Mammalian 5-Formyluracil−DNA Glycosylase. 2. Role of SMUG1 Uracil−DNA Glycosylase in Repair of 5-Formyluracil and Other Oxidized and Deaminated Base Lesions†

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ABSTRACT: In the accompanying paper [Matsubara, M., et al. (2003) *Biochemistry 42*, 4993−5002], we have partially purified and characterized rat 5-formyluracil (fU)−DNA glycosylase (FDG). Several lines of evidence have indicated that FDG is a rat homologue of single-strand-selective monofunctional uracil−DNA glycosylase (SMUG1). We report here that rat and human SMUG1 (rSMUG1 and hSMUG1) expressed from the corresponding cDNAs indeed excise fU in single-stranded (ss) and double-stranded (ds) DNA. The enzymes also excised uracil (U) and uracil derivatives bearing an oxidized group at C5 [5-hydroxyuracil (hoU) and 5-hydroxymethyluracil (hmU)] in ssDNA and dsDNA but not analogous cytosine derivatives (5-hydroxycytosine and 5-formylcytosine) and other oxidized damage. The damage specificity and the salt concentration dependence of rSMUG1 (and hSMUG1) agreed well with those of FDG, confirming that FDG is rSMUG1. Consistent with the damage specificity above, hSMUG1 removed damaged bases from Fenton-oxidized calf thymus DNA, generating abasic sites. The amount of resulting abasic sites was about 10% of that generated by endonuclease III or 8-oxoguanine glycosylase in the same substrate. The HeLa cell extract and hSMUG1 exhibited a similar damage preference (hoU·G > hmU·A, fU·A), and the activities for fU, hmU, and hoU in the cell extract were effectively neutralized with hSMUG1 antibodies. These data indicate a dual role of hSMUG1 as a backup enzyme for UNG and a primary repair enzyme for a subset of oxidized pyrimidines such as fU, hmU, and hoU.

DNA constantly suffers damage from hydrolytic degradation and reactive oxygen species generated by aerobic metabolism and other sources (*1*). The aberrant bases produced by hydrolysis or reactive oxygen species are primarily restored by the base excision repair (BER)[1] pathway that involves sequential actions of damage-specific DNA glycosylase, apurinic/apyrimidinic (AP) endonuclease, gap-filling DNA polymerase, and DNA ligase (*2*). In the study reported in the accompanying paper (*3*), we have partially purified and characterized an enzyme from rat liver that removes 5-formyluracil (fU), a potentially genotoxic lesion (*4*−*14*), from DNA. The enzyme [5-formyluracil−DNA glycosylase (FDG)] was a monofunctional DNA glycosylase and excised fU from single-stranded (ss) as well as double-stranded (ds) DNA. Interestingly, FDG also excised uracil (U), 5-hydroxyuracil (hoU), and 5-hydroxymethyluracil (hmU) from both ssDNA and dsDNA. The results of the competitive inhibition experiments showed that all of the activities for fU, U, hoU, and hmU resided on the same enzyme.

Mammalian cells contain two enzymes that remove U from ssDNA as well as dsDNA, i.e., uracil−DNA glycosylase (UNG) (*15*, *16*) and single-strand-selective monofunctional uracil−DNA glycosylase (SMUG1) (*17*). Recent biochemical analyses of the cryptic repair activity for U in UNG nullizygous mice suggest that SMUG1 might be a backup enzyme for UNG concerning the antimutagenic function (*18*, *19*). In the accompanying paper (*3*), we have tentatively concluded that FDG is a rat homologue of SMUG1 since the enzyme recognized base damage both in ssDNA and in dsDNA and the activity was effectively neutralized by hSMUG1 antibodies. However, the reported damage specificity of human SMUG1 (hSMUG1) other than for U is rather controversial. Two groups have shown that hSMUG1 releases hmU from DNA (*20*, *21*), whereas another has concluded that hmU-excising activity in HeLa cells is distinct from

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[1] Abbreviations: BER, base excision repair; U, uracil; hoU, 5-hydroxyuracil; fU, 5-formyluracil; hmU, 5-hydroxymethyluracil; hoC, 5-hydroxycytosine; fC, 5-formylcytosine; Tg, thymine glycol; dhT, 5,6-dihydrothymine; 8oxoG, 7,8-dihydro-8-oxoguanine; 7mG, 7-methylguanine; AP, apurinic/apyrimidinic; FDG, 5-formyluracil−DNA glycosylase; r(h)SMUG1, rat (human) single-strand-selective monofunctional uracil−DNA glycosylase; NTH1, endonuclease III homologue; NEH1/NEI1 and NEH2/NEI2, endonuclease VIII homologues; Endo III, endonuclease III; OGG1, 8-oxoguanine−DNA glycosylase; Ec(h)UNG, *Escherichia coli* (human) uracil−DNA glycosylase; Ugi, uracil−DNA glycosylase inhibitor; TDG, thymine−DNA glycosylase; EcMUG, *E. coli* mismatched uracil glycosylase; ss, single stranded; ds, double stranded.

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Table 1: Oligonucleotide Substrates Used in This Study

substrate	damage (X)	sequence (5′ → 3′)	paired base[a]
19T	T	ACAGACGCCAXCAACCAGG	ss, A, G
19TG	Tg	ACAGACGCCAXCAACCAGG	ss, A
19DHT	dhT	ACAGACGCCAXCAACCAGG	ss, A
19U	U	ACAGACGCCAXCAACCAGG	ss, A, G
25TpG	T	CATCGATAGCATCCGXGACAGGCAG	G[b]
25HOC	hoC	GAAACACTACTATCAXGGAAGAGAG	ss, A, G
25HOU	hoU	GAAACACTACTATCAXGGAAGAGAG	ss, A, G
25HMU	hmU	GAAACACTACTATCAXGGAAGAGAG	ss, A, G
25FU	fU	CATCGATAGCATCCGXCACAGGCAG	ss, A, G
25FC	fC	CATCGATAGCATCCGXCACAGGCAG	ss, A, G
25MC	mC	CATCGATAGCATCCGXCACAGGCAG	ss, G
25C	C	CATCGATAGCATCCGXCACAGGCAG	G
25OG	8oxoG	CATCGATAGCATCCTXCCTTCTCTC	C
25MG	7mG	CATCGATAGCATCCTXCCTTCTCTC	C
25FP	FapyG	CATCGATAGCATCCTXCCTTCTCTC	C

[a] The base opposite the lesion (X). ss = single-stranded DNA. [b] In CpG.

hSMUG1 (*22*). Moreover, in contrast to our data for FDG (*3*), hSMUG1 exhibited no detectable activity for hoU (*21*). Also, purified rat SMUG1 (rSMUG1) and hSMUG1 have not been tested for fU in the previous study (*3*).

In view of the absence of activity data of SMUG1 for fU and the apparently conflicting results on the activity for other oxidized base lesions (hmU and hoU), we have carried out extensive biochemical analyses of hSMUG1, rSMUG1, and the HeLa cell extract for repair of oxidized bases and U. We report here that both purified hSMUG1 and rSMUG1 excise fU, hmU, and hoU as well as U from dsDNA and ssDNA, showing that FDG is indeed a rat homologue of SMUG1. The activities of hSMUG1 and the HeLa cell extract for fU, hmU, and hoU vary in parallel depending on salt concentrations, and those of the extract were effectively neutralized by hSMUG1 antibodies, indicating that the observed cellular activities for fU, hmU, and hoU are mostly attributable to hSMUG1. Finally, hSMUG1 removes damaged bases from Fenton-oxidized calf thymus DNA, generating AP sites. These data indicate a dual role of SMUG1 as a backup enzyme for UNG and a primary repair enzyme for a subset of oxidized pyrimidine bases such as fU, hmU, and hoU.

