c oxidase subunit IV (20E8, Molecular Probes) and His_6 (BD Biosciences) antibodies. For visualization, the membranes were developed with ECL plus Western Blotting Detection System (Amersham) and exposed to HyperFilm ECL (Amersham). In the case of human Ogg1 antibody, SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) was used.

Prediction of secondary structure of human Ogg1 proteins

Secondary structure of α- and β-Ogg1 were predicted from the amino acid sequence using the GOR Secondary Structure Prediction program (http://molbiol.soton.ac.uk/compute/GOR.htm) (39). The prediction of a transmembrane domain in β-Ogg1 was done using the SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/sosui_submit.html) (32).

Sequence alignments of various Ogg1 proteins

Amino acid sequences of Ogg1 were obtained from the Entrez Protein entries in NCBI; *Arabidopsis thaliana* (Accession no.: CAC19363), *Drosophila melanogaster* (Q9V3I8), *Saccharomyces cerevisiae* (NP_013651), *Rattus norvegicus* (NP_110497), *Mus musculus* (NP_035087), human Ogg1 isoform 1a (NP_002533) and 2a (NP_058214). Obtained sequences were aligned by Clastal W.

RESULTS

β-Ogg1 protein levels and 8-oxoG DNA glycosylase activity in human mitochondria

Previously we reported that mitochondria from cultured human ρ^0 cells that lack mtDNA have only slightly reduced levels of 8-oxoG DNA glycosylase activity relative to WT cells (37). In the present study, we measured β-Ogg1 protein levels in mitochondria isolated from those cells. Despite similar 8-oxoG incision activities, β-Ogg1 protein level was significantly reduced (~80%) in ρ^0 cells as determined by western blot analysis (Figure 2A and C). These contradictory results suggested that either β-Ogg1 is regulated differentially in ρ^0 or is not the major 8-oxoG DNA glycosylase in human mitochondria. Thus, we decided to characterize the biochemical properties of recombinant β-Ogg1.

Purification of human β-Ogg1 protein

Human β-Ogg1 has a putative membrane-binding domain in the C-terminal portion of the protein (Figure 1). In addition, immunohistochemical studies have suggested that the protein localizes to the inner mitochondrial membrane (40). In the present study, human β-Ogg1 was expressed as hexa histidine-(His-) and glutathione S-transferase (GST)-tagged proteins, with the tags fused to the N-terminus of the protein. The solubility of the recombinant protein was very low, particularly for the His-tagged protein, possibly due to the acidic and hydrophobic nature of the C-terminus, and it often accumulated in inclusion bodies. To circumvent this problem, His–β-Ogg1 (Figure 3A, lane 4) was co-expressed with the bacterial chaperones GroEL–GroES. His–α-Ogg1, His–β-Ogg1 and GST–β-Ogg1 were then purified from *E.coli* expression systems to near homogeneity (Figure 3A, lanes 1, 4 and 2, respectively). The estimated molecular masses, as determined from Coomassie blue-stained gels, were in agreement with the calculated molecular masses for the fusion proteins (Figure 3A). The identity of the recombinant proteins was also verified by western blot with His-, GST-, or mtOgg1-antibodies (data not shown).

Biochemical characterization of β-Ogg1 protein

α-Ogg1 removes 8-oxoG from DNA and cleaves the DNA strand at the 3′ side of the resulting AP site via its AP lyase activity (β-elimination). In order to compare the enzymatic activities of α- and β-Ogg1, we performed an incision assay with an 8-oxoG containing oligonucleotide (Supplementary Table I). While His–α-Ogg1 efficiently incised the substrate

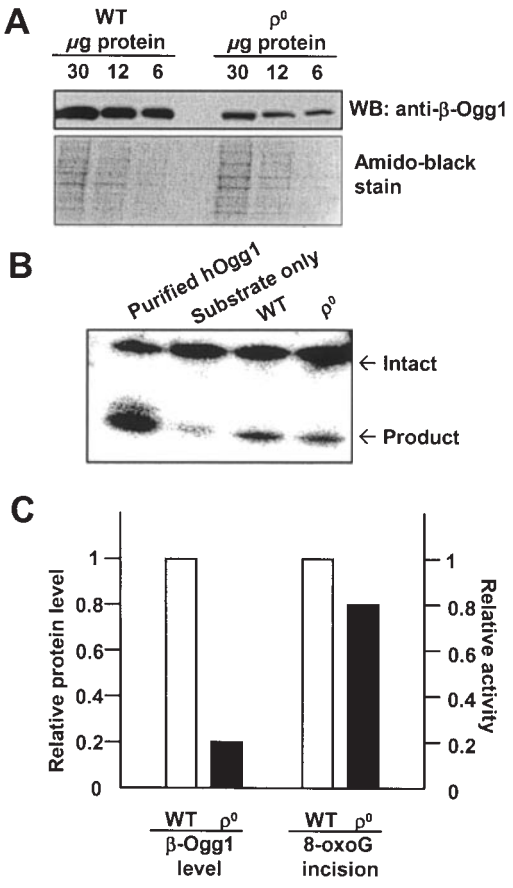

Figure 2. β-Ogg1 protein levels in human mitochondria lacking mtDNA. (**A**) β-Ogg1 protein levels in mitochondria from WT and ρ^0 cells were analyzed by western blot using β-Ogg1 antibody. An amido black-stained PVDF membrane is also presented to show equal loading of protein. (**B**) Incision of an 8-oxoG-containing substrate by WT and ρ^0 mitochondrial extracts was measured as described in Materials and Methods. (**C**) β-Ogg1 protein levels and incision activity are presented relative to the values observed with WT cells. 8-oxoG DNA glycosylase activity was measured in our previous study (37).

at the 8-oxoG position, GST–β-Ogg1 did not generate any cleaved products, even when 100-fold more recombinant β-Ogg1 was used (Figure 3B). Longer incubation times resulted only in the appearance of non-specific cleavage products (Figure 3C). Similar results were obtained with His–β-Ogg1 (data not shown). No cleavage products were detected from oligonucleotides containing 8-oxoG/G, 8-oxoG/A, 8-oxoG/T, 8-oxoG/C, G/C and AP site/C in the β-Ogg1 (data not shown). We also failed to detect β-Ogg1 8-oxoG DNA glycosylase activity following various modifications of the assay conditions, including length of oligonucleotides (up to 50mer), pH, NaCl, Zn^{2+} and Mg^{2+} concentrations (data not shown). Recombinant β-Ogg1 expressed in a baculovirus expression system was also partially purified and analyzed. Again, no incision activity could be detected (data not shown), suggesting that the lack of activity is not due to some required post-translational modification specific to eukaryotic cells.

