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The high binding affinity of human ribosomal protein S3 to 7,8-dihydro-8-oxoguanine is abrogated by a single amino acid change

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ABSTRACT

Previous studies have shown that human ribosomal protein S3 (hS3) has a high apparent binding affinity for 7,8-dihydro-8-oxoguanine (8-oxoG) residues in DNA and interacts with the human base excision repair (BER) proteins OGG1 and APE/Ref-1. We used a combination of computational and experimental approaches to understand the role of hS3 in BER and its potential to hinder repair of 8-oxoG lesions by OGG1 and APE/Ref-1. Sequence analysis was employed to identify hS3 residues likely to be involved in binding to 8-oxoG. One putative site, lysine 132 (K132), located in a helix-hairpin-helix DNA binding motif, was mutated to alanine (K132A). The hS3-K132A mutant retained the ability to cleave abasic DNA, but its capacity to bind 8-oxoG was abrogated completely. The ability of OGG1 to cleave an 8-oxoG-oligonucleotide substrate pre-incubated with hS3 or hS3-K132A was also tested. Pre-incubations with wild-type hS3 and 8-oxoG-containing oligonucleotides completely prevented the subsequent removal of 8-oxoG by OGG1. On the other hand, OGG1 incubations combined with hS3-K132A stimulated cleavage of 8-oxoG in excess of two-fold, confirming previous observations that hS3 positively interacts with OGG1, but only under conditions in which the binding of hS3 to 8-oxoG is limited. Overall, the ability of OGG1 to repair 8-oxoG is compromised when hS3 is bound to 8-oxoG sites. Conversely, in the absence of DNA binding, hS3 interacts positively with OGG1 to produce a more robust removal of 8-oxoG residues in DNA.

1. Introduction

The formation of 7,8-dihydro-8-oxoguanine (8-oxoG) in DNA is primarily through the action of reactive oxygen species (ROS). The 8-oxoG adduct in DNA has the potential to mispair with A during DNA replication [1–3] leading to G → T transversion mutations [2,4] that can predispose cells to a number of pathological states including cancer. All organisms, however, have the ability to remove this lesion via base excision repair (BER), in which the first step is the liberation of 8-oxoG by an *N*-glycosylase activity. In many instances *N*-glycosylases possess an intrinsic apurinic/apyrimidinic (AP) lyase activity, and still others are capable of additionally carrying out a delta-elimination reaction depending upon the glycosylase involved.

In humans, the removal of 8-oxoG is predominantly through the action of the *N*-glycosylase/AP lyase activity possessed by OGG1. We, and others [5–7] have examined this acti-

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vity and its ability to cleave 5′-end-labeled oligonucleotides possessing a single 8-oxoG residue. Notably, these studies have found that the human protein performs rather poorly when compared to *N*-glycosylases found in other organisms. For example, the k(cat)/K(m) for human OGG1 was found to be 80-fold lower for the removal of 8-oxoG as compared to the *Escherichia coli* enzyme Fpg [8]. We have shown using surface plasmon resonance (SPR) that the apparent binding affinity (K_D) for 8-oxoG was two orders of magnitude greater for *Drosophila* S3 *N*-glycosylase/AP lyase compared to human OGG1 [7].

Several studies have shown that the poor catalytic activity of OGG1 can be somewhat improved when it is combined with other BER proteins [9,10]. In our own studies, we have found using SPR analysis that the human ribosomal protein S3 (hS3) positively interacts with OGG1 and APE/Ref-1 [11]. Furthermore, co-immunoprecipitation experiments done with purified proteins showed that hS3 was physically associated with APE/Ref-1 or OGG1.

The human S3 ribosomal protein was also shown in a previous study to have a very high apparent binding affinity for 8-oxoG [7], even though it lacks *N*-glycosylase activity capable of liberating this DNA lesion. It was concluded from that study that the binding of hS3 to 8-oxoG could in fact lead to adverse consequences on the ability of OGG1 to access its DNA target for removal of 8-oxoG. This notion was supported by both SPR analysis and assays using 5′-end-labeled 8-oxoG-containing oligonucleotides, in which the positive interactions observed between hS3 and APE/Ref-1 or OGG1 only occurred when the purified proteins were mixed prior to exposure to DNA [11].