EXPERIMENTAL PROCEDURES

Oligonucleotides. Oligonucleotide substrates used for repair assays were summarized in Table 1. Oligonucleotides containing fU, hmU, hoU, U, thymine glycol (Tg), 7,8-dihydro-8-oxoguanine (8oxoG), and 7-methylguanine (7mG) have been described in the accompanying paper (*3*). The synthesis of oligonucleotides containing 5,6-dihydrothymine (dhT) (*23*) and 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (FapyG) (*24*, *25*) has been also reported previously. 5-Formylcytosine (fC) and 5-methylcytosine (mC) were introduced using the corresponding special phosphoramidite monomers (*26*). 5-Hydroxycytosine (hoC) was site-specifically incorporated into the oligonucleotide by the DNA polymerase reaction in the presence of 5-hydroxy-2′-deoxycytidine 5′-triphosphate (TriLink BioTechnologies) (*27*). The strand containing hoC was separated from the template by denaturing PAGE, extracted from the gel, and purified by a Sep-Pak cartridge (Waters). The oligonucleotides containing base lesions were 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, purified by a Sep-Pak cartridge, and used as single-stranded (ss) substrates. Alternatively, they were annealed to matched or mismatched complementary strands and used as double-stranded (ds) substrates.

Cloning of hSMUG1 and rSMUG1 cDNA. On the basis of the published sequence (*17*), hSMUG1 cDNA was amplified from the human liver cDNA library (Nippon Gene) by the polymerase chain reaction (PCR). Since the sequence of rSMUG1 cDNA has not been reported, it was PCR-amplified from rat kidney cDNA prepared by the standard method (*28*) using several primer sets adapted from the cDNA sequences of mouse and human SMUG1 (*17*, *18*). A primer set of MM1 (5′-AAGGAAGCGGAAGCTGCATGG) and MM5 (5′-TACGTTGTGACCGACCCCAGAG) yielded a DNA band with an expected size of the rSMUG1 gene (1 kbp). The amplified DNA fragments (hSMUG1 and rSMUG1 cDNA) were ligated into the pGEM-T Easy vector (Promega) by TA cloning. *Escherichia coli* XL1-Blue cells were transformed by the vectors, and the plasmids containing the hSMUG1 and rSMUG1 genes were purified and sequenced. The hSMUG1 and rSMUG1 genes in the plasmids were PCR-amplified using primers containing *Bam*HI and *Xho*I sites. The PCR product was digested with *Bam*HI and *Xho*I and ligated into the *Bam*HI/*Xho*I site of pGEX4T-1 (Amersham Biosciences) so that hSMUG1 and rSMUG1 could be expressed as an N-terminal glutathione *S*-transferase (GST) fusion protein for affinity purification. The recombinant plasmids for hSMUG1 and rSMUG1 cDNA were designated pGEX-hSMUG1 and pGEX-rSMUG1, respectively. *E. coli* BL21(DE3) was transformed with pGEX-hSMUG1 or pGEX-rSMUG1. The original sequence of the inserts was confirmed by sequencing the inserted regions of pGEX-hSMUG1 and pGEX-rSMUG1purified from *E. coli* BL21(DE3).

Expression and Purification of hSMUG1 and rSMUG1. hSMUG1 and rSMUG1 were purified by the same procedure. *E. coli* BL21(DE3) harboring pGEX-hSMUG1 (or pGEX-rSMUG1 for rSMUG1) was grown in LB media (500 mL) containing ampicillin (50 μg/mL) at 37 °C until the OD_{600} reached 0.6. After addition of isopropyl β-D-thiogalactopyranoside (final concentration 0.5 mM), the cell culture was continued at 20 °C for 8 h. The following procedures were performed at 4 °C or on ice. Cells (ca. 1.8 g) were harvested by centrifugation, resuspended in 5 mL of buffer A [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 0.02% Triton X-100], and disrupted by sonication (4 × 15 s at 73 W) on ice using a ultrasonic disruptor UR-200P (Tomy Seiko). The cell lysate was centrifuged (14000*g*, 20 min), and the supernatant was loaded onto a glutathione−Sepharose 4B column (Amersham Biosciences, 6 mL) preequilibrated with buffer A. The column was washed with buffer A (50 mL), and the fusion protein was eluted with buffer A containing 15 mM glutathione (125 mL). The eluted fraction was dialyzed against buffer B [20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM EDTA, 0.02% Triton X-100, 6 mM 2-mercaptoethanol]. The GST-hSMUG1 fusion protein was treated with thrombin (3.9 units/mg of fusion protein, Amersham Biosciences) at 4 °C for 16 h. The digested sample was applied to a glutathione−Sepharose 4B column (13 mL) preequilibrated with buffer C [20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 6 mM 2-mercaptoethanol, 10% glycerol]. The column was eluted with buffer C (40 mL), and fractions were collected every 5 mL. The fractions containing

hSMUG1 without the GST tag were identified by SDS−PAGE analysis and pooled. The pooled fraction was dialyzed against buffer D [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 6 mM 2-mercaptoethanol, 10% glycerol] for 3 h and loaded onto a HiTrap SP-Sepharose HP column (Amersham Biosciences, 1 mL). The column was washed with buffer D (20 mL) and eluted with a linear gradient of NaCl (50−500 mM) in buffer D (20 mL). The fractions containing hSMUG1 (around 300 mM NaCl, total 3 mL) were identified by SDS−PAGE analysis, combined, dialyzed against buffer E [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2 mM dithiothreitol (DTT), 0.005% Triton X-100, 50% glycerol] overnight, and stored at −20 °C. The protein concentration was determined with a BCA protein assay kit (Pierce) using BSA as a standard. The purified hSMUG1 and rSMUG1 proteins contained two extra amino acid residues (Gly-Ser) from the GST linker on the N terminus. SDS−PAGE analysis of the purified hSMUG1 and rSMUG1 proteins showed a single band with an apparent molecular mass of about 31 kDa, which agreed with the size expected from cDNA.

Analysis of Salt Concentration Effects on the Activity of SMUG1 and UNG. ssDNA and dsDNA substrates (50 fmol) containing U, hoU, hmU, or fU were incubated with hSMUG1 or rSMUG1 (1 and 5 ng for ssDNA and dsDNA substrates, respectively) in 25 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 2 mM DTT, 0.1 mg/mL BSA, and 3−150 mM NaCl (total volume 10 μL) at 37 °C for 5 min. The reaction was terminated by the addition of 1 M NaOH (final concentration 0.1 M). The sample was heated at 70 °C for 5 min to cleave AP sites, neutralized with 1 M acetic acid, and mixed with gel loading buffer (0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM EDTA, and 98% formamide). After being heated at 70 °C for 5 min, products were separated by 16% denaturing polyacrylamide gel electrophoresis (PAGE), and the radioactivity in the gel was analyzed on a phosphorimaging analyzer, Fuji BAS2000. Alternatively, the gel was autoradiographed at −80 °C. The activity of *E. coli* UNG (EcUNG, USB) for ssDNA and dsDNA substrates containing U was assayed in a similar manner except that the amount of EcUNG was 0.02 unit and the buffer composition was 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 3−150 mM NaCl.

Analysis of Damage Specificity of SMUG1. ssDNA and dsDNA substrates containing various base lesions listed in Table 1 were incubated with hSMUG1 or rSMUG1 (10 ng), and the products were analyzed as described above. The NaCl concentration of the reaction buffer was 3 mM.

Comparison of Excision Activities of hSMUG1 for U, hoU, hmU, and fU. ssDNA and dsDNA substrates containing U, hoU, hmU, or fU were incubated with varying amounts of hSMUG1 (0−10 ng), and the products were analyzed as described above. The NaCl concentrations in the reaction buffer were 3, 20, and 50 mM. Enzymatic parameters for U·G and fU·A in dsDNA were determined by incubating hSMUG1 (1 ng) and varying concentrations of substrates at 37 °C for 3 min. Parameters (k_{cat} and K_m) were evaluated from S−V plots using a hyperbolic curve-fitting program.

