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Substrate Specificity of Homogeneous Monkeypox Virus Uracil-DNA Glycosylase[†]

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ABSTRACT: Weak or nonexistent smallpox immunity in today's human population raises concerns about the possibility of natural or provoked genetic modifications leading to re-emergence of variola virus and other poxviruses. Thus, the development of new antiviral strategies aimed at poxvirus infections in humans is a high priority. The DNA repair protein uracil-DNA glycosylase (UNG) is one of the viral enzymes important for poxvirus pathogenesis. Consequently, the inhibition of UNG is a rational therapeutic strategy for infections with poxviruses. Monkeypox virus, which occurs naturally in Africa, can cause a smallpox-like disease in humans. Here, the monkeypox virus UNG (mpUNG) is characterized and compared to vaccinia virus UNG (vUNG) and human UNG (hUNG). The mpUNG protein excises uracil preferentially from single-stranded DNA. Furthermore, mpUNG prefers the U·G pair over the U·A pair and does not excise oxidized bases. Both mpUNG and vUNG viral proteins are strongly inhibited by physiological concentrations of NaCl and $MgCl_2$. Although the two viral DNA repair enzymes have similar substrate specificities, the k_{cat}/K_M values of mpUNG are higher than those of vUNG. The mpUNG protein was strongly inhibited by 5-azauracil and to a lesser extent by 4(6)-aminouracil and 5-halogenated uracil analogues, whereas uracil had no effect. To develop antiviral drugs toward mpUNG, we also validated a repair assay using the molecular beacons containing multiple uracil residues. Potential targets and strategies for combating pathogenic orthopoxviruses, including smallpox, are discussed.

Since the worldwide eradication of smallpox at the end of the 1970s, the discontinuation of vaccination resulted in most of the current population becoming susceptible to infection. Therefore, variola virus is a potential agent for bioterrorism. The monkeypox virus can cause a smallpox-like disease in humans. This disease is a viral zoonosis endemic in central and western Africa and has emerged in the United States in 2003 (*1*−*3*). Due to the loss of specific immunity in a substantial proportion of the population, it is important to prevent and treat such infections. One of the viral enzymes important for poxvirus pathogenesis is the uracil-DNA glycosylase which is mandatory for DNA replication and therefore for the replication of the virus. Consequently, this enzyme represents a potential target for antiviral compounds (*4*).

Incorporation of uracil into DNA can arise through two mechanisms: either the misincorporation of dUTP by a DNA polymerase during replication or the spontaneous deamination of cytosine present in DNA. The resulting mismatches are U·A and U·G base pairs, respectively, and the last one leads to transition mutations after a further round of replication. To prevent the potential cytotoxic and mutagenic impact of the uracil residues, living organisms ranging from *Escherichia coli* to yeast and humans encode UNGs[1] that excise uracil base from DNA (*5*). UNGs excise uracil from single- and double-stranded DNA, leaving an apyrimidinic (AP) site and thus initiating the base excision repair (BER) pathway (*6*).

UNG enzymes have been identified in a wide variety of cellular organisms and viruses, including poxviruses. The hUNG protein belongs to the family of highly conserved UNGs typified by *E. coli* UNG. The *hUNG* gene encodes both mitochondrial (UNG1) and nuclear (UNG2) forms of the enzyme using differentially regulated promoters and alternative splicing to produce two proteins with unique N-terminal sorting sequences (*7*, *8*). A recombinant hUNG from the *hUNG* gene lacking the first 84 nonconserved amino acids at the amino terminus has been extensively studied (hUNGΔ84) (*9*). The three-dimensional structure of the hUNGΔ84 protein complexed to its DNA substrate was resolved, thus providing valuable information for the molecular design of specific inhibitors toward the viral family of UNGs (*10*−*13*). In this study, two forms, hUNG2 and hUNGΔ84, were used for comparison with the viral protein. Physiological concentrations of magnesium stimulate hUNG2 but at the same time inhibit hUNGΔ84 DNA glycosylase activities (*7*, *14*).

A homologue of the mammalian DNA repair enzyme UNG is encoded by herpesviruses and poxviruses (*15*, *16*).

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[1] Abbreviations: UNG, uracil-DNA glycosylase; hUNG, human uracil-DNA glycosylase; mpUNG, monkeypox virus uracil-DNA glycosylase; vUNG, vaccinia virus uracil-DNA glycosylase; hUNG2, full-length human uracil-DNA glycosylase protein; hUNGΔ84, catalytic fragment of human uracil-DNA glycosylase.