We created and inspected a multiple sequence alignment of a protein superfamily that includes OGG1 and hS3 and whose hallmark is a helix-hairpin-helix (HhH) structural element followed by a Gly/Pro rich loop and a conserved Asp (HhH-GPD family) (Fig. 1). Given its location in the HhH DNA binding motif and its positive charge, we focused on a specific, fairly well conserved lysine residue (Fig. 1, OGG1 residue in green and labeled K249; hS3 residue in cyan and labelled hS3-K132). In *Drosophila melanogaster* ribosomal protein S3 (dS3), an alanine mutant of the equivalent OGG1 lysine (dS3-K134A) is shown to have no 8-oxoG-DNA binding activity. We also present studies on the alanine mutant of this lysine residue in human ribosomal protein S3 (hS3-K132A). We show that a change at this site (hS3-K132A) completely abrogates DNA binding to 8-oxoG. Notably, the combination of hS3-K132A and OGG1 *in vitro* results in a greater than two-fold

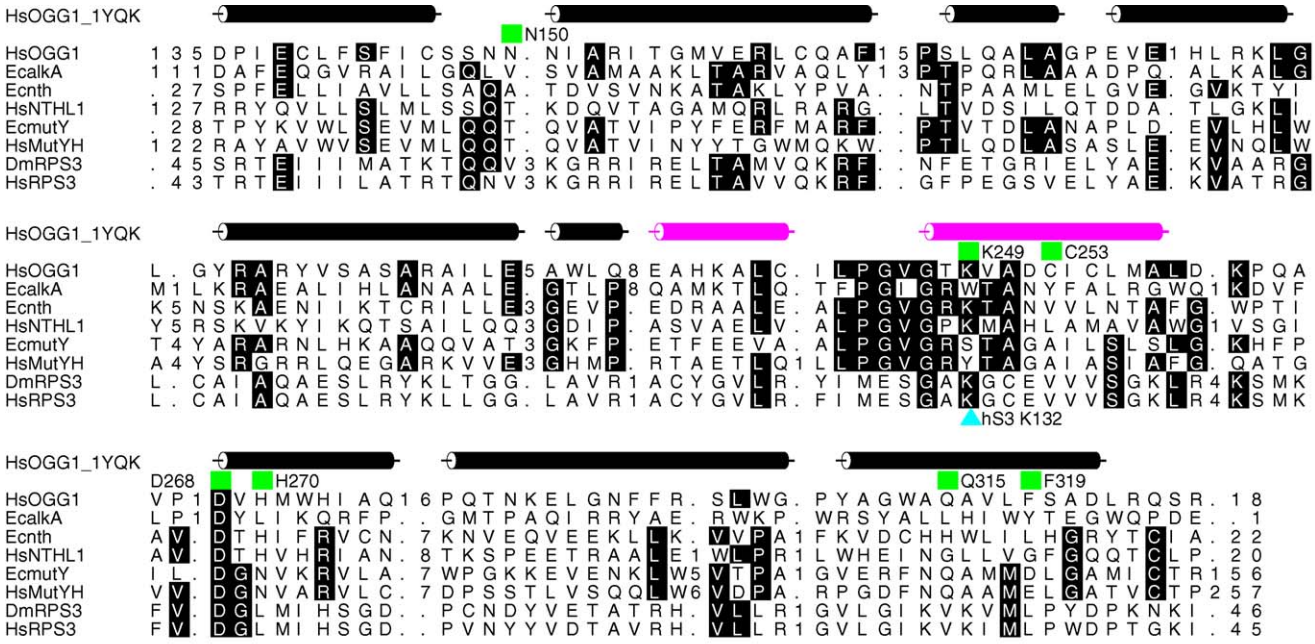

Fig. 1 – An HMM-generated multiple sequence alignment of eight members of the HhH-GPD protein family. A column in black indicates residues that are conserved in at least four family members and numbers indicate the number of amino acids not shown explicitly (regions at the N- and C-termini were not part of the HhH-GPD HMM and so are not shown in the alignment). The sequences shown are as follows: HsOGG1, *Homo sapiens* 8-oxoguanine DNA glycosylase isoform 1a (RefSeq NP_002533); EcalkA, *Escherichia coli* 3-methyl-adenine DNA glycosylase II (NP_416572); Ecnth, *E. coli* endonuclease III/DNA glycosylase/apyrimidinic (AP) lyase (NP_416150); HsNTHL1, *H. sapiens* nth endonuclease III-like 1 (NP_002519); EcmutY, *E. coli* adenine DNA glycosylase (NP_417436); HsMutYH, *H. sapiens* mutY homolog (NP_036354); DmRPS3, *Drosophila melanogaster* 40S ribosomal protein S3 (Q06559) and HsRPS3, *H. sapiens* ribosomal protein S3 (NP_000996). For the sequence labeled "HsOGG1_1YQK", the location of secondary structure elements and key sites are taken from the X-ray crystal structure of *H. sapiens* OGG1 cross-linked with guanine-containing DNA (RCSB entry 1YQK). Cylinders represent α-helices (the HhH motif is in magenta), and the green positions and residue numbers refer to OGG1 amino acids that contact DNA. The Hs RPS3 residue mutated in this study is equivalent to HsOGG1 K249 located in the second magenta helix of the HhH motif and its position is marked by a cyan triangle (hS3-K132A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