Activity Assays with HeLa Cell Extracts. The cell extract was prepared from confluent HeLa cells. All procedures were performed on ice or at 4 °C. The cell pellet (ca. 1.7 g) was suspended in 3 volumes of 50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 5 mM $Mg(CH_3COO)_2$, 3 mM 2-mercaptoethanol, 300 mM KCl, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. The cells were disrupted with 30 strokes of a tight-fitting Dounce homogenizer and centrifuged at 100000*g* for 30 min. The supernatant was dialyzed against 25 mM Tris-HCl (pH 7.7), 20 mM NaCl, and 2 mM DTT. The extract was stored as aliquots at −80 °C. The protein concentration of the extract was determined with the BCA protein assay kit. The extract (0.5 or 2 μg as protein) was preincubated without or with 1.5 units of Ugi (New England BioLabs) in 25 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 2 mM DTT, and 0.1 mg/mL BSA at 37 °C for 10 min. dsDNA substrates (50 fmol) containing U, hoU, hmU, or fU were incubated with the cell extract at 37 °C for 10 min (0.5 μg of protein) or 30 min (2 μg of protein). The final volume of the reaction mixture was 10 μL, and the buffer composition was the same as that for the activity assay with hSMUG1 (NaCl concentration 2−160 mM). The reaction was terminated by the addition of 1 M NaOH, and products were analyzed as described for hSMUG1 protein. The addition of extra Ugi (up to 5 units) did not change the residual uracil-excision activity from the ssDNA substrate in the nuclear extract, indicating that the amount of Ugi used was saturating under the present conditions. To assess the effects of hSMUG1 antibodies, the cell extracts (2 μg as protein) were incubated without or with hSMUG1 antibodies [1 μL of antiserum (*3*)] for 10 min at 20 °C. After incubation, the activity for dsDNA containing fU·A, hmU·A, hoU·A, and Tg·A was assayed as described above.

Quantitation of Enzyme Sensitive Sites in Fenton-Treated DNA by the ARP Assay. DNA containing oxidative lesions was prepared by the Fenton reaction as follows. Genomic DNA extracted from calf thymus was incubated with 10 μM H_2O_2 and 10 μM $FeSO_4$ in 10 mM Tris-HCl (pH 7.5) at 37 °C for 10 min. The reaction was terminated by the addition of 2,2,6,6-tetramethylpiperidineoxyl (final concentration 15 mM; Wako). DNA was purified by ethanol precipitation and dissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The oxidized DNA (10 μg in 60 μL of reaction buffer) was incubated with varying amounts of hSMUG1 (0−1 μg) or EcUNG (0−3 units) at 37 °C for 1 h in 25 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 2 mM DTT, and 50 mM NaCl (hSUMG1) or 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10 mM NaCl (EcUNG). In separate experiments, the DNA was also treated with a saturating amount (1 μg of enzyme/10 μg of DNA) of *E. coli* endonuclease III (Endo III) (*23*, *24*) and human 8-oxoguanine glycosylase (hOGG1) (*25*) in a similar manner. AP sites formed by the Fenton reaction and subsequent treatment with the repair enzymes were tagged by the aldehyde reactive probe (ARP; Dojindo) (*29*, *30*) and quantified by the method of Nakamura et al. (*31*, *32*) with some modifications. Further details of the quantitation of AP sites and base damage by the ARP assay in combination with Endo III and hOGG1 treatment will be published elsewhere.

RESULTS

Amino Acid Sequence of rSMUG1. Since the rSMUG1 cDNA that likely encoded FDG was not reported, it was cloned from rat kidney cDNA. The rSMUG1 cDNA (GenBank accession number: AY191521) encoded a protein of 278 amino acids (Figure 1). The deduced amino acid sequences of rSMUG1 (this study) and hSMUG1 (*17*) were

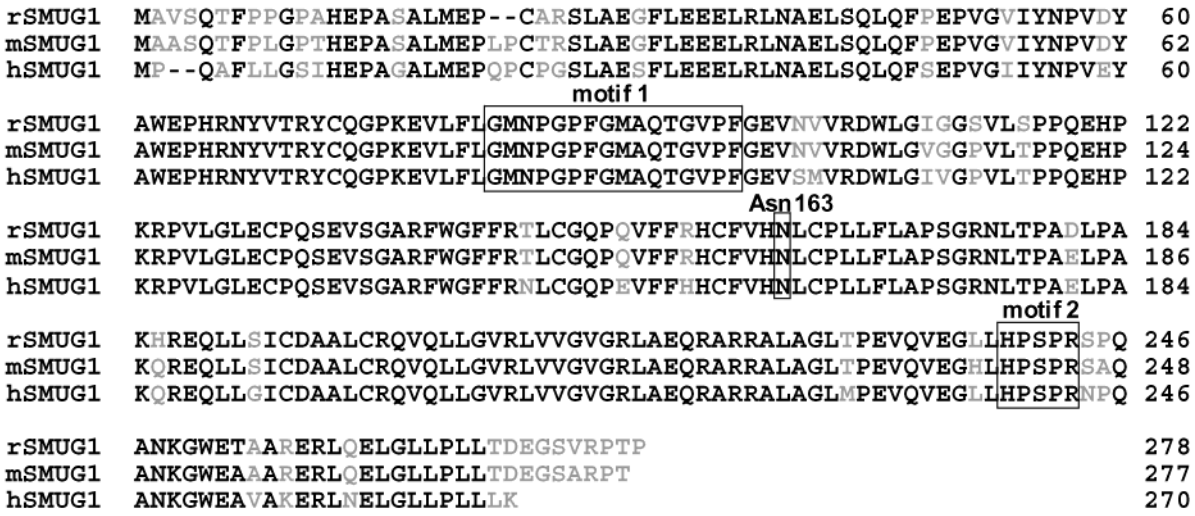

FIGURE 1: Alignment of deduced amino acid sequences of rat (r), mouse (m), and human (h) SMUG1. Amino acid residues presumably involved in damage recognition and catalysis [motifs 1 and 2 and Asn163 (for rSMUG1)] are boxed. Amino acid residues that are not conserved in rat, mouse, and human are shown in gray. GenBank accession numbers: AY191521 (rSMUG1), AK004735 (mSMUG1), and NM014311 (hSMUG1).

84% identical, and those of rSMUG1 (this study) and mSMUG1 (*18*) were 94% identical. Furthermore, the key amino acid residues (Figure 1, motifs 1 and 2 and Asn163) presumably involved in damage recognition and catalysis (*17*, *33*) were also conserved in rSMUG1.

Effects of Salt Concentrations on U-Excising Activity of SMUG1. The substrates containing ssU, U·A, and U·G (Table 1) were incubated with hSMUG1 in the presence of varying concentrations of NaCl (3−150 mM). After cleavage of the resulting AP sites by NaOH treatment, products (a mixture of β- and δ-elimination products) were analyzed by PAGE (Figure 2A−C). hSMUG1 exhibited distinct salt concentration dependences for ssU, U·A, and U·G substrates. The activity for ssU was the highest at 3 mM NaCl and declined sharply with the increasing concentration of NaCl (Figure 2D). Conversely, the activity for U·A and U·G pairs favored higher salt concentrations than for ssU and showed fairly broad optima around 20 mM and 50 mM NaCl for U·A and U·G pairs, respectively. rSMUG1 exhibited similar salt concentration dependences for the substrates containing ssU, U·A, and U·G (data not shown). The salt concentration effects on the activity of EcUNG for the same substrates (ssU, U·A, U·G) were quite different from those for hSMUG1 and rSMUG1, showing narrow optima around 10 mM NaCl for all of the substrates (Figure 2E).