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The poxvirus genome consists of a large double-stranded DNA, and their entire life cycle, including replication, occurs exclusively within the cytoplasm of the host cell (*17*). Cytoplasmic localization provides a significant independence from cellular nuclear functions to the virus, and consequently, the virus encodes most of the enzymes and factors for transcription and replication. Many, but probably not all, of the proteins required for viral DNA synthesis have been identified, including the essential replicative DNA polymerase, the stoichiometric component of the processivity factor, a single-strand DNA-binding protein, the Holliday junction resolvase, a Ser/Thr protein kinase, the DNA-independent nucleotide triphosphatase, topoisomerase, DNA ligase, several enzymes involved in nucleotide metabolism, and a UNG (*4*, *18*−*20*). This latter enzyme stoichiometrically interacts with a component of the processivity factor to form a heterodimeric processivity factor of the viral DNA polymerase (*21*).

Poxviral UNG was first identified in Shope fibroma virus (*16*) and then identified in all poxviruses, including vaccinia, monkeypox, and variola viruses which belong to the *Orthopoxvirus* genus (*22*−*24*). Recently, De Silva and Moss (*4*) demonstrated that the enzymatic activity of the vaccinia virus vUNG protein is not required for virus viability, and it has become clear that vUNG plays a role in virus DNA replication that is independent of its UNG activity. Nevertheless, these authors also reported that vaccinia virus mutants lacking vUNG activity have reduced virulence in an intranasal infection mouse model, suggesting that vUNG plays a role in viral pathogenesis. Previously, we have compared the enzymatic properties of the recombinant vUNG protein with those of hUNG2 and hUNGΔ84 (*14*). We have demonstrated that vUNG is strongly inhibited in the presence of 7.5 mM $MgCl_2$, in contrast to hUNG2. Also, vUNG is not inhibited by the uracil-DNA glycosylase peptide inhibitor (UGI) from the *Bacillus subtilis* bacteriophages (*25*). Indeed, a conserved leucine (L272) residue in UDG superfamily-1, which is part of the DNA binding groove that interacts with UGI within a hydrophobic pocket, is replaced with an arginine residue in vUNG (*10*). Altogether, the structural and biochemical differences between viral and human UNGs provide a base for the search of specific viral enzyme inhibitors.

In this study, we characterized and compared enzymatic properties of the monkeypox virus UNG, i.e., mpUNG, to those of the related human enzymes, i.e., hUNGΔ84 and hUNG2, and vaccinia virus one, i.e., vUNG. The development of assays for the screening of antiviral compounds is discussed.

MATERIALS AND METHODS

Plasmids and Enzymes. The pTUNGΔ84 plasmid encoding the catalytic domain of the hUNG protein (hUNGΔ84) and the full-length hUNG2 protein were gifts from H. Krokan (Norwegian University of Science and Technology, Trondheim, Norway). The recombinant hUNG protein (27 kDa, 230 amino acids) was overexpressed and purified as described previously (*14*). UGI was purchased from New England Biolabs (OZYME, Saint Quentin Yvelines, France).

Oligonucleotides. All oligodeoxyribonucleotides were purchased from Eurogentec (Seraing, Belgium), including molecular beacons, those containing modified residues and complementary oligonucleotides, containing either dA, dG, dC, or T opposite from the adduct. The oligonucleotide sequences were as follows: 45 mer PS-U, d(AGCTACCATGCCTGCACGAAUTAAGCAATTCGTAATCATGGTCAT), where U is a uracil; modified 30 mer oligonucleotide X, d(TGACTGCATAXGCATGTAGACGATGTGCAT), where X is either 3,N^4-ethenocytosine (ϵC), 5-hydroxycytosine (5OHC), 5,6-dihydrouracil (DHU), 5-hydroxyuracil (5OHU), thymine glycol (Tg), 7,8-dihydro-8-oxoguanine (8oxoG), or 1,N^6-ethenoadenine (ϵA); and molecular beacon, FD-UDG, (FITC)-d(GCACUUAAGAAUUCACGCCATGTCGAAAUUCUUAAGUGC)-Dabcyl, where FITC is fluorescein isothiocyanate and Dabcyl is 4-[4′-(dimethylamino)phenylazo]benzoic acid. Oligonucleotides were 5′-end labeled by T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Biomedicals, SARL, Orsay, France). The 5′-^{32}P-labeled oligonucleotide was annealed to complementary nonlabeled oligonucleotides in a buffer containing 60 mM NaCl and 20 mM Tris-HCl (pH 8.0) at 65 °C for 3 min and then slowly cooled to room temperature. The resulting oligonucleotide duplexes are termed X·C, X·G, X·A, and X·T, respectively, where X is a modified base.