β-Ogg1 also did not bind to an oligonucleotide containing 8-oxoG/C, as determined by EMSA (Figure 3D). Significant amounts of shifted DNA were observed in the lanes incubated with α-Ogg1, while incubation with β-Ogg1 did not produce any significant complex formation. From these observations, we concluded that the recombinant form of β-Ogg1 does not have 8-oxoG DNA glycosylase activity.

Effect of the long C-terminal tail of β-Ogg1 on 8-oxoG DNA glycosylase activity

To investigate the possibility that the longer C-terminal domain of β-Ogg1 inhibits catalytic activity, we generated a chimeric protein comprised of the α-Ogg1 catalytic domain (residues 1–327) fused to the β-Ogg1 C-terminal tail (317–424) (Figure 4A). Due to the cloning strategy, two amino acids (Val–Asp) were also inserted into the junction of the MixAB fusion protein. The recombinant chimera (total length 437 amino acids, calculated molecular mass 51.5 kDa) was partially purified as a His-tagged protein (Figure 4B) and quantified by western blot, using a known concentration of His–α-Ogg1 as a standard. In the DNA incision assay, the MixAB protein showed similar 8-oxoG DNA glycosylase activity to that of WT α-Ogg1 (Figure 4C). The partially purified MixAB preparation showed two bands that cross-reacted with the anti-Ogg1 antibody, suggesting that part of the recovered protein had been cleaved into smaller species. To determine whether the glycosylase activity was due to the full length, 51.5 kDa MixAB chimera, or to the smaller, faster migrating species, we performed a $NaBH_4$-trapping assay (Figure 4D). In the lanes incubated with MixAB protein, we detected a DNA–protein complex of slower mobility (lanes 5–7) than WT α-Ogg1 (lanes 2–4), suggesting that the MixAB protein retains the 8-oxoG DNA glycosylase activity. These results indicate that the longer C-terminal tail of the recombinant β-Ogg1 *per se* does not cause the lack of 8-oxoG DNA glycosylase activity.

The role of the phenylalanine residue at position 319 in the C-terminal helix domain of Ogg1

The function of the unique C-terminus of β-Ogg1 (108 amino acid residues) is not known (Figure 1). Using a secondary structure prediction program (39), we predicted the formation of an α-helix (αO) in the C-terminus of α-Ogg1, but no formation of this α-helix in β-Ogg1 (Figure 5A). The crystal structure of α-Ogg1 (28) confirmed the presence of an αO-helix domain in this region (amino acids 313–323). The study of Bruner and colleagues also showed that a phenylalanine residue at position 319 interacts with 8-oxoG, and may be critical for 8-oxoG recognition by the Ogg1 protein (Figure 5B, adapted from Entrez Structure pdb code 1HU0). Van der Kemp and co-workers (2004) recently reported that a F319A mutant α-Ogg1 is deficient in 8-oxoG DNA glycosylase and binding activities (38). β-Ogg1 has a leucine at position 319, and thus may not form a complete α-helix in the C-terminus (Figure 5A and B). We aligned the Ogg1 amino acid sequences from various organisms (Figure 5A) and found that Phe-319 is highly conserved in all Ogg1 orthologues except in human β-Ogg1.

In order to determine whether the phenylalanine to leucine substitution in β-Ogg1 renders it inactive, we mutated Phe-319 in α-Ogg1 to the aliphatic amino acid glycine (F319G), disrupting the aromatic ring of phenylalanine, and to leucine (F319L), as found in β-Ogg1. We purified the His-tagged mutant proteins F319G and F319L. The DNA sequence at