Damage Specificity of SMUG1. The damage specificity of hSMUG1 was determined using the base lesions in ssDNA and dsDNA shown in Table 1. The substrate (50 fmol) was incubated with an excessive amount of hSMUG1 (10 ng, ca. 330 fmol) in the presence of 3 mM NaCl, and products were analyzed by PAGE. hSMUG1 excised hmU, fU, and hoU in ssDNA and dsDNA substrates (Figure 3A−C). hSMUG1 recognized the lesions paired with both A and G. The lesions recognized were all uracil derivatives bearing an oxidized group (−CH_2OH, −CHO, and −OH) at the C5 position (Figure 4). In contrast, hSMUG1 did not excise intact cytosine, cytosine bearing an oxidized group (hoC and fC) or a methyl group (mC) at the C5 position, C5−C6 double bond saturated thymine (Tg and dhT), and intact thymine in an A·T pair and a G·T mispair (Figure 4). Typical gel data for hoC, fC, and Tg are shown in Figure 3D−F, respectively. Purine lesions such as 8oxoG, FapyG, and 7mG were not the substrates for hSMUG1 either. The salt concentration dependences for hmU, fU, and hoU in ssDNA and dsDNA were essentially similar to those for U (Figure 2F−H). The damage specificity of rSMUG1 was similarly determined. Like hSMUG1, rSMUG1 recognized fU, hmU, hoU, and U in ssDNA and dsDNA (Figure 3G−J) but not other lesions listed in Table 1. The salt concentration dependences for the recognized lesions were virtually the same as those of hSMUG1 (data not shown).

Comparison of the Activities of hSMUG1 for U, hoU, hmU, and fU. ssDNA and dsDNA substrates containing U, hoU, hmU, and fU were incubated with the varying amounts of hSMUG1 (0−10 ng) in the presence of 3, 20, or 50 mM NaCl. After NaOH treatment, the amount of nicked products (i.e., excised bases) was quantified by PAGE analysis. Panels A−C of Figure 5 show typical data obtained for sshmU, hmU·A, and hmU·G, respectively, where the amount of nicked products was plotted against that of hSMUG1. Similar plots were obtained for U, hoU, and fU (data not shown). From the slope of the linear part of the plot (0.1−0.5 ng of hSMUG1), the activity for individual substrates was calculated (Figure 5D−F). Although there were some exceptions for hoU·A, the general order of the preference for ssDNA and dsDNA substrates was the following: ssDNA > dsDNA (G pair) ≈ dsDNA (A pair) at 3 mM NaCl (Figure 5D); ssDNA ≈ dsDNA (G pair) ≈ dsDNA (A pair) at 20 mM NaCl (Figure 5E); dsDNA (G pair) > dsDNA (A pair) > ssDNA at 50 mM NaCl (Figure 5F).

hoU is derived from cytosine glycol via deamination and dehydration and exists as an hoU·G pair in cellular DNA, whereas hmU and fU are derived from oxidation of the methyl group of thymine and exist as hmU·A and fU·A pairs, respectively, in cellular DNA. Accordingly, hoU·G, hmU·A, and fU·A pairs in dsDNA and hoU, hmU, and fU in ssDNA are potentially important substrates under physiological conditions. At the low salt concentration (3 mM NaCl), hSMUG1 exhibited a slight preference for hoU (and U) over hmU and fU in the ssDNA substrates (Figure 5D). At the

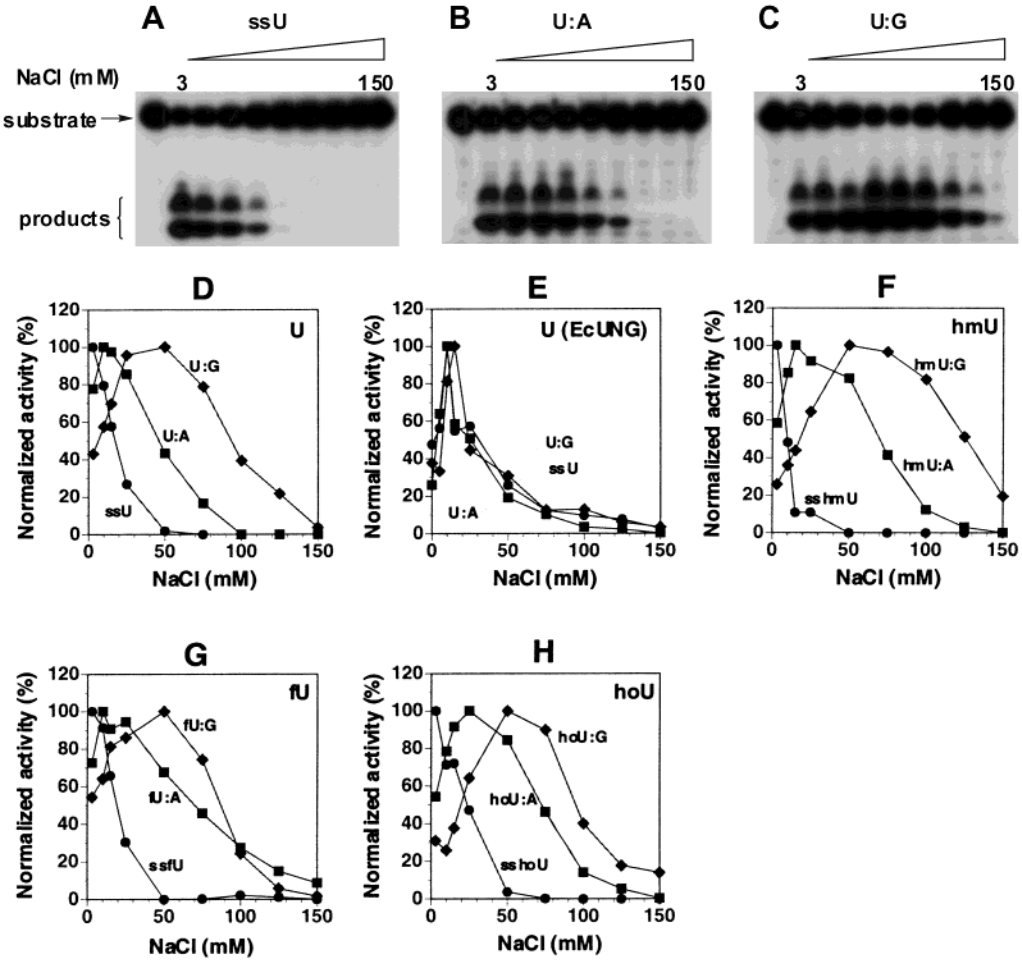

FIGURE 2: Salt concentration dependences of the activities of hSMUG1 for U, hmU, fU, and hoU. ssDNA and dsDNA substrates containing U (50 fmol, the U-containing strand was 5′-^{32}P labeled) were incubated with hSMUG1 (1 and 5 ng for ssDNA and dsDNA, respectively) in the presence of varying concentrations of NaCl. After incubation, AP sites resulting from excision of U were cleaved by NaOH treatment, and products (a mixture of β- and δ-elimination products) were separated by denaturing PAGE. Gel data for (A) ssDNA containing U (ssU), (B) dsDNA containing a U·A pair, and (C) dsDNA containing a U·G mispair. The leftmost lane in each gel shows untreated substrates. The NaCl concentrations were 3, 10, 15, 25, 50, 75, 100, 125, and 150 mM from left to right lanes. (D) Salt concentration dependences of the U-excising activity of hSMUG1 and (E) those of EcUNG. The activities of hSMUG1 and EcUNG were determined as described above, and the normalized activities were plotted against the salt concentration. Salt concentration dependences of the excision activity of hSMUG1 for (F) hmU, (G) fU, and (H) hoU in ssDNA and dsDNA (damage paired with A or G). The activities for hmU, fU, and hoU were determined as described for U, and the normalized activities were plotted against the salt concentration. Symbols: ssDNA (●); dsDNA containing a damage·A pair (■); dsDNA containing a damage·G pair (◆).

high salt concentration (50 mM NaCl), hoU·G (and U·G) was the notably preferred substrate over hmU·A and fU·A in the physiologically relevant dsDNA substrates (Figure 5F).