Construction of the Expression Plasmid. The *E4R* gene was PCR amplified from monkeypox virus strain *Zaire 1996* (*23*) (kindly provided by H. Meyer, Bundeswehr Institute of Microbiology, Munich, Germany) genomic DNA using the following oligonucleotide pair: 5′-oligo E4R, d(ATGAATTCAGTGACTATATCACAC); and 3′-oligo E4R, d(ATAAATAAACCCTTGAGC). The PCR DNA fragment was cloned into pGEMT-easy vector according to the manufacturer's instructions (Promega, Charbonnières-les-Bains, France). The resulting pGEMT-E4R plasmid was used to PCR amplify the *E4R* gene using the following oligonucleotide pair: 5′-oligo attE4R, d(GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAATTCAGTGACTATATCACACGCA); and 3′-oligo attE4R, d(GGGGACCACTTTGTACAAGAAAGCTGGGTTTAATAAATAAACCCTTGAGCCC) (sequence of attB site underlined). The PCR attB−attB fragment of the *E4R* gene was inserted into the pDEST17 vector according to the specification of Gateway Cloning Technology (Invitrogen, Cergy Pontoise, France). The resulting plasmid, pDEST17-E4R, encodes a 27.604 kDa E4R UNG with an N-terminal His tag. All plasmid constructions were sequenced to confirm the absence of mutations in the *E4R* gene according to the manufacturer's instruction (Genomexpress, Meylan, France).

Expression and Purification of mpUNG. E. coli strain BL21(DE3) pLysS transformed with pDEST17-E4R was grown at 37 °C (in LB supplemented with 1% glucose, 34 μg/mL chloramphenicol, and 100 μg/mL ampicillin) until the A_{600} reached 0.8. The mpUNG protein was induced overnight with 1 mM IPTG at 15 °C. Cells were pelleted by centrifugation at 4500 rpm for 30 min at 4 °C and resuspended in lysis buffer [500 mM NaCl, 0.3% NP-40, and 50 mM Tris-HCl (10%) (pH 8.0)]. Cells were disrupted by sonication at 2.4 kV and 4 kΩ (6 × 20 s bursts) on ice. Bacterial cell lysate was centrifugated at 10 000 rpm for 30 min at 4 °C. The cell lysate was loaded on a HiTrap-Ni^{2+} ion chelating column (Amersham Biosciences, Orsay, France) pre-equilibrated with buffer A [500 mM NaCl and 50 mM Tris-HCl (pH 8.0)]. The column was washed with 2 bed

volumes of buffer A, and proteins were eluted from the column with 15 bed volumes of a 0 to 400 mM imidazole gradient in buffer A. Aliquots from the A_{280} peak fractions were pooled and loaded onto a HiTrap Heparin 1 mL column (Amersham Biosciences) pre-equilibrated with buffer C [50 mM NaCl and 20 mM HEPES-KOH (pH 7.6)]. The column was washed with 2 bed volumes of buffer C, and mpUNG was eluted using a 15 bed volume gradient from 50 to 600 mM NaCl in buffer C. The A_{280} peak fractions were analyzed by 12% SDS−PAGE, and aliquots exhibiting the highest concentration of mpUNG were then pooled and stocked at −20 °C in 50% glycerol and 1 mM DTT.

Enzyme Assays. The release of uracil residues was assessed by the cleavage of an oligonucleotide containing a single lesion at a defined position. The standard assay for UNG activity (20 μL) contained 0.2 pmol of the 5′-^{32}P-end-labeled single-stranded or duplex oligonucleotide, 20 mM Tris-HCl (pH 7.5), 60 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 μg/mL BSA, and limiting amounts of enzyme, unless otherwise stated. The optimal reaction conditions for viral enzymes included 20 mM Tris (pH 7.0), 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA, unless otherwise stated. Incubations were carried out at 37 °C for 5 min. Reaction mixtures were incubated in the presence of 0.15% SDS and 10% piperidine for 15 min at 37 °C to reveal the abasic sites. Reaction products were analyzed by electrophoresis in denaturing 20% (w/v) polyacrylamide gels (PAGE) (20:1, 7 M urea, 0.5× TBE), visualized with a Fuji FLA-3000 Phosphor Screen, and analyzed using Image Jauge, version 3.12.

Molecular Beacon Assay. The standard enzyme assay with molecular beacons was performed at room temperature in 96-well plates containing 200 nM molecular beacon, limiting amounts of pure protein (mpUNG, vUNG, or hUNGΔ84), 20 mM Tris (pH 7.0), 1 mM EDTA, 1 mM DTT, and 100 μg/mL BSA.