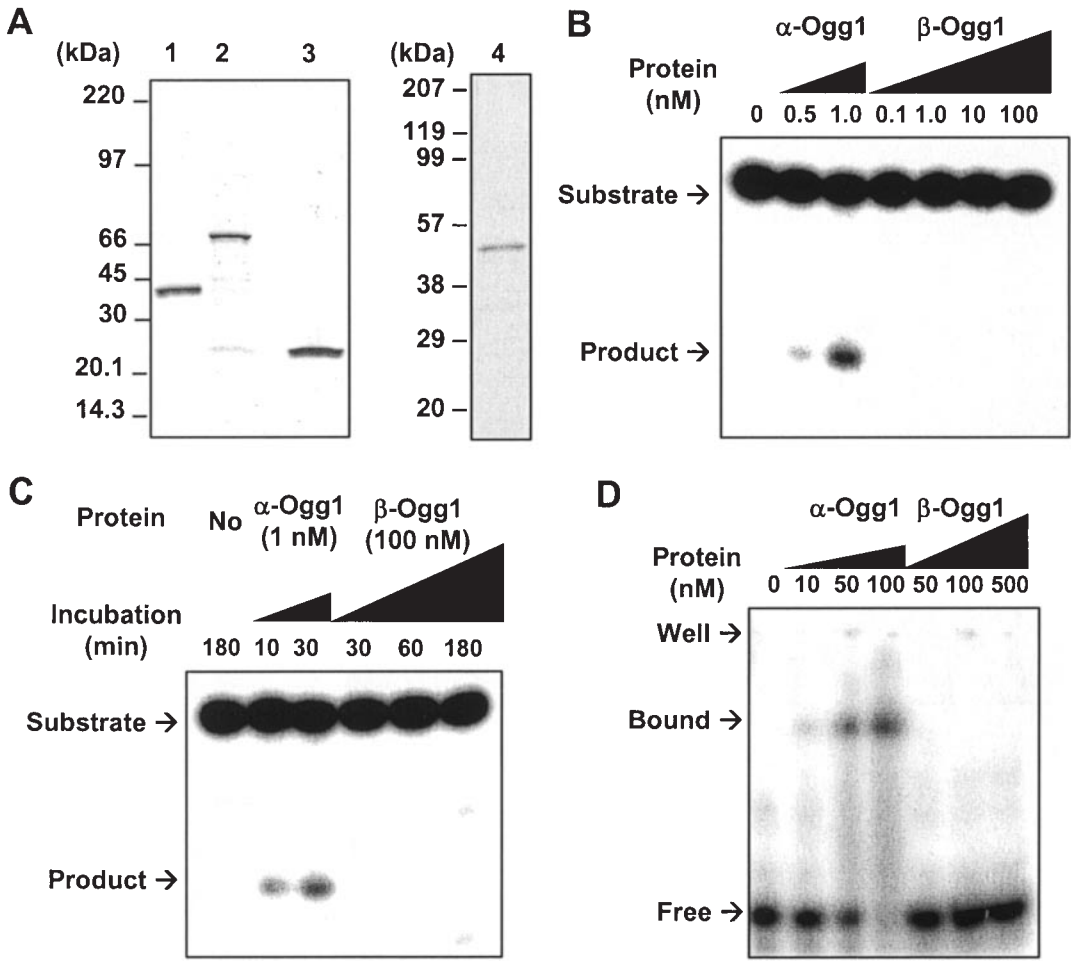

Figure 3. Purification and characterization of the recombinant β-Ogg1 proteins. (**A**) Gels from SDS–PAGE analysis with Coomassie blue staining are presented. Lane 1: His–α-Ogg1 (41.6 kDa, 0.5 μg), lane 2: GST–β-Ogg1 (73 kDa, 0.5 μg), lane 3: GST (26 kDa, 0.5 μg) and lane 4: His–β-Ogg1 (49.8 kDa, 0.5 μg). (**B**) DNA incision activity using different concentrations of enzyme. Indicated amounts of α- and β-Ogg1 proteins were incubated with 10 nM of 30mer oligonucleotide containing 8-oxoG/C at 37°C for 30 min, as described in Materials and Methods. Incision products were analyzed on polyacrylamide gels containing 7 M urea. (**C**) DNA incision activity with various incubation times. α-Ogg1 (1 nM) and β-Ogg1 (100 nM) proteins were incubated with 10 nM oligonucleotide containing 8-oxoG/C at 37°C for the indicated times. (**D**) EMSA with oligonucleotide containing 8-oxoG/C. 10 nM oligonucleotide were mixed with the indicated amounts of Ogg1 proteins, and DNA binding was detected by EMSA, as described.

the mutated sites of each expression vector was verified (Supplementary Figure 1A), and each protein was purified to homogeneity (Figure 6A). A mutant β-Ogg1 protein, in which Leu-319 was substituted by phenylalanine was also generated, and partially purified (Figure 6B).

Glycosylase activity of the mutant proteins was determined using oligonucleotides containing 8-oxoG/C (Figure 6C). The F319G substitution in α-Ogg1 completely abolished 8-oxoG incision activity in the mutant α-Ogg1 protein (lanes 5–7), similarly to the F319A mutant reported by van der Kemp *et al*. (38). Interestingly, the F319L substitution in α-Ogg1, as found in β-Ogg1, only partially decreased the activity, as incubation of the substrate with this mutant protein resulted in the generation of incision products (lanes 8–10), although the specific activity was 2.1-fold weaker than WT α-Ogg1 (lanes 2–4). Conversely, the mutant β-Ogg1 L319F did not show any significant activity (lanes 11–13), suggesting that some other amino acid changes in β-Ogg1 cause the lack of glycosylase function. The DNA binding activity of the mutant proteins to 8-oxoG was also characterized (Figure 6D). Surprisingly, the F319L mutant α-Ogg1 protein did not stably bind to the 8-oxoG/C-containing substrate (lanes 8–10), even though this mutant showed 8-oxoG incision activity. On the other hand, the lanes incubated with β-Ogg1 L319F mutant showed some DNA binding activity. To confirm that the signal observed in the EMSA gel was indeed from L319F β-Ogg1 mutant protein, an EMSA with cold substrate was resolved in a polyacrylamide gel and transferred to a PVDF membrane, and the membrane hybridized with anti-mtOgg1 antibody. As shown in Supplementary Figure 1B, the shifted signal in a radioactive EMSA corresponded to the western blot signal of β-Ogg1. These observations suggest that Phe-319 is important, but its substitution alone does not explain the lack of 8-oxoG DNA glycosylase activity in β-Ogg1.

The role of the αO helix domain for 8-oxoG glycosylase activity

Since the L319F substitution did not render β-Ogg1 an active glycosylase, we systematically mutated each amino acid in the