Interestingly, the activity of hSMUG1 for U, hoU, hmU, and fU in dsDNA was increased when the base opposite a lesion was changed from a matched one (A) to a mismatched one (G) (50 mM NaCl, Figure 5F). The fold increase in the activity was 4.6 (U), 1.9 (hoU), 4.1 (hmU), and 1.9 (fU). This result may account for the reported preference of partially purified human hmU−DNA glycosylase (*22*) and the HeLa cell extract (*34*) for mismatched hmU (hmU·G) over matched hmU (hmU·A), though the authors claimed that human hmU−DNA glycosylase was distinct from hSMUG1.

Activities for U, hoU, hmU, and fU in HeLa Cell Extracts. The activity for U, hoU, hmU, and fU in the HeLa cell extract was assayed using dsDNA substrates containing the physiologically relevant base pairs [U (deaminated C)·G, hoU·G, hmU·A, and fU·A]. The substrates were incubated with the cell extract (0.5 or 2 μg as protein) in the presence of varying concentrations of NaCl. Preincubation of the extract with a saturating quantity of Ugi almost abolished the ssU-excising activity. The residual activity for ssU was about 0.5% of that observed in the absence of Ugi (data not shown). The activity for a U·G pair in dsDNA was also decreased by Ugi when assayed with 0.5 μg of protein (Figure 6A). However, unlike ssU, there was a significant residual activity for a U·G pair. At the optimal salt concentration for the residual activity (60 mM NaCl), approximately 70% of the cellular U·G activity was Ugi-sensitive, therefore attributable to human UNG (hUNG), and the rest (30%) was Ugi-insensitive. To compare the activities for U, hoU, hmU, and fU quantitatively, dsDNA substrates were incubated with 2 μg of the cell extract (Figure 6B−E). The extract exhibited activities for hoU·G, hmU·A, and fU·A as well as U·G, and the activities for the former three substrates were not sensitive to Ugi. The profiles of salt concentration dependence for U·G (+Ugi), hoU·G, hmU·A, and fU·A (Figure 6B−E) closely resembled those of hSMUG1 (Figure 2). Furthermore, the magnitude relation of the cellular activities (U·G, hoU·G > hmU·A, fU·A) at relatively high salt conditions (e.g.,

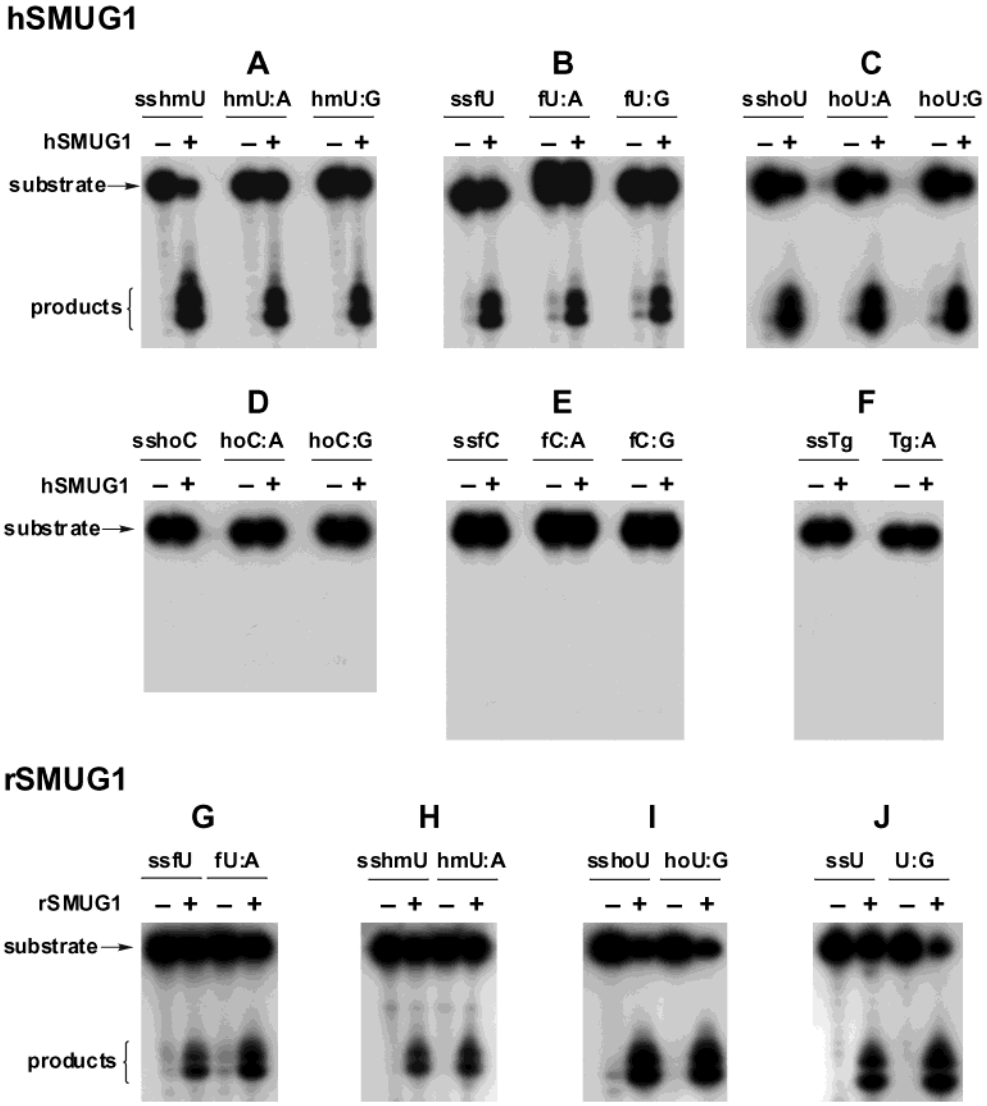

FIGURE 3: Analysis of damage specificity of hSMUG1 and rSMUG1. ssDNA and dsDNA substrates (damage paired with A or G) (50 fmol) were incubated with hSMUG1 or rSMUG1 (10 ng, ca. 330 fmol) in the presence of 3 mM NaCl for 5 min. After incubation, AP sites resulting from excision of damage were cleaved by NaOH treatment, and products (a mixture of β- and δ-elimination products) were separated by denaturing PAGE. PAGE data for (A) hmU, (B) fU, (C) hoU, (D) hoC, (E) fC, and (F) Tg with hSMUG1 and those for (G) fU, (H) hmU, (I) hoU, and (J) U with rSMUG1.

50 mM NaCl) correlated fairly well with those of hSMUG1 (Figure 5F). When the cell extract was pretreated with hSMUG1 antibodies, the activities for fU·A and hmU·A were completely inhibited, while that for Tg·A was not affected (Figure 7). Interestingly, the activity for hoU·G was also effectively neutralized by hSMUG1 antibodies, and the residual activity for hoU·G was only about 10% of that without antibody. The results of salt concentration effects and antibody inhibition show that hSMUG1 accounts for the major activities for fU, hmU, and hoU in HeLa cells.

Excision of Base Damage from Oxidized Calf Thymus DNA by hSMUG1. To further examine the activity of hSMUG1 for oxidized base lesions, calf thymus DNA was treated by the Fenton reaction and incubated with hSMUG1. The AP sites resulting from removal of oxidized bases were tagged by the aldehyde reactive probe (ARP) and quantified by an ELISA-like assay as described in the Experimental Procedures. Both intact and 3′-nicked AP sites (products formed by β-elimination) can be measured by the ARP assay (*29*−*32*). The amount of AP sites increased with the increasing amount of hSMUG1 and reached a plateau with 1 μg of hSMUG1 (Figure 8). Conversely, similar treatment with EcUNG did not result in any detectable increase in the amount of AP sites (Figure 8), indicating that the damage excised by hSMUG1 was not U. Table 2 summarizes the amount of AP sites formed by the Fenton reaction and those (= enzyme sensitive sites) by the subsequent treatment with a saturating quantity of hSMUG1, Endo III, and hOGG1 (all 1 μg of enzyme for 10 μg of oxidized DNA). hSMUG1 excised a small but significant amount of damaged bases from the oxidized DNA, which corresponded to 13% of the Endo III-sensitive sites (mostly oxidized pyrimidines) and to 11% of hOGG1-sensitive sites (mostly oxidized purine damage), respectively. In view of the substrate specificity of hSMUG1 (Figure 4) and the result of the ARP assay with EcUNG (Table 2), the released bases were not U, C5−C6 oxidized/saturated thymine (Tg and dhT), C5-oxidized cytosine (hoC and fC), or oxidized purines (8oxoG and FapyG) but were likely uracil derivatives bearing an oxidized group at C5.