RESULTS

Expression and Purification of mpUNG. To overexpress the His-tagged recombinant mpUNG protein, the pDETS17-E4R plasmid was transfected into the *E. coli* BL21(DE3) pLysS strain and grown as described in Materials and Methods. The mpUNG protein was found in the soluble fraction after an overnight induction at 15 °C. The purification of the soluble protein, from a 1 L batch culture, was performed in two chromatographic steps by HiTrap-Ni^{2+} ion chelating and HiTrap-Heparin columns. The purified mpUNG protein migrated as a single band corresponding to a molecular mass of 28 kDa via SDS−PAGE. The identity of the recombinant protein was confirmed by Western blot analysis using polyclonal antibodies specific to the vUNG protein, which cross-react with the monkeypox virus enzyme (data not shown).

Purified mpUNG Excises Uracil Residues from ssDNA and dsDNA. Substrate specificities of the mpUNG protein were investigated using single-stranded, PS-U, and duplex U·G oligonucleotides containing a single uracil residue. Light piperidine treatment was used to reveal abasic sites generated by DNA glycosylase activity. As shown in Figure 1 under physiological conditions [60 mM NaCl (pH 7.5)], increasing amounts of mpUNG (0.1−1 nM) cleaved both 45 mer U·G and PS-U oligonucleotides, generating a fragment migrating

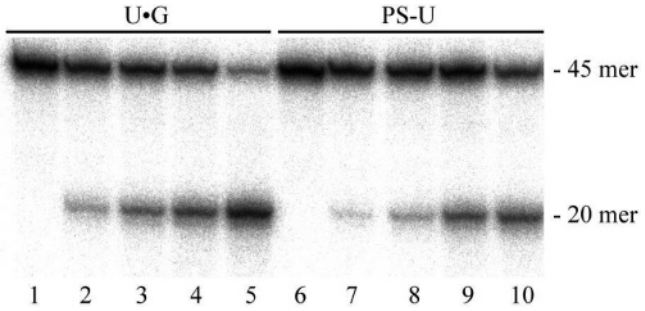

FIGURE 1: Activity of mpUNG on single-stranded PS-U and duplex U·G oligonucleotides containing a single uracil residue. The 5′-^{32}P-labeled U·G (lanes 1−5) and PS-U (lanes 6−10), each at 5 nM, were incubated with increasing amounts of mpUNG under physiological conditions: lanes 1 and 6, no enzyme; lanes 2−5, 0.1, 0.2, 0.5, and 1 nM mpUNG, respectively; and lanes 7−10, 0.05, 0.1, 0.2, and 0.5 nM mpUNG, respectively. Reaction products were analyzed as described in Materials and Methods.

at the position of the 20 mer (lanes 2−5 and 7−10). At high protein concentrations, mpUNG excised uracil more efficiently from duplex than from single-stranded DNA (lanes 5 and 10). Piperidine treatment alone did not cleave oligonucleotides (lanes 1 and 6), showing no detectable AP sites in the substrates. Under the reaction conditions that were used, similar amounts of hUNGΔ84 (data not shown) were required to cleave both PS-U and U·G oligonucleotides compared to mpUNG (0.1 nM for both enzymes). Interestingly, the vUNG protein was much less active under these conditions than mpUNG (*14*).

We investigated whether the mpUNG protein was able to excise modified bases induced by the oxidative stress in cellular DNA. No detectable activity was observed when an excess of mpUNG was incubated with oligonucleotide duplexes ϵC·G, 5OHC·G, DHU·G, 5OHU·G, ϵA·T, 8oxoG·C, 8oxoG·A, and Tg·A, indicating that the viral enzyme is highly specific for uracil (data not shown).

Reaction Condition Requirements for the mpUNG-Catalyzed Activity. To characterize the ionic strength dependence of the viral UNGs, we measured DNA glycosylase activity on U·G at varying concentrations of NaCl. As shown in Figure 2, both vUNG and mpUNG were strongly inhibited by only 10 mM NaCl and the maximal activity was observed in the absence of NaCl. At 20 mM NaCl, the DNA glycosylase activity was less than 10% for both viral enzymes, and at >60 mM NaCl, the activity was essentially abolished. The pH profiles of vUNG and mpUNG activities were also examined in the absence of NaCl. As shown in Figure 3, we observed a dramatic difference between two viral DNA glycosylases; the pH profile of mpUNG-catalyzed activity was very well defined with an optimal pH of 7.0, whereas vUNG had a broad pH optimum of 7.0−8.5. Taken together, these data suggest a striking difference between physiological conditions and an optimal reaction buffer with pH 7.0 and low ionic strength for both viral enzymes on the dsDNA substrate.