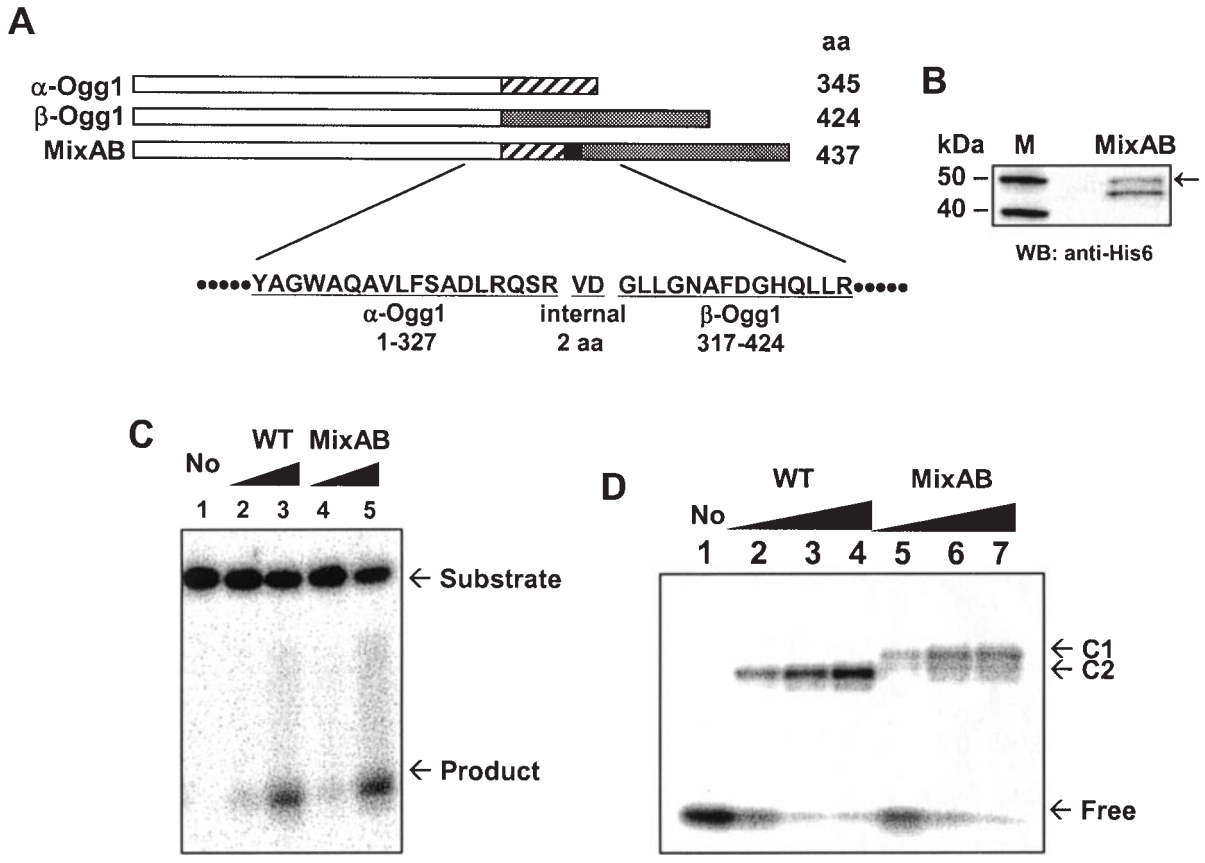

Figure 4. Effect of the long C-terminal tail of β-Ogg1 on 8-oxoG DNA glycosylase activity. (**A**) The catalytic domain of α-Ogg1 (position 1–327) was fused to the long C-terminal tail of β-Ogg1 (317–424). At the junction, two internal amino acids (Val–Asp) were inserted because of the cloning strategy, to generate restriction endonuclease SalI site. This engineered protein was named as MixAB. (**B**) The MixAB protein was partially purified as a His-tagged protein from *E.coli fpg*-deficient cells, and the amount of the MixAB protein was quantified by western blot using a known concentration of purified His–α-Ogg1. The arrowhead indicates the signal corresponding to His–MixAB protein (calculated molecular mass 51.5 kDa). (**C**) 8-oxoG DNA glycosylase activity of MixAB protein. WT α-Ogg1 (lanes 2 and 3) and MixAB (lanes 4 and 5) protein were incubated with a 30mer oligonucleotide (10 nM) containing 8-oxoG/C, at 37°C for 30 min. The concentrations of protein added were 1 nM (lanes 2 and 4) and 10 nM (lanes 3 and 5). (**D**) $NaBH_4$-mediated DNA trapping assay: 100 mM $NaBH_4$ was added to the DNA glycosylase assay, in order to covalently link the complex between substrate and reacting protein. The products were separated on 8–16% Tris–glycine SDS–polyacrylamide gel. C1 and C2 indicate substrate complexes with MixAB and WT α-Ogg1, respectively. The concentrations of protein added were 50 nM (lanes 2 and 5), 100 nM (lanes 3 and 6) and 500 nM (lanes 4 and 7).

317–323 region of α-Ogg1 to the corresponding amino acid in the β-Ogg1 sequence, generating the mutants V317G, F319L, S320G, A321N, D322A, L323F (Figure 7A). A mutant α-Ogg1 in which the entire 317–323 stretch was substituted for the β-Ogg1 sequence was also generated (317–323/α-β). To further test the effect of end-capping of the αO-helix domain, the R324D mutant protein was also prepared. In order to investigate a possible contribution of the C-terminal portion (amino acids 326–345), a mutant protein lacking this region was generated (Stop325). These proteins were then expressed, partially purified and quantified by western blot. In $NaBH_4$-mediated DNA trapping assays with 8-oxoG/C (Figure 7B), the mutant proteins V317G and F319L showed significantly weaker complex formation with 8-oxoG/C, indicative of lower DNA glycosylase activity. DNA–enzyme complex trapping was completely eliminated in the 317–323/α-β mutant protein. Accordingly, 8-oxoG incision (Figure 7C) and DNA binding (Figure 7D) activity were much reduced in the V317G mutant compared with WT, and completely abolished in the 317–323/α-β mutant. These results suggest that Val-317 is a critical residue for 8-oxoG DNA glycosylase activity, a residue that had not been identified previously.

We next generated the corresponding β-Ogg1 mutant proteins in which positions 317 and 319 contained the amino acids found in α-Ogg1, G317V and G317V/L319F. However, neither substitution significantly increased 8-oxoG DNA incision or binding activities in these mutants protein (data not shown). It is possible that the αO-helix formation is still incomplete in these mutant proteins.

To evaluate the DNA binding activity of α-Ogg1 mutant proteins, EMSA was performed using a synthetic AP site analogue tetrahydrofran (THF)-containing oligonucleotide (Supplementary Figure 2). WT, F319G and F319L proteins showed efficient binding activity to THF, while V317G and 317–323/α-β proteins did not. A K249Q mutant protein, previously shown to lack 8-oxoG DNA glycosylase activity and AP lyase activity (29), still retained significant binding activity to THF, suggesting that this mutation does not disrupt the recognition/binding mechanism (summarized in Table 1). Altogether, our results suggest that Val-317 is crucial for