increase in the processing of 8-oxoG lesions compared to OGG1 alone.

2. Experimental procedures

2.1. Bacterial strains for overexpression

RPC501 nfo-1::kanΔ(xth-pncA) 90 [12], a strain defective for AP endonuclease IV (*nfo*) and the AP endonuclease activity associated with exonuclease III (*xth*), was used to overexpress GST fused proteins as described previously [7]. This resulted in homogeneous preparations of wild-type hS3, hS3-K132A, dS3, and dS3K134A as judged by SDS-PAGE and Coomassie staining.

2.2. Site-directed mutagenesis

The human ribosomal protein S3 gene was mutated at lysine 132 (K132) to alanine (A) using Pfu Turbo DNA polymerase and the QuickChange mutagenesis approach (Stratagene). The following oligonucleotide primer set was used in the generation of the hS3-K132A mutant: sense-ggagagtggggccgcaggctgcgagg and anitsense-cctcgcagcctgcggccccactctcc. Mutants were selected in *E. coli* XL10-Gold ultracompetent cells and confirmed by sequencing.

2.3. Purified proteins

GST-hS3, GST-hS3-K132A and GST-hAPE/Ref-1 were purified as previously described [7,11]. GST-hOGG1 was purchased from Trevigen, Gaithersburg, MD.

2.4. Surface plasmon resonance analysis

Interactions of wild-type hS3 and mutant hS3-K132A with 8-oxoG or abasic DNA were monitored using a surface plasmon resonance biosensor instrument, BIAcore 3000 (Biacore Inc., Uppsala, Sweden). For preparation of the biosensor surface with DNA, 5′-biotinylated 37-mer duplex DNA's containing specific damages 5′-CTT GGA CTG GAT GTC GGC ACX AGC GGA TAC AGG AGC A-3′ where X = 8-oxoguanine opposite a cytosine residue (8-oxoG-37mer) or uracil opposite an adenine residue (Sigma Genosys, The Woodlands, Texas) at nucleotide position 21 (biotinylated strand). The uracil-containing oligonucleotide was treated with *E. coli* uracil DNA glycosylase (Epicenter, 2 units) to form an apyrimidinic (AP) site in place of the uracil residue [13]. The damaged duplex DNA's were diluted in a buffer containing 10 mM sodium acetate, pH 4.8, and 1.0 M NaCl and manually injected over a streptavidin-coated surface of a BIAcore sensor chip (BIA sensor chip SA) to the desired density in two different flow cells. One flow cell was left underivatized, and another immobilized with undamaged duplex DNA to allow for refractive index change corrections and nonspecific DNA binding, respectively.