Previously, we found a strong inhibitory effect of $MgCl_2$ on vUNG activity (*14*). Here, mpUNG- and vUNG-mediated cleavage of U·G and PS-U were assessed in the presence of varying concentrations of $MgCl_2$. As shown in Figure 4, 0.5 mM $MgCl_2$ strongly inhibits mpUNG and vUNG activities down to 20−40%, and 3 mM $MgCl_2$ totally abolishes the DNA glycosylase activities of both enzymes toward U·G,

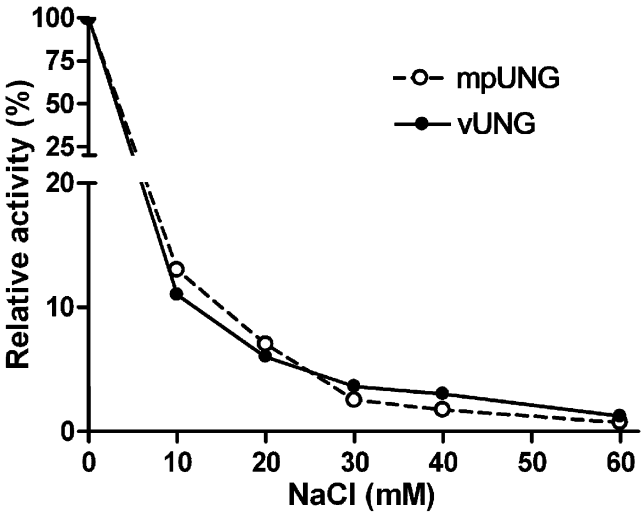

FIGURE 2: Effect of NaCl concentration on mpUNG (○) and vUNG (●) activities toward the U·G oligonucleotide duplex. The 5′-^{32}P-labeled U·G (5 nM) was incubated for 5 min at 37 °C with 5 pM mpUNG or 10 pM vUNG in the buffer containing 20 mM Tris (pH 7.0), 1 mM EDTA, 1 mM DTT, 0.1 mg/mL BSA, and varying amounts of NaCl. Reaction products were analyzed as described in Materials and Methods.

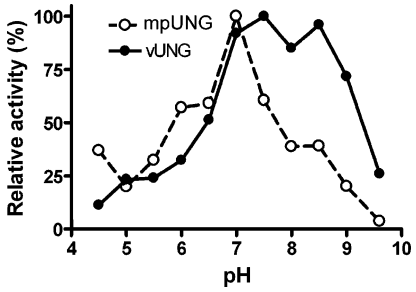

FIGURE 3: Effect of pH on mpUNG (○) and vUNG (●) activities toward the U·G oligonucleotide duplex. The 5′-^{32}P-labeled U·G (5 nM) was incubated with 5 pM mpUNG or 10 pM vUNG in the buffer containing 20 mM Tris (varying pH), 1 mM EDTA, 1 mM DTT, and 0.1 mg/mL BSA at 37 °C for 5 min. Reaction products were analyzed as described in Materials and Methods.

indicating that divalent cations are not required for the viral DNA glycosylase. Indeed, maximal activity (100%) was observed with 1 mM EDTA, whereas in the absence of EDTA, 25 and 50% decreases were observed for mpUNG and vUNG, respectively. Unexpectedly, a low concentration of 0.1 mM $MgCl_2$ stimulated activities of both enzymes compared to buffer without magnesium. As expected, a similar effect of $MgCl_2$ was observed for the viral enzymes when using PS-U oligonucleotide (Figure 4). However, in the absence of EDTA, a greater decrease in activity was observed with a ssDNA substrate than with dsDNA (70% vs 25% for mpUNG and 80% vs 50% for vUNG). Altogether, these results show that the optimal reaction condition for mpUNG-catalyzed DNA glycosylase activity requires a low ionic strength, pH 7.0, and 1 mM EDTA.

Steady State Kinetic Studies and Base Pair Specificity of mpUNG and vUNG. To further characterize and compare the substrate specificities of the viral DNA glycosylases, the kinetic constants for uracil excision in PS-U and U·G and the influence of the base opposite from it were determined under optimal reaction conditions. As shown in Table 1, comparison of the kinetic constants shows that k_{cat}/K_M values of mpUNG and vUNG toward ssDNA substrate were 30- and 50-fold, respectively, higher compared to those for a dsDNA substrate. Interestingly, the apparent K_M values for mpUNG and vUNG toward U·G oligonucleotide duplex were the same, whereas the catalytic constant (k_{cat}) of mpUNG was 6-fold higher compared to that of vUNG. The apparent K_M and k_{cat}/K_M values measured for mpUNG and vUNG when they are acting upon a U·G duplex indicate that in vitro mpUNG is more efficient than vUNG.