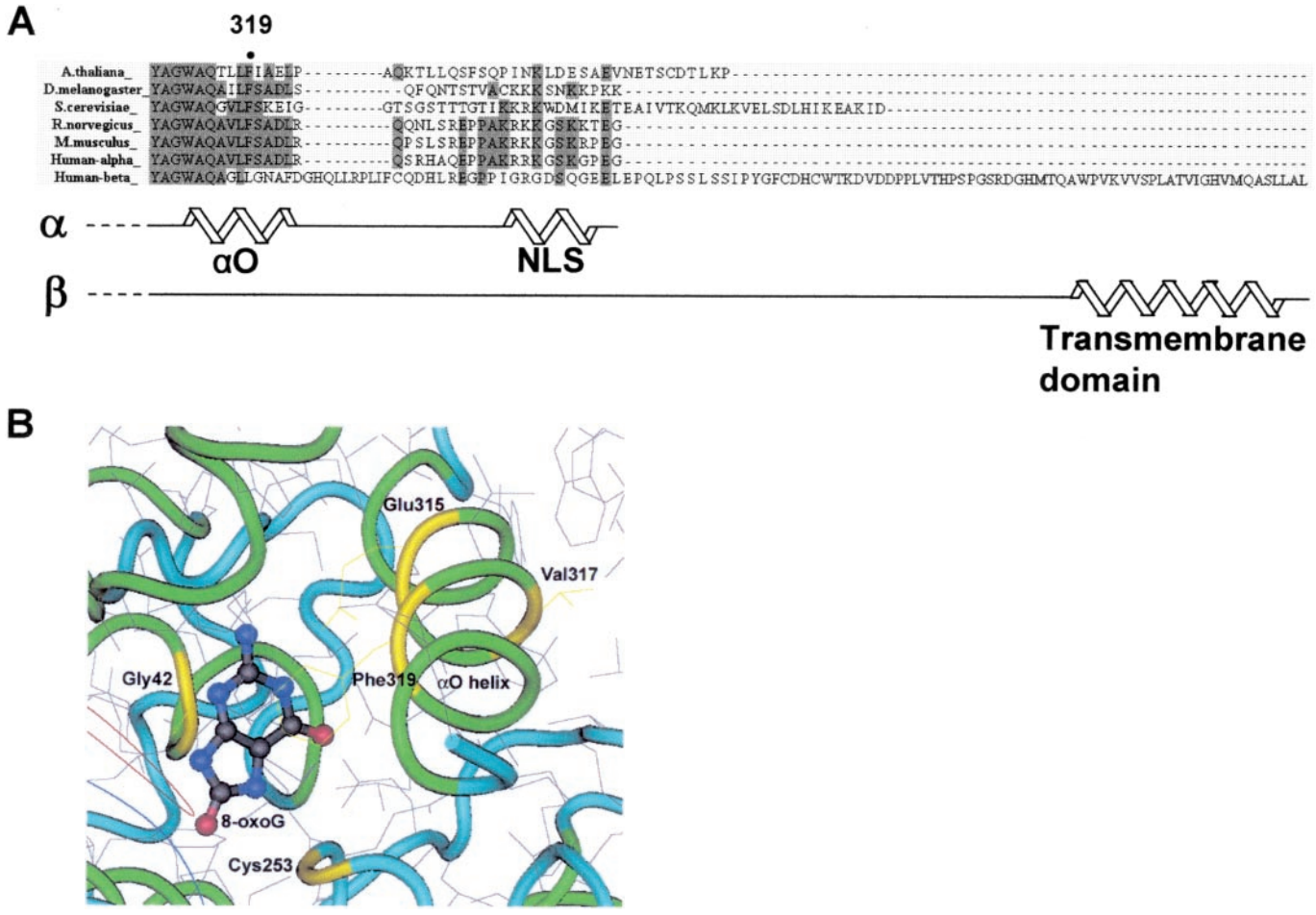

Figure 5. Structure of the C-terminus of Ogg1. (**A**) Sequence alignments of Ogg1 orthologues from various species. The C-terminal amino acid sequences were obtained from Entrez Protein entries in NCBI, and aligned by ClastalW. The conserved phenylalanine (amino acid 319) in the human α-Ogg1 is marked. The other conserved amino acids are shaded. Secondary structure of human α- and β-Ogg1 proteins was predicted using the computer-based GOR secondary structure prediction program and the SOSUI system, as described in Materials and Methods, and the predicted helix domain is illustrated. The NLS (335–342) in α-Ogg1 and the transmembrane domain (400–422) in β-Ogg1 were expressed as helix domains. The formation of the αO helix domain (313–323 in α-Ogg1) has been found previously (28). (**B**) Relative location of αO helix and 8-oxoG residue in the active pocket. This figure was obtained from Entrez Structure (pdb code 1HU0) and modified by Cn3D. Important amino acids residues are marked in yellow.

DNA binding activity. However, since the Val-317 mutant protein loses the ability to bind to the substrate in the first place, these analyses do not allow us to speculate whether it is also critical for catalytic activity.

Quantification of Ogg1 protein level in human mitochondria

The results above clearly indicate that β-Ogg1 lacks 8-oxoG glycosylase activity, and thus this protein likely does not account for this activity in human mitochondria. We next purified mitochondria from GM1310 cells in order to evaluate the relative levels of Ogg1 isoforms in mitochondria *in vivo*. Before freeze-storage, mitochondria were purified as described in Materials and Methods and treated with Proteinase K to eliminate any cross-contamination of nuclear and cytosolic proteins. Purity of the mitochondrial suspensions was assessed by western blot with the abundant nuclear protein Lamin B1. As shown in Supplementary Figure 3, LaminB1 was readily detected in nuclear extracts but not detected in the mitochondrial fraction. β-Ogg1 protein levels in GM1310 mitochondria were quantified by western blot using polyclonal anti-β-Ogg1 antibody and purified GST-β-Ogg1 as standards (Figure 8A). We estimated that 100 μg GM1310 mitochondria contained 218.5 fmol of β-Ogg1. Nishioka *et al.* (26) detected about 5 ng of β-Ogg1 in 100 μg of mitochondria from other human cell lines, corresponding to about 100 fmol β-Ogg1 (Table 2). Since α-Ogg1 also contains a mitochondrial localization signal in its N-terminus, we measured α-Ogg1 levels in GM1310 mitochondria by western blot (Figure 8B). Possible nuclear contamination was again assessed and no LaminB1 signal was detected in up to 200 μg of mitochondria. In the blot with anti-Ogg1 polyclonal antibody, we detected a significant signal of ∼39 kDa protein in the mitochondrial fraction. We also detected a strong signal at ∼25 kDa in the mitochondrial fraction, which is likely a non-specific cross-reacting band, since there is no report of Ogg1 isoforms of such molecular weight. From those blots, we estimated that mitochondria contain 12.3 fmoles of α-Ogg1 per 100 μg protein.