2.4.1. Activity on 8-oxoG or abasic site-containing DNA

A 37 bp oligomer 5′-CTT GGA CTG GAT GTC GGC ACX AGC GGA TAC AGG AGC A-3′ where X = 8-oxoguanine (8-oxoG-37mer) (Sigma Genosys) or uracil (The Midland Certified Reagent Co.) at nucleotide position 21 (labeled strand) was 5′ ^{32}P end-labeled with DNA polynucleotide kinase. Following complementary strand annealing and gel purification the DNA fragment was used as a substrate either directly, or treated (20 pmol) with *E. coli* uracil DNA glycosylase (Epicenter, 2 units) to form an apyrimidinic (AP) site in place of the uracil [13]. Following phenol/chloroform extraction, the AP site-containing oligonucleotide (AP-37mer) was precipitated with cold ethanol.

Reaction mixtures (20 μl) contained ∼1 pmol of 5′-end-labeled 37mer; in addition, reactions with purified GST-hS3, GST-hS3-K132A, GST-dS3, GST-dS3-K134A, or GST-OGG1 contained 30 mM HEPES, pH 7.4, 50 mM KCl, 1 μg/ml bovine serum albumin (BSA), 0.05% TritonX-100, 1 mM DTT and 0.5 mM EDTA. The DNA reaction products were separated on a 16% polyacrylamide gel containing 7 M urea. Dried gels were subjected to autoradiography for visualization and densitometric analysis (ChemiImager^{TM} 4000, Alpha Innotech Corporation). The purified DNA repair proteins used to perform SPR analysis were tested for activity on 5′-end-labeled 37mers containing either 8-oxoG or an abasic site.

2.5. Sequence analysis

We updated our previous hidden Markov model (HMM)-based investigation [14] of a protein superfamily that is characterized by a helix–hairpin–helix (HhH) structural element followed by a Gly/Pro rich loop and a conserved Asp (HhH-GPD family). The parameters of the HhH-GPD HMM were estimated from eight protein sequences and the ensuing HMM was used to generate a multiple sequence alignment of these eight family members. The alignment was inspected in the context of the X-ray crystal structure of one family member [15]. The results are shown in Fig. 1.

3. Results

3.1. Site-directed mutagenesis strategy

Our structure-based analysis of HhH-GPD superfamily members suggests that sites in the HhH element are a logical choice to begin studies on eliminating the DNA binding activity possessed by hS3 (Fig. 1). The human S3 residue K132 is especially noteworthy because our studies of the equivalent position in the *Drosophila* protein (dS3-K134) were shown to be important in the formation of an imine (Schiff) base intermediate that can be trapped *in vitro* with sodium borohydride. Specifically, construction of a Lys to Ala mutant in *Drosophila* causes a complete loss of GST-dS3-K134A binding to 8-oxoG residues residing within a 37mer (Fig. 2). We therefore directed our attention to the same Lys position in human S3, namely K132, and mutated this site to an alanine residue to produce hS3-K132A.

3.2. DNA binding affinity of wild-type GST-hS3 and GST-hS3-K132A for 8-oxoG and abasic sites as determined by SPR

We previously used SPR analysis to show that hS3 has an extremely high apparent binding affinity for both 8-oxoG and abasic sites in DNA [7]. SPR analysis is extremely robust and

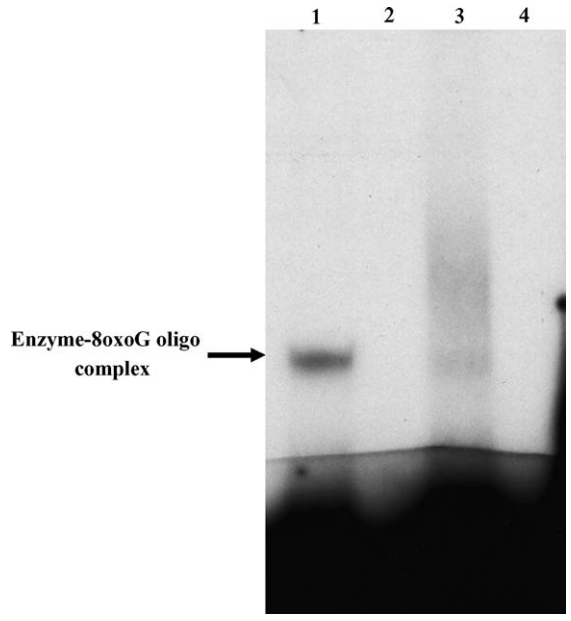

Fig. 2 – $NaBH_4$-mediated trapping of GST-dS3, GST-dS3-K134A and 8oxoG-37mer. Individual reaction mixtures (40 μl) contained 8-oxoG (20 pmol) and purified fusion proteins (10 pmol). Reaction samples were applied to a 10% non-denaturing gel, electrophoresed, vacuum dried and subjected to autoradiography. Lanes 1 and 2 is GST-dS3 and GST-dS3-K134A. Control reactions in lanes 3 and 4 were incubated in the absence of $NaBH_4$ or enzyme, respectively.