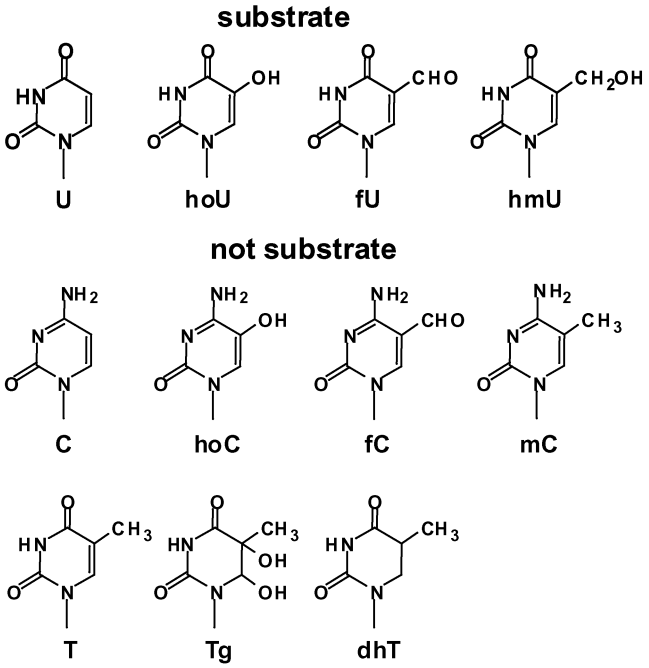

FIGURE 4: Base lesions recognized by hSMUG1 and rSMUG1. The structures of pyrimidine lesions that were the substrates and not the substrates of hSMUG1 and rSMUG1 are shown. The damage specificity of hSMUG1 and rSMUG1 was determined using the substrates shown in Table 1. The substrates recognized by hSMUG1 and rSMUG1 were uracil or uracil derivatives bearing an oxidized group at the C5 position. The purine lesions (7mG, 8oxoG, and FapyG) that were not substrates for hSMUG1 and rSMUG1 are not shown.

DISCUSSION

In this study, it has been shown that purified hSMUG1 and rSMUG1 excise fU, hmU, and hoU from ssDNA and dsDNA. Conversely, Tg, 8oxoG, 7mG, and T·G mismatches in CpG and non-CpG contexts are not the substrate of hSMUG1 and rSMUG1 (Figures 3 and 4). Thus, the damage specificity of rSMUG1/hSMUG1 is fully consistent with the results of FDG reported in the accompanying paper (*3*). In addition, the profiles of the salt concentration dependence of rSMUG1/hSMUG1 for ssDNA (ssU, sshoU, sshmU, ssfU) and dsDNA (U·G and hoU·G) (Figure 2) agree well with those of FDG (*3*). It is noted that the optimal salt concentration of rSMUG1/hSMUG1 for dsDNA substrates containing fU·A and hmU·A (NaCl = 20 mM, Figure 2) was somewhat lower than that of FDG (NaCl = 50 mM) for unknown reasons (*3*). Considering the extent of FDG purification (160-fold), the FDG preparation might have contained some proteins or contaminants that might alter the salt effects.

The HeLa cell extracts contained Ugi-resistant damage-excising activities for U, hoU, hmU, and fU in dsDNA (Figure 6B−E, left panels), which were also recognized by hSMUG1 (Figure 4). The salt concentration dependences of these cellular activities (Figure 6B−E, right panels) closely resembled those of hSMUG1 (Figure 2). Also, the magnitude relation of the cellular activities for U·G, hoU·G, hmU·A, and fU·A (=7.8:5.6:1:1; data standardized to hmU·A at 50 mM NaCl in Figure 6B−E) correlated fairly well to those of hSMUG1 (=5.1:6.7:1:0.5, Figure 5F). More importantly, the activities for fU·A, hmU·A, and hoU·G in the cell

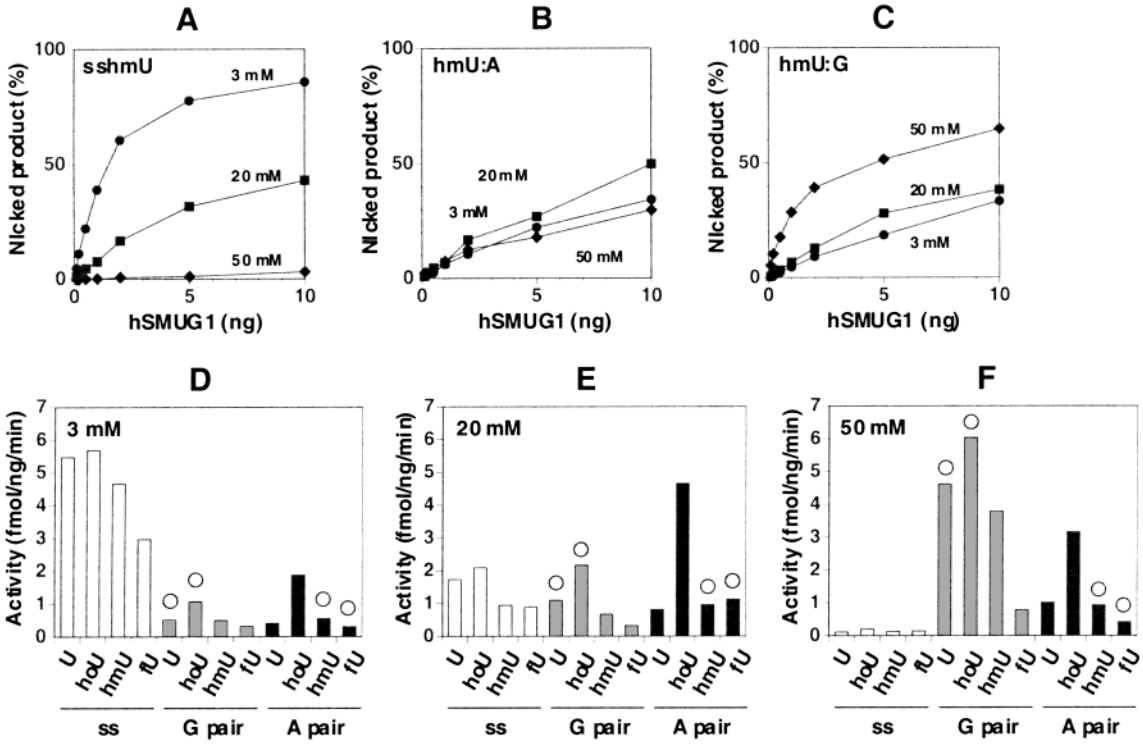

FIGURE 5: Comparison of the activities of hSMUG1 for U, hoU, hmU, and fU. (A) Typical data showing the relationship between the amount of hSMUG1 and that of nicked products for ssDNA containing hmU (sshmU), (B) those for dsDNA containing an hmU·A pair, and (C) those for dsDNA containing an hmU·G mispair. The reactions were performed with 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng of hSMUG1 for 5 min in the presence of 3 mM (●), 20 mM (■), and 50 mM (◆) NaCl. Similar relationships were also determined for U, hoU, and fU (data not shown). The activities for U, hoU, hmU, and fU in ssDNA and dsDNA (damage paired with G or A) were calculated from the initial slopes of the plots, such as panels A−C. The activities in the presence of (D) 3 mM NaCl, (E) 20 mM NaCl, and (F) 50 mM NaCl were plotted against the substrates. The circle on the bar indicates the physiologically relevant dsDNA substrates formed by oxidative stress (hoU·G, hmU·A, fU·A) or hydrolytic deamination (U·G).