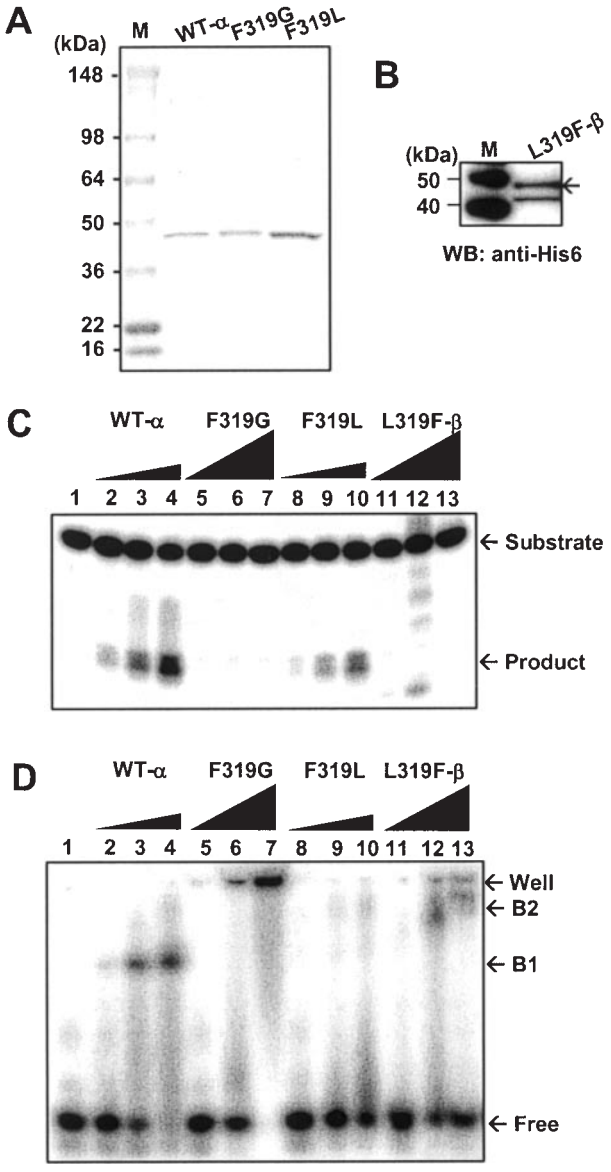

Figure 6. Enzymatic activity of human His–Ogg1 WT and mutant proteins. (**A**) Purification of α-Ogg1 WT and mutant proteins. Purified WT-α, F319G and F319L mutant proteins (0.5 μg each) were analyzed by SDS–PAGE with Coomassie blue staining. Lane M represents molecular weight marker. (**B**) L319F-β protein was partially purified and visualized by western blot with anti-His_6 antibody. The arrow-head indicates L319F-β protein signal. (**C**) DNA incision assay. 30mer OG/C oligonucleotide (10 nM) were incubated with WT and F319L (5, 10 and 100 nM) and F319G and L319F-β (10, 100 and 1000 nM) proteins at 37°C for 30 min, and reaction products were analyzed on denaturing-polyacrylamide gel. Lane 1, no protein added. (**D**) DNA binding assay. The binding activity to 30mer OG/C oligonucleotide (10 nM) of WT and F319L (10, 50 and 100 nM) and F319G and L319F-β (10, 100 and 1000 nM) was analyzed by EMSA. B1 and B2 indicate DNA plus α-Ogg1 complex and DNA plus β-Ogg1 complex, respectively.

To determine the relative distribution of α-Ogg1 between nuclear and mitochondrial compartments, we calculated the ratio of protein content between nuclear and mitochondrial fractions in GM1310 cells, using the ratio of nuclear LaminB over mitochondrial CoxIV from the whole cell extract. The levels of these two proteins were measured in whole cell (25 μg), nuclear (5 μg) and mitochondrial (50 μg) extracts by western blot (Table 3). A calculated ratio of 4.2:1 (nuclear:mitochondrial) was obtained. After standardization, we calculated that the semi-quantified amounts of α-Ogg1 were 132.3 and 12.3 fmol in 420 and 100 μg of nucleus and mitochondria, respectively (Table 2). This suggests that about 10% of α-Ogg1 localizes to mitochondria.

DISCUSSION

We report that recombinant β-Ogg1 does not show 8-oxoG DNA glycosylase activity. Two residues that are not conserved in β-Ogg1, Val-317 and Phe-319, were identified as necessary for DNA binding and 8-oxoG DNA glycosylase activity in α-Ogg1. These results explain the observation that ρ^0 mitochondria contained only 20% of β-Ogg1 protein compared with WT, despite displaying similar 8-oxoG DNA glycosylase activity. We detected significant levels of α-Ogg1 in human GM1310 mitochondria, implying that this isoform may be responsible for 8-oxoG repair in mitochondria.

β-Ogg1 has been considered to be the major 8-oxoG DNA glycosylase in human mitochondria since the observation by Nishioka *et al*. (26) that it localizes solely to this compartment. While cloning the human Ogg1 gene, Roldan-Arjona and colleagues purified β-Ogg1 (25), and in that study, incision activity was detected on an 8-oxoG-containing oligonucleotide. However, those experiments employed very high concentrations of recombinant β-Ogg1 (1 μg, 20 pmol) compared to substrate (75 fmol), resulting in a specific activity of β-Ogg1 that was 100-fold lower than that of Fpg. We did not detect any glycosylase activity of β-Ogg1 in this study under any of the numerous conditions tested (see below). Our experiments with ρ^0 cells (Figure 2) showed that β-Ogg1 protein levels can decrease by 80% with no concomitant change in mitochondrial 8-oxoG incision activity. These observations suggest that β-Ogg1 may not be the major mitochondrial 8-oxoG DNA glycosylase. It should also be noted that β-Ogg1 is not present in rat and mouse [reviewed in (22)]. Using western blot detection of endogenous α- and β-Ogg1 in human GM1310 mitochondria (Figure 8), we detected 12.3 fmol of α-Ogg1 and 218.5 fmol of β-Ogg1 per 100 μg of mitochondria (Table 2). Thus, our data suggest that α-Ogg1 performs the 8-oxoG DNA glycosylase activity in human mitochondria. Among rat, mouse and human, the α-isoform of Ogg1 is highly conserved (84% identity in amino acid sequence) (22), underscoring the importance of α-Ogg1 in genomic stability.

Recombinant β-Ogg1 was expressed and purified with two different fusion epitopes, GST and hexa-histidine, and from an *fpg*-deficient cell line in order to avoid contamination with endogenous *E.coli* Fpg protein. The His-fused β-Ogg1 was also co-expressed with the chaperones, GroEL and GroES, which are bacterial homologues of the human mitochondria chaperones Hsp60 and Hsp10, to facilitate proper protein folding during expression (41). To address the possibility that a post-translational modification specific to eukaryotic cells was required for activity, we also purified β-Ogg1 from an insect cell expression system. However, in all cases, no significant 8-oxoG DNA glycosylase activity was detected with recombinant β-Ogg1, although α-Ogg1 expressed in parallel was active under all conditions. The β-Ogg1 also did not display any AP-lyase activity. To investigate the strand scission, after