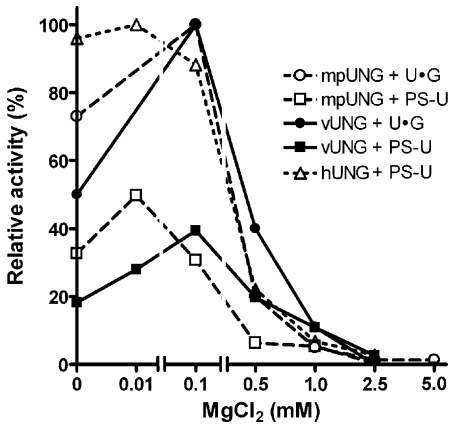

FIGURE 4: mpUNG and vUNG DNA glycosylase activities depending on $MgCl_2$ concentration. The 5′-^{32}P-labeled U·G and PS-U, each at 5 nM, were incubated with 5 and 1 pM mpUNG or 10 and 5 pM vUNG, respectively, in the buffer containing 20 mM Tris (pH 7.0), 1 mM DTT, 0.1 mg/mL BSA, and varying concentrations of $MgCl_2$ at 37 °C for 5 min. The highest activity of each enzyme was normalized to 100%. Human and viral enzymes have the highest activities on duplex DNA at 0.01 and 0.1 mM $MgCl_2$, respectively. Reaction products were analyzed as described in Materials and Methods.

Table 1: Kinetic Parameters for the Excision of a Single Uracil Residue by the mpUNG and vUNG Proteins under Optimal Reaction Conditions

protein[a]	substrate	K_M (μM)[b]	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
mpUNG	PS-U	0.015 ± 0.004	280 ± 22	18245 ± 4700
	U·G	0.03 ± 0.006	18 ± 1	590 ± 175
vUNG	PS-U	0.0087 ± 0.003	53 ± 4	6095 ± 133
	U·G	0.03 ± 0.006	3 ± 0.3	124 ± 50

[a] Enzyme activity was determined under optimal conditions for the UNG assay (see Materials and Methods). [b] All experiments were carried out in triplicate.

The kinetic constants of mpUNG toward PS-U and U·G were also determined under physiological conditions [60 mM NaCl and 1 mM EDTA (pH 7.5)]. As shown in Table 2, mpUNG, like vUNG, preferentially excises a uracil residue when present in ssDNA (k_{cat}/K_M values of 73 and 56 min^{-1} μM^{-1}) compared to a U·G dsDNA substrate (k_{cat}/K_M values of 58 and 19 min^{-1} μM^{-1}). Interestingly, comparison of K_M values of mpUNG suggests that the viral enzyme, like the human one, has a stronger affinity for a dsDNA substrate (1.0 μM) than for a ssDNA substrate (1.7 μM).

Kinetic parameters of the viral enzymes were dramatically dependent on the reaction conditions, with the highest k_{cat}/K_M and lowest K_M values observed in the absence of NaCl (Tables 1 and 2). However, under both reaction conditions, the viral enzymes preferentially excise uracil when present in ssDNA rather than in dsDNA. In contrast, the human

Table 2: Kinetic Parameters for the Excision of a Single Uracil Residue by the mpUNG, vUNG, and hUNG Proteins under Physiological Reaction Conditions

protein[a]	substrate	K_M (μM)[b]	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
mpUNG	PS-U	1.7 ± 0.4	131 ± 11	73
	U·G	1.0 ± 0.2	58 ± 3.5	58
	U·C	1.6 ± 0.3	143 ± 10	89
	U·T	1.4 ± 0.3	110 ± 10	78
	U·A	1.0 ± 0.1	2.5 ± 0.1	2.5
vUNG[c]	PS-U	0.5 ± 0.2	28 ± 0.6	56
	U·G	2.6 ± 0.7	49 ± 0.9	19
hUNG[c]	PS-U	2.9 ± 0.6	11800 ± 80	4000
	U·G	1.6 ± 0.7	11860 ± 130	7000

[a] Enzyme activity was measured under physiological reaction conditions for the UNG assay (see Materials and Methods). [b] All experiments were carried out in triplicate. [c] Data taken from ref *14*.

enzyme preferentially excises uracil when present in dsDNA rather than in ssDNA (k_{cat}/K_M values of 7000 and 4000 min^{-1} μM^{-1}, respectively).