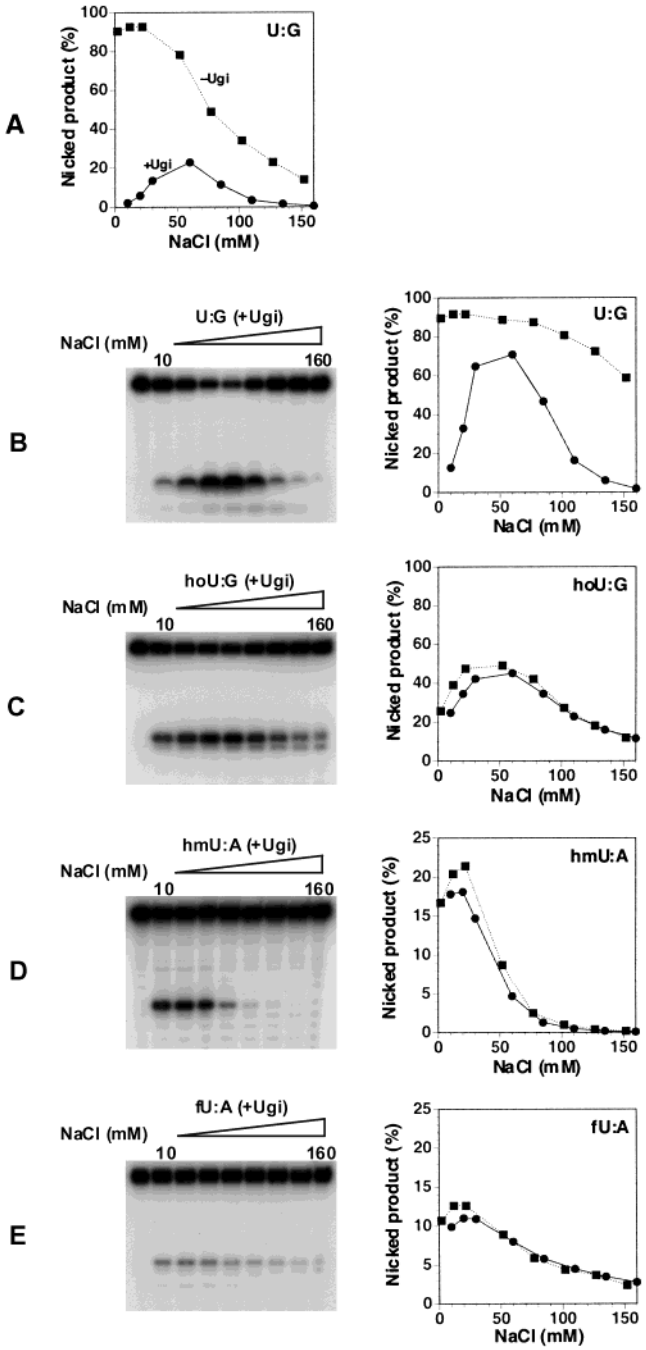

FIGURE 6: Analysis of cellular activities for U, hoU, hmU, and fU. (A) The dsDNA substrate containing U·G was incubated with the HeLa cell extracts (0.5 μg as protein) that were preincubated without or with Ugi. The concentrations of NaCl were 2, 12, 22, 52, 77, 102, 127, and 152 mM for the reaction mixtures without Ugi and 10, 20, 30, 60, 85, 110, 135, and 160 mM for the reaction mixtures with Ugi. The amount of nicked products was analyzed by PAGE and plotted against the NaCl concentration. Symbols: cell extracts without Ugi treatment (■); those with Ugi treatment (●). dsDNA substrates containing (B) U·G, (C) hoU·G, (D) hmU·A, and (E) fU·A were incubated with the HeLa cell extracts (2 μg as protein), and products were analyzed as described in panel A. The left panels show the PAGE data for the cell extracts treated with Ugi. The leftmost lane in each gel shows the untreated substrate, and the NaCl concentration in the reaction mixture was increased from 10 to 160 mM (left to right lanes). The right graphs show the plots of the amount of nicked products against the NaCl concentration. Symbols: cell extracts without Ugi treatment (■); those with Ugi treatment (●).

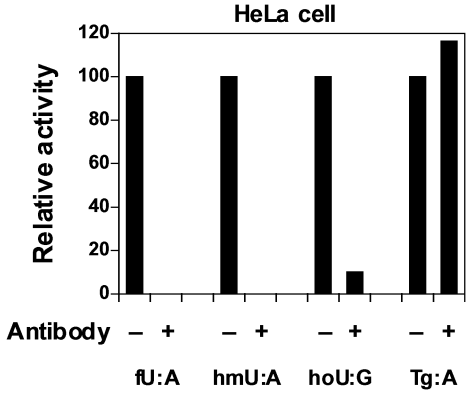

FIGURE 7: Inhibition of cellular activities for fU, hmU, and hoU by hSMUG1 antibodies. The HeLa cell extracts (2 μg as protein) were incubated without or with hSMUG1 antibodies (1 μL of antiserum) for 10 min at 20 °C. After incubation, the extracts were incubated with dsDNA substrates containing fU·A, hmU·A, hoU·A, and Tg·A, and products were quantified by denaturing PAGE. The activity for individual substrates was standardized to that without hSMUG1 antibodies and plotted against the type of substrates.

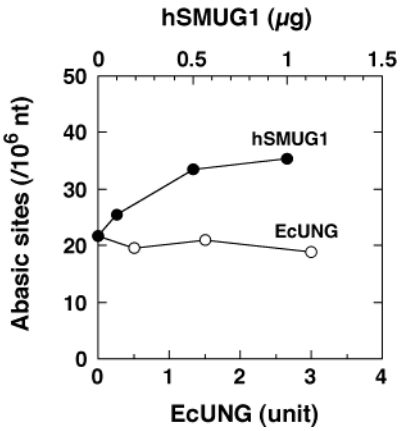

FIGURE 8: Generation of AP sites in oxidized calf thymus DNA by hSMUG1. Calf thymus DNA was oxidized by the Fenton reaction and treated with varying amounts of hSMUG1 or EcUNG. The amount of AP sites [per 10^6 nucleotides (nt)] resulting from removal of damaged bases by the enzymes was quantified by the aldehyde reactive probe (ARP) assay as described in the Experimental Procedures and was plotted against the amount of hSMUG1 (●) and EcUNG (○) used in the treatment. The top and bottom abscissa axes are for hSMUG1 and EcUNG, respectively.

Table 2: Enzyme Sensitive Sites (ESS) in Calf Thymus DNA Treated by the Fenton Reaction

enzyme[a]	detectable damage	ESS (per 10^6 nt)[b]
Endo III	oxidized pyrimidine	109
hOGG1	oxidized purine	131
hSMUG1	uracil/uracil with a C5-oxidized group	14
EcUNG	uracil	<1.5
none	AP sites	22

[a] After the Fenton reaction, DNA was treated by a saturating amount of the indicated enzyme, and the resulting AP sites (both intact and 3′-nicked AP sites) were quantified by the ARP assay as described in the Experimental Procedures. [b] Enzyme sensitive sites per 10^6 nucleotides (nt).

extracts were effectively neutralized by hSMUG1 antibodies (Figure 7). These results indicate that hSMUG1 mostly, if not all, accounts the cellular activities for fU·A, hmU·A, and hoU·G, hence substantially contributing to repair of the lesions in cells. This notion is also consistent with the previous studies where the activities for hmU and fU in cells were individually assessed using hSMUG1-neutralizing antibodies (*3*, *21*) and chromatographic separation (*3*), respectively. In addition to hSMUG1, human cells contain several enzymes that excise hoU from DNA (NTH1, NEH1/NEI1, NEH2/NEI2) (*35*−*39*). Although the role of the latter three enzymes in repair of hoU in cells remains to be determined, they make relatively minor contributions (at most 10% of the total cellular activity) as judged from the analysis of the cellular activities (Figures 6 and 7). This was further supported by the chromatographic separation of the major Tg and hoU activities in HeLa cells. With a High S column, the activity for hoU·G in the cell extract comigrated with the Ugi-resistant U·G activity (i.e., hSMUG1) but was mostly separated from that for Tg·A (a hallmark of NTH1 and possibly that of NEH1/NEI1and NEH2/NEI2) (data not shown).