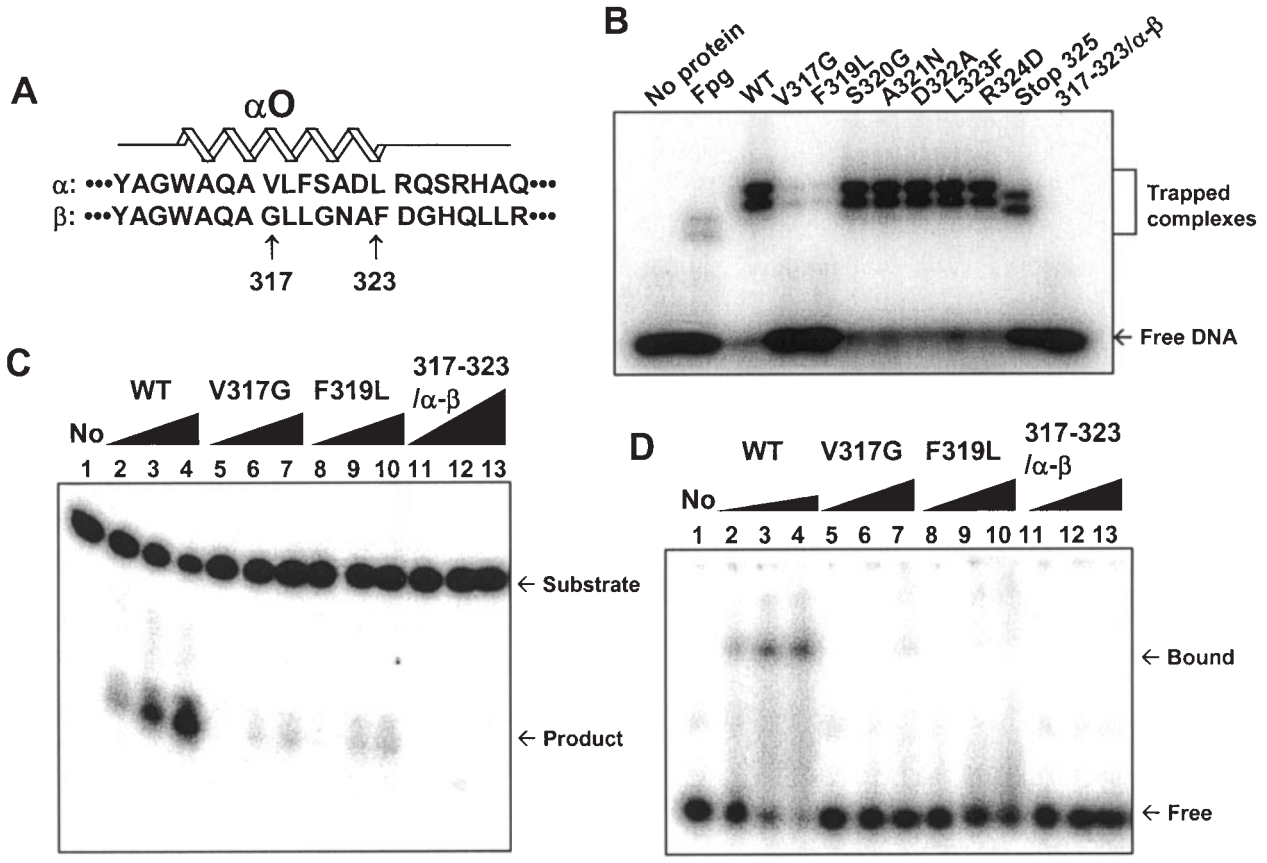

Figure 7. Screening of 8-oxoG DNA glycosylase-deficient mutant α-Ogg1 proteins. (**A**) Amino acid sequence alignment of the αO helix domain from α- and β-Ogg1 proteins. αO helix domain is located between amino acids 313–323 in α-Ogg1. Since the first 316 amino acids are common between α- and β-Ogg1 proteins, only positions 317–323 were targeted for site-directed mutagenesis. Each amino acid in α-Ogg1 was substituted with the corresponding amino acid in β-Ogg1. As a result, V317G, F319L, S320G, A321N, D322A, L323F His–α-Ogg1 mutant proteins were generated. R324D was also generated to test the effect of αO helix-end capping. In addition, all seven amino acids in α-Ogg1 were substituted at once for those in β-Ogg1 (317–323/α-β). To exclude the effect of positions 326–345 in α-Ogg1 on the activity, a Stop325 protein was also generated, in which positions 326–345 were deleted. (**B**) DNA trapping assay: 1 pmol of each protein was applied to a $NaBH_4$-mediated DNA trapping assay with oligonucleotide containing 8-oxoG/C, as described earlier. Purified Fpg protein (30.2 kDa) was used as positive control. (**C**) DNA incision assay with mutant proteins. WT, V317G, F319L and 317–323/α-β Ogg1 proteins were used for DNA incision assay with 10 nM of oligonucleotides containing 8-oxoG/C. The protein amounts added were 0 (lane 1), 1 nM (lanes 2, 5 and 8), 5 nM (lanes 3, 6 and 9) and 10 nM (lanes 4, 7 and 10). For lanes 11–13, 10 nM (lane 11), 100 nM (lane 12) and 500 nM (lane 13) of 317–323/α-β α-Ogg1 mutant proteins were added to reactions. (**D**) DNA binding assay: 10 nM of oligonucleotide containing 8-oxoG/C was incubated with WT (10, 50 and 100 nM), V317G, F319L and 317–323/α-β (10, 100 and 500 nM). Products were analyzed by EMSA.

Table 1. Summary of the activity of α-Ogg1 WT and mutants proteins

Protein	8-oxoG binding	8-oxoG incision	THF binding	AP lyase
WT	++	++	++	++
F319A[a]	–	–	++	+
F319G	–	–	++	n.d.
F319L	–	+	++	n.d.
V317G	–	+	–	n.d.
317–323/α-β	–	–	–	n.d.
K249Q[b]	++	–	++	–

n.d. = not determined.
[a]van der Kemp *et al*. (38).
[b]Nash *et al*. (29).

incision reaction, we also stopped the reaction in the presence of NaOH with heat, and no cleaved product was detected (data not shown), suggesting no uncoupled glycosylase activity in β-Ogg1. We therefore conclude that recombinant β-Ogg1 may in fact not have a physiologically significant 8-oxoG DNA glycosylase activity. We have tested a wide range of relevant concentrations, buffers, conditions, and substrates.

The three-dimensional structure of the catalytic core of human Ogg1 in complex with 8-oxoG containing oligonucleotides has been solved at a resolution of 2.1 Å (28). This study revealed that residues Cys-253 and Phe-319 interact with opposite π-faces of 8-oxoG, suggesting that these amino acids form an 8-oxoG recognition pocket. 8-oxoG also forms hydrogen bonds with Gly-42, Gln-315 and two water molecules (Figure 5B). β-Ogg1 lacks the phenylalanine residue at position 319 (Figure 5A). We used a site-directed mutagenesis approach to determine how the sequence differences between α- and β-isoforms of Ogg1 affect 8-oxoG recognition and catalysis of its removal. During the preparation of this manuscript, van der Kemp *et al*. (38) reported that a F319A mutant α-Ogg1 lost 8-oxoG incision ability, similar to the F319G mutant we constructed (Figure 6C and D). Bacterial AlkA, MutY, Nth and human Ogg1 DNA glycosylases are all members of the endonuclease III family of proteins, with Trp-272 in AlkA, Met-185 in MutY and Ile-180 in Nth DNA glycosylases corresponding to Phe-319 of human Ogg1 (42–44). All these amino acids have hydrophobic side