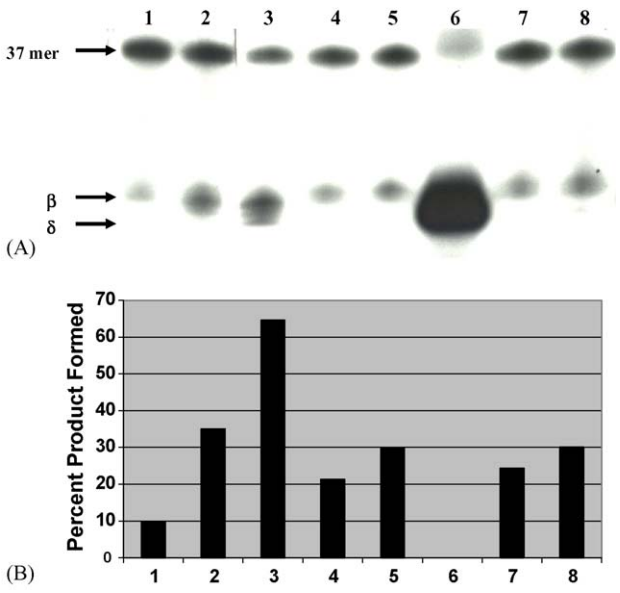

Fig. 4 – Activity on abasic site-containing DNA with different amounts of GST-dS3, GST-hS3 and GST-hS3-K132A. (A) Reactions contained ~1 pmol of AP site-containing oligonucleotide (AP-37mer) incubated with purified enzymes at 37 °C for 30 min. Lane 1 is the no enzyme control; lanes 2–3, incubation with 0.1 and 0.2 pmol of GST-dS3; lanes 4–5, incubation with 0.1 and 0.2 pmol of GST-hS3, respectively; lanes 7–8, incubations with 0.1 and 0.2 pmol of GST-hS3-K132A, respectively; Lane 6 is hot piperdine (HA) treated AP-37mer to generate a β–δ elimination product. (B) Densitometric analysis of the autoradiogram presented as percent product formed was calculated from the sum of the substrate and product. Numbers on the X-axis indicate respective lanes from (A). The autoradiogram was scanned using the AlphaEase™ software, Alpha Innotech Corporation.

allows for macromolecular interactions to be measured in real time. We therefore turned to this technology to determine the outcome of a mutation at hS3-K132 and its subsequent behavior on DNA sensor chips containing either an 8-oxoG lesion (Fig. 3A) or an abasic site (Fig. 3B). We also included wild-type hS3, in which sensograms of 8-oxoG- and abasic-containing oligonucleotides once again showed a high apparent binding affinity of GST-hS3 for these damaged DNA sites (Fig. 3A and B).