The present study has also confirmed the role of hSMUG1 as a backup enzyme of hUNG for removal of U from a U·G pair that results from deamination of C (*18*, *19*). At the optimal salt concentration (around 60 mM NaCl in Figure 6A), the ratio of the Ugi-sensitive (i.e., hUNG) and Ugi-resistant (i.e., hSMUG1) U-excising activities from U·G mismatches was 7:3, demonstrating a significant contribution of hSMUG1 to the cellular activity. Therefore, hSMUG1 has a dual repair role for deaminated cytosine (i.e., U) and a subset of oxidized pyrimidine bases. The latter role was further supported by generation of a significant amount of AP sites in oxidized calf thymus DNA upon treatment with hSMUG1 (Table 2).

Despite sharing limited overall sequence homology (less than 10%), hUNG, hSMUG1, hTDG (human thymine−DNA glycosylase), and EcMUG (*E. coli* mismatched uracil glycosylase) commonly excise U from DNA. Crystal structures of hUNG and EcMUG have revealed that they have a common fold of the core domains and interact with the flipped-out uracil base and the DNA backbone in similar ways (reviewed in ref *40*). Two conserved motifs in hUNG and EcMUG (motif 1 and motif 2, Figure 9A) are essential for base recognition and catalysis, and these motifs are also present in hSMUG1 (*17*). Figure 9B shows the interactions in the active site pocket of hUNG (*40*) and putative ones of hSMUG1 deduced from functional similarity. The interactions involved in base recognition of hSMUG1 are π-stacking of Phe98 and a bifurcate hydrogen bond of Asn163. These interactions provide the molecular basis as to how Tg and dhT that lost aromaticity and hoC and fC that have oxidized groups at C5 but retain the amino group at C4 cannot be accommodated in the active site pocket of hSMUG1 and, therefore, are not substrates (Figure 4). More importantly, the substantial difference in damage specificity between hSMUG1 and hUNG arises from the replacement of Tyr147 in hUNG by Gly87 in hSMUG1. In hUNG the bulky Tyr147 excludes T from the active site pocket through steric hindrance to the methyl group of T (*40*). In contrast, an open space created by replacement with Gly87 allows binding of hoU, hmU, and fU bearing a bulky substituent at C5 (−OH, $-CH_2OH$, −CHO) to the active site pocket. This situation

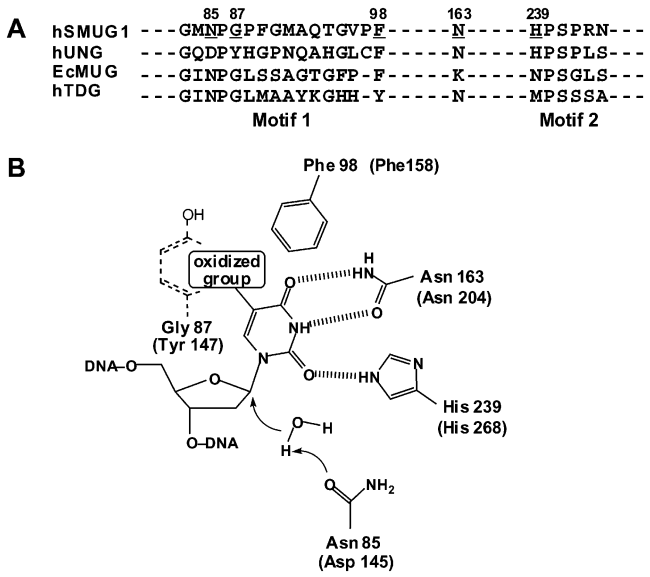

FIGURE 9: Conserved motifs and putative interactions in the active site pocket of hSMUG1. (A) Conserved motifs (motif 1 and motif 2) in hSMUG1, human UNG (hUNG), *E. coli* MUG (EcMUG), and human TDG (hTDG). The amino acid residues of hSMUG1 that appeared in panel B were underlined, and their positions from the N-terminus were indicated on the top. (B) Putative interactions in the active site pocket of hSMUG1 deduced from functional similarity to hUNG. The amino acid residues that have been shown to interact with U in hUNG are shown in parentheses. Tyr147 in hUNG that is replaced by Gly87 in hSMUG1 is shown by a structure with broken lines.

is similar to EcMUG and hTDG that have Gly at this position (Figure 9A). Both enzymes excise T from T·G mispairs in addition to U from U·G mispairs, although EcMUG excises mispaired T very poorly (*41*). A puzzling question is why hSMUG1 did not accept T as a substrate despite having a putative open space to accommodate the C5 substituent groups of hoU, hmU, and fU. The substrates accepted by hSMUG1 (hoU, hmU, and fU) have hydrophilic (polar) substituents (−OH, $-CH_2OH$, −CHO) in common, whereas T has hydrophobic (nonpolar) one ($-CH_3$). Accordingly, it is tempting to speculate that binding of hoU, hmU, and fU to the active site pocket is precisely controlled by a hydrophilic interaction of the polar substituents at C5 with an amino acid residue or a bound water molecule surrounding the open space. Whether this is the case will have to await determination of the three-dimensional structure of hSMUG1.

The activity of hSMUG1 for U, hoU, hmU, and fU in ssDNA was sensitive to the salt concentration and declined sharply with the increasing salt concentration (Figure 2). The activity was essentially negligible at 50 mM NaCl. If the sharp drop of the activity with the salt concentration is due to dissociation of hSMUG1 from DNA, hSMUG1 may need to interact with other protein to excise base lesions in ssDNA (possibly in the replication fork) under physiological conditions. The nuclear form of human hUNG (UNG2) binds to proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) via an overlapping motif and is localized to replication foci (*42*). However, such a PCNA/RPA binding motif is not present in hSMUG1 (*17*, *18*). Therefore, the physiological role of the ssDNA specific activity of hSMUG1 remains equivocal at this moment. In contrast, the dsDNA specific activity of hSMUG1 was more salt-resistant than the ssDNA specific activity (Figure 2). Although the optimal

concentration was 20 and 50 mM for the A and G pairs, respectively, hSMUG1 was partially active at least up to 100 mM NaCl due to the considerably broad salt concentration optima. This was also the case for hSMUG1 in the HeLa cell extract (Figure 6). Thus, it is likely that hSMUG1, without interacting with other proteins, can excise U, hoU, hmU, and fU from relevant base pairs (U·G, hoU·G, hmU·A, fU·A) under physiological conditions. For both purified and cellular hSMUG1 (Figures 5F and 6), the order of the excision capacity was U·G, hoU·G > hmU·A, fU·A, showing a preference of hoU and U over hmU and fU as substrates. The enzymatic parameters of hSMUG1 were $k_{cat} = 0.48$ min^{-1} and $K_m = 12$ nM for U·G and $k_{cat} = 0.68$ min^{-1} and $K_m = 96$ nM for fU·A. The k_{cat} values are much lower than that of hUNG that exhibits unusually high k_{cat} (280 min^{-1}) (*43*) to act in concert with the fast-moving DNA replication machinery (*42*). However, they are comparable to or higher than those of other mammalian DNA glycosylases such as NTH1 and OGG1 (*23*, *25*, *44*, *45*), thus consistent with the role of hSMUG1 in surveillance of damage in nonreplicating DNA regions.

During the revision of the present and previous (*3*) papers, a paper has been published by Liu et al., showing that the fU-excising activity in HeLa cells is distinct from hTDG and MBD4 (methyl-CpG binding domain) (*46*). This result is consistent with those reported in the present and previous (*3*) papers.

ACKNOWLEDGMENT

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