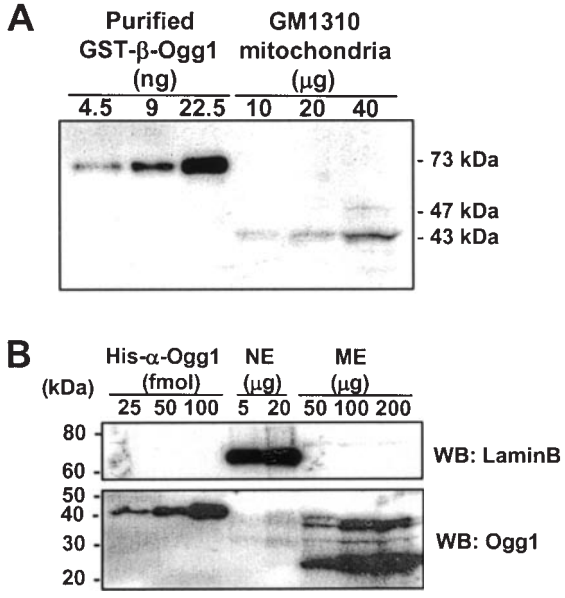

Figure 8. Quantification of α- and β-Ogg1 proteins in human GM1310 cells. (**A**) Quantification of β-Ogg1 protein. Purified GST–β-Ogg1 (73 kDa) and GM1310 mitochondria were fractionated by SDS–PAGE, and western blot analysis was done with mtOgg1 antibody. The signal around 43 kDa in the mitochondrial fraction indicates the β-Ogg1 processed after translocation into mitochondria. (**B**) Quantification of α-Ogg1 (~39 kDa) in GM1310 mitochondria. Purified His–α-Ogg1 (41.6 kDa), nucleus and mitochondria from GM1310 cells were fractionated by SDS–PAGE and analyzed by western blot with polyclonal Ogg1 antibody.

Table 2. Quantification of amount of Ogg1 proteins in cultured human cell lines

Cell line	Fraction	Protein	Amount[a]
GM1310	Nucleus	α-Ogg1	132.3
GM1310	Mitochondria	α-Ogg1	12.3
GM1310	Mitochondria	β-Ogg1	218.5
HeLa S3[b]	Mitochondria	β-Ogg1	144.2
Jurkat[b]	Mitochondria	β-Ogg1	79.1

[a]fmol protein per 420 and 100 μg of nuclear and mitochondrial proteins, respectively.
[b]Nishioka *et al*. (26).

chains, suggesting that hydrophobic interactions are necessary for the formation of α-helix structures and for interactions with lesions in DNA. The absence of this phenylalanine residue in β-Ogg1 suggests that this protein lacks the critical 8-oxoG recognition structure. This conclusion is further supported by the observation that the F319L α-Ogg1 mutant protein lost DNA binding activity, although it retained some DNA glycosylase activity (Figure 6C and D). Moreover, the L319F β-Ogg1 mutant protein showed slightly recovered DNA binding activity to 8-oxoG, but no detectable 8-oxoG DNA glycosylase activity. These observations can be reconciled if Phe-319 is critical for 8-oxoG recognition, with other critical amino acid residues present in the C-terminal αO helix playing a more prominent role in the base excision reaction. In this study, we found that the V317G mutant α-Ogg1 also lost DNA glycosylase activity. Val-317 is located in the middle of the αO helix, and its side chain does not face 8-oxoG, whereas the residues Gln-315 and Phe-319 do (Figure 5B). It is likely

Table 3. Relative amount of nuclear Lamin B and mitochondrial CoxIV in GM1310 whole cell extract

Fraction	Lamin B	CoxIV
WCE (25 μg)	1.91	0.046
Nuclear extract (5 μg)	1	N/A
Mitochondrial extract (50 μg)	N/A	1

N/A, not applicable.

that Val-317 does not directly interact with 8-oxoG, however, since valine is a hydrophobic amino acid contributing to α-helix stabilization, V317G mutant protein likely causes disruption of the αO helix, preventing correct positioning of the 8-oxoG for Ogg1 activity. As summarized in Table 1, our results suggest that stable formation of the αO helix is critical not only for 8-oxoG DNA glycosylase activity but also for DNA binding activity.

In order to excise 8-oxoG from DNA, Ogg1 is thought to initially bend the DNA at the minor groove of undamaged DNA, searching for modifications that can promote drastic kinking (45). 8-oxoG stimulates the bending of DNA, causing a greater distortion of the DNA double helix structure at the damage site (46). Ogg1 then finds this distortion and likely flips the 8-oxoG residue out, allowing for the interaction of the lesion with a specific pocket in the protein. These interactions then allow the catalytic activity of Ogg1 to take place, cleaving the N-glycosylic bond and releasing 8-oxoG. Norman *et al*. (47) investigated the structure of a catalytically inactive Ogg1 mutant (K249Q) with oligonucleotides containing either 8-oxoG or an abasic site analogue (THF) that is not cleaved by Ogg1. They suggested that substrate recognition and catalysis were coupled, observing that the αO-helix slightly shifted away from the active site residue Asp-268. This phenomenon would explain why the F319A mutant retains about 60% of AP lyase activity compared with WT (38) and that the F319L mutant (Figure 6) looses binding activity but retains some 8-oxoG incision activity. Based on these findings, we conclude that β-Ogg1 lacks DNA glycosylase activity because its amino acid sequence does not support the correct assembly of the C-terminal αO helix. Further support for this conclusion comes from the observation that substituting seven amino acids in α-Ogg1 in the αO helix region, positions 317–323, with the corresponding sequence in β-Ogg1 (317–323/α-β protein, Figure 7) completely abrogates 8-oxoG DNA glycosylase activity.

In this study, we presented evidence that recombinant β-Ogg1 does not have DNA glycosylase activity due to the lack of the C-terminal αO helix (Figure 7), and α-Ogg1 is present in human mitochondria (Figure 8) and probably accounts for the 8-oxoG incision activity. The question still remains whether β-Ogg1 has glycosylase activity *in vivo*, and if not, what its biological role is. For instance, the possibility exists that β-Ogg1 has repair activities for novel substrates, such as base lesions and RNA substrate, or requires yet unidentified co-factors, including other proteins. It should be noted that β-Ogg1 contains a long tail located in the C-terminus of the protein. This tail was predicted to form a long coiled-coil structure, spanning a transmembrane domain (Figure 5A), which may suggest a role in anchoring the protein in membranous structures. The long tail *per se* did not affect 8-oxoG DNA glycosylase activity as shown in Figure 4. It is also

possible that β-Ogg1 participates in another step of base excision repair since it retains the HhH-GPD motif which is highly conserved in EndoIII DNA glycosylase family, and this is currently under investigation. We are now developing β-Ogg1 knock-down and/or over-expressing cell lines to obtain a better understanding of the biological roles of β-Ogg1.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

We thank Drs Erling Seeberg, Serge Boiteux and Giuseppe Attardi for supplying Ogg1 expression vectors, *E.coli* and ρ^0 human cell lines. We also thank Drs Jeanine A. Harrigan and David M. Wilson III for the critical reading of this manuscript.

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