Effect of the Nature of the Base Opposite from the Uracil Residue. To further characterize the substrate specificities of mpUNG under physiological conditions, we measured the K_M, k_{cat}, and k_{cat}/K_M values using duplex oligonucleotides containing one of the four possible bases opposite uracil. As shown in Table 2, the apparent k_{cat}/K_M values indicate that the mpUNG excises uracil residues with a very low efficiency opposite from dA compared to uracil residues opposite from dC, T, and dG. The relative order of the opposite base preference for mpUNG was as follows: dC > T > dG ≫ dA [which is the same for vUNG and hUNG (*14*)].

To validate the concept of selective inhibition, we compared the relative sensitivities of mpUNG and hUNG2 to uracil and uracil analogues: 5-bromouracil, 5-fluouracil, 5-azauracil, and 4(6)-aminouracil. As shown in Figure 5, in agreement with previous data, we observed that uracil, 5-azauracil, and 4(6)-aminouracil efficiently inhibit hUNG2 whereas 5-bromouracil and 5-fluouracil had a slight inhibitory effect (Figure 5). In contrast to the human enzyme, mpUNG exhibited different patterns of inhibition by uracil analogues. Uracil and 4(6)-aminouracil had a weak effect against the viral enzyme, whereas 5-bromouracil and 5-fluouracil had an inhibitory effect significantly higher that that with hUNG2. Finally, 5-azauracil inhibited both enzymes but more strongly mpUNG than hUNG2.

Uracil Analogues Discriminate between Human and Viral Uracil DNA Glycosylases. It has already been shown that at high concentrations uracil analogues inhibit hUNG (*26*). Interestingly, herpes simplex virus type 1 uracil-DNA glycosylase is selectively inhibited by 6-(*p*-*n*-octylanilino)-uracil in the micromolar range and without an effect on human UNG (*27*). Here, we were interested in whether the mpUNG and hUNG2 proteins have different sensitivities to uracil and its analogues: 5-bromouracil, 5-fluouracil, 5-azauracil, and 4(6)-aminouracil. As shown in Figure 5, 60 and 80% inhibition by uracil, 5-azauracil, and 4(6)-aminouracil were observed for hUNG2, whereas 5-bromouracil and 5-fluouracil have slight inhibitory effects, i.e., 20−30% inhibition at 5 mM. In contrast, mpUNG exhibited different patterns of inhibition by uracil analogues. Indeed, 5 mM 5-bromouracil and 5-fluouracil efficiently decrease vUNG activity by 50%, whereas 5 mM uracil and 4(6)-aminouracil inhibit only 30% of mpUNG activity (Figure 5). Finally, 5 mM 5-azauracil strongly inhibited vUNG activity by 90% compared to a 50% inhibition for hUNG2. These results further substantiate the difference between viral and human UNG and provide a basis for the search of specific viral enzyme inhibitors.

Molecular Beacon Assay. In this work, we used a direct method to assay DNA excision repair on the basis of the fluorescence quenching mechanism of molecular beacons (*28*). The molecular beacon consists of a single-stranded DNA oligonucleotide labeled with a 5′-fluorescein (F) and a 3′-Dabcyl (D) in which the fluorophore, F, is held in the proximity of the quencher, D, by the stem−loop structure design of the oligonucleotide. Following removal of the modified base or incision of the oligonucleotides, the fluorophore is separated from the quencher and fluorescence can be detected as a function of time. Figure 6shows a time-dependent increase in fluorescence for all three enzymes. Furthermore, a similar $MgCl_2$ inhibition profile, previously seen in Figure 4, was also observed with the molecular beacon assay (data not shown). These results indicate that the molecular beacon assay is suitable for the automated screening of antiviral substances against these two viral DNA glycosylases.

DISCUSSION

Human monkeypox is an emergent viral zoonosis, endemic in central and western Africa. Recent human outbreaks of monkeypox and the threat about the use of variola virus as a biological weapon have highlighted the need to develop effective anti-orthopoxvirus therapeutics to address these problems and concerns. Indeed, there is currently no proven treatment for human monkeypox, and questions about its potential as an agent of bioterrorism persist. Currently, one of the antiviral agents allowed for use as an emergency treatment in the case of smallpox outbreak is cidofovir (Vistide), which acts by interfering with the viral DNA polymerase and has been shown to be active in several animal models (*29*, *30*). Another promising antiviral drug involved in the inhibition of the release of orthopoxviruses out of the cell has also been described (*31*, *32*). Nevertheless, it is important to continue screening for new antiviral compounds due to the recommendation of the World Health Organization (WHO) to stockpile at least two antiviral molecules that work by distinct synergistic mechanisms, to combat a possible smallpox outbreak (*33*, *34*).