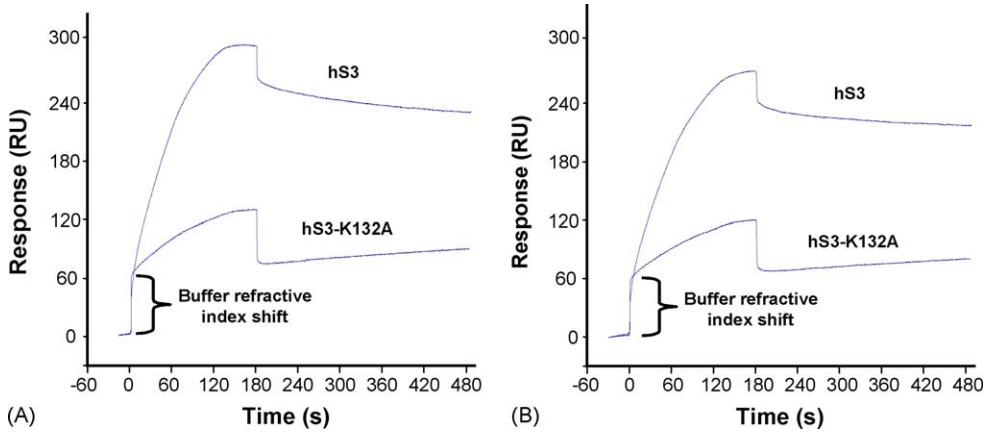

Fig. 3 – Binding characteristics of hS3 and hS3-K132A on an 8-oxoG or an abasic site modified DNA substrate as revealed by SPR analysis. Protein (45 nM) was injected onto an 8-oxoG or abasic site substrate (~2200 response units surface) at 30 μl/min using the KINJECT function of BIAcore 3000. The association phase was allowed for 180-s followed by a 300-s buffer injection period for dissociation. The initial refractive index shifts due to different buffer conditions are indicated in the figures. (A) 8-oxoG substrate. (B) Abasic substrate.

On the other hand, GST-hS3-K132A showed no binding affinity for either of these two sites whatsoever (Fig. 3A and B).

3.3. *Activity of wild-type GST-hS3 and GST-hS3-K132A on an abasic-site containing oligonucleotide*

It was of interest to learn if the abrogation of DNA binding activity of hS3 affected other activities possessed by this protein. As has been established by others, and us, hS3 contains AP lyase activity. Several studies have shown that this activity is directed towards heavily irradiated DNA [16], and several other DNA modifications [17]. We therefore tested whether GST-hS3-K132A retained its ability to cleave an abasic site embedded in a 5′-end-labeled oligonucleotide. We included in our analysis *Drosophila* GST-S3, which produces a β–δ elimination product, and wild-type human S3, which produces a single β-elimination product. As can be seen in Fig. 4, GST-hS3-K132A (lanes 7 and 8) was equally effective in cleaving the abasic substrate as was its wild-type counterpart (lanes 4 and 5). Furthermore, GST-hS3-K132A produced a β-elimination product similar to wild-type GST-hS3, suggesting that the change we made at hS3-K132 had no effect on the processing of AP sites in DNA.

3.4. *Preincubations of wild-type GST-hS3 and GST-hS3-K132A with an 8-oxoG containing oligonucleotide substrate prior to GST-OGG1 addition*

Based upon our previous observations, we believed that hS3 might create an obstacle to the liberation of 8-oxoG by OGG1. To test this, we used a 5′-end-labeled oligonucleotide containing a single 8-oxoG residue that was used as a substrate to measure OGG1 activity. As can be seen in Fig. 5, lane 2, GST-OGG1 was able to efficiently process this substrate. However, the formation of the β-elimination product by GST-OGG1 seen in lane 2 was completely inhibited by pre-incubation with increasing amounts of wild-type GST-hS3 (lanes 3 and 4). Conversely, reactions that were pre-incubated with GST-hS3-K132A did not inhibit the removal of 8-oxoG by OGG1. In fact, the absence of DNA binding by GST-hS3-K132A resulted in a greater than two-fold stimulation (determined by densitometric analysis) for the removal of 8-oxoG (lanes 5 and 6) when compared to reactions with GST-OGG1 alone (lane 2).

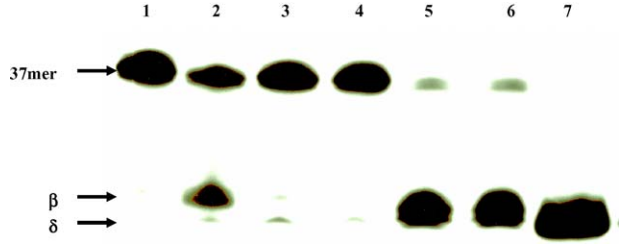

Fig. 5 – GST-hS3 inhibits OGG1 activity, whereas GST-hS3-K132A appears to stimulate GST-OGG1. Reactions with GST-hS3 and GST-hS3-K132A contained ∼1 pmol of 8-oxoG-37mer pre incubated with the respective purified protein for 10 min at room temperature followed by addition of GST-OGG1 and incubated at 37 °C for 16 min. Lane 1 is the no enzyme control; lane 2 is incubation with 0.4 pmol of GST-OGG1; lanes 3–4, incubation with 0.8 and 1.6 pmol of GST-hS3, respectively followed by 0.4 pmol of GST-OGG1; lanes 5–6, incubations with 0.8 and 1.6 pmol of GST-hS3-K132A, respectively followed by 0.4 pmol of GST-OGG1; lane 7 is hot piperdine (HA) treated 37mer to generate a β–δ elimination product. The electrophoretic mobilities of DNA cleavage products corresponding to β- and δ-elimination reactions are indicated.

4. Discussion

Previous studies have shown that human ribosomal protein S3 is a remarkably versatile protein. It was first shown that hS3 can be cross-linked to eukaryotic initiation factor eIF-2 [18] and eIF-3 [19], and appears to be directly involved in mRNA-aminoacyl tRNA interactions during protein synthesis [20]. The multifunctional characteristics possessed by S3 began to emerge through the studies of Kuhnlein et al. [21]. Their seminal studies on the presence of AP endonuclease activity in human cells led to the discovery that one of the two AP endonuclease activities being studied by the Linn group turned out to be S3 [16].

Additional studies showed that *Drosophila* S3 possessed an extremely robust activity for the liberation of 8-oxoG residing in 5′-end-labeled oligonucleotide substrates [7,14,22]. Human S3, on the other hand, was found to contain only AP lyase activity for abasic sites in DNA, even though it is 80% identical in sequence to *Drosophila* S3. Nevertheless, hS3 was found to possess a remarkably high binding affinity for 8-oxoG [7], leaving open its possible involvement in BER. Studies being conducted elsewhere on proteins stimulating the less than robust removal of 8-oxoG by OGG1 [9,10,23] led us to investigate whether hS3 could similarly stimulate the removal of 8-oxoG. For the most part our studies failed to show that hS3 was able to facilitate a more rapid removal of 8-oxoG, that is, until we began to mix hS3 with BER proteins prior to their exposure to DNA. Under these reaction conditions, it was found that hS3 positively interacted with both OGG1 and APE/Ref-1. Through co-immunoprecipitation experiments, we were also able to establish that a physical relationship existed between hS3 and other purified proteins participating in BER.

Based upon these observations, we felt that the expression of hS3 could lead to either of two outcomes. In the absence of hS3 binding to 8-oxoG, our results suggested that the protein–protein interactions between hS3 and OGG1 and APE/Ref-1 would lead to the more robust removal of 8-oxoG. Conversely, the binding of hS3 to 8-oxoG could result in a stagnant complex that we expected would prevent access of OGG1 to 8-oxoG, thereby inhibiting the removal of 8-oxoG. The construction of an hS3 mutant that no longer binds to 8-oxoG proved that our expectations were correct. As shown here, when wild-type hS3 is pre-incubated with 8-oxoG substrates, the removal of this lesion by OGG1 is for the most part totally inhibited. On the hand, pre-incubation with the binding mutant hS3-K132A resulted in a greater than two-fold stimulation of OGG1 to process 8-oxoG. This result is consistent with our previous observations by SPR that showed that mixtures of hS3 with OGG1 or APE/Ref-1, prior to introducing

DNA, resulted in an increased SPR response by 1.5- to 2-fold above that expected if the proteins acted independently. Co-immunoprecipitation assays also support the contention that hS3 is physically involved with these BER proteins. Overall, an abundance of *in vitro* data now exists to show that hS3 is an active participant in BER.

The binding of hS3 to 8-oxoG on the other hand presents a conundrum that appears to negate the overall beneficial effects it provides to stimulating BER. If indeed hS3 remains bound to 8-oxoG and thereby produces a blockade to DNA repair, a question arises as to the outcome of this stagnant complex. One scenario that seems reasonable is that hS3 transmits a signal that results in apoptosis. Notably, published accounts [24] and our own studies (unpublished observations) show that hS3 overexpression leads to the activation of apoptotic caspases. There is also the possibility that hS3 is removed from 8-oxoG through ubiquitination [25]. In any event, the possible stable binding of hS3 to 8-oxoG, regardless of its subsequent removal, could represent a deleterious consequence that could make cells more susceptible to the damaging effects of free radical attack on DNA.

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