The UNG protein, a component of a multiprotein replication complex of poxviruses, represents a target for the development of effective antiviral drugs. Previously, we have studied vUNG (*14*). Here, in an attempt to select specific inhibitors against variola virus, we characterized the mpUNG protein of the related emergent human pathogen, the monkeypox virus. Indeed, the amino acid sequence of the variola virus is 98% homologous with that of monkeypox virus. Therefore, the aim of this study was to characterize and compare the biochemical properties of the mpUNG and hUNG proteins which are highly homologous to variola UNG. This may help in the development of an in vitro system suitable for the screening of antiviral compounds specifically against variola and the related viruses.

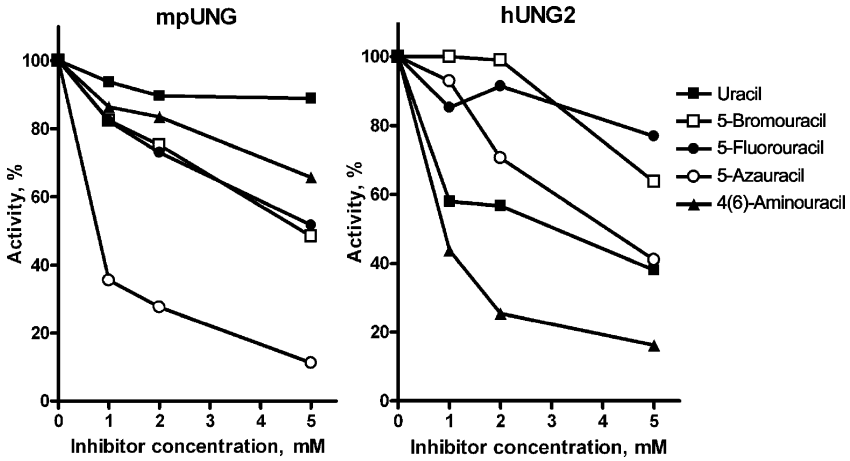

FIGURE 5: Effect of uracil and uracil analogues on the activity of UNGs. mpUNG (0.2 nM) or hUNG2 (0.025 nM) was incubated with 5 nM 5′-^{32}P-labeled U·G in the presence of inhibitors. For details, see Materials and Methods.

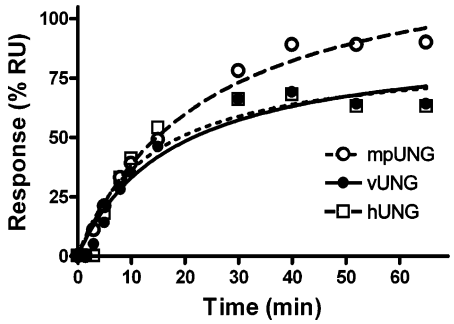

FIGURE 6: Activity of the viral and human UNGs toward molecular beacons containing multiple uracil residues. Fluorescence was expressed as response units (RU). Molecular beacons (200 nM) were incubated with 10 nM mpUNG, 25 nM vUNG, or 0.5 nM hUNGΔ84 under optimal reaction condition for the viral enzymes. For details, see Materials and Methods.

We have purified the homogeneous recombinant mpUNG protein. The enzyme is active on ssDNA and dsDNA substrates, containing a single uracil residue, in a concentration-dependent manner (Figure 1). Interestingly, the human thymine-DNA glycosylase and the single-strand, mismatch-specific UNG (TDG and SMUG1) from the UNG superfamily have broad substrate specificity and can excise bases other than uracil-modified bases, whereas the mpUNG protein does not excise oxidized bases and etheno adducts when present in DNA. Although the active site of viral UNGs differs significantly from that of hUNG, viral UNGs did not acquire broad range substrate specificity during evolution. These observations strongly suggest an important biological role and high selective pressure on the viral enzyme to keep its substrate specificity.

Optimal activity of mpUNG toward uracil residues was observed at pH 7.0, 1 mM EDTA, and very low ionic strengths. Most interestingly, the presence of 10 mM NaCl caused a 10-fold inhibition of the mpUNG-catalyzed activity. In addition, a concentration of $MgCl_2$ of >0.5 mM strongly inhibited viral DNA glycosylase activity toward ssDNA and dsDNA. Quite surprisingly, in the absence of EDTA, we detected a 70% inhibition of mpUNG activity, and the addition of 0.1 mM $MgCl_2$ restored its activity toward ssDNA (Figure 4). We found that vUNG has similar properties except for the optimal pH (Figure 3). It has been shown that the hUNG protein requires a pH optimum at pH 8.0 and 10 mM NaCl to have an optimal activity (*9*). Here, we found that the hUNG in contrast to the viral enzymes does not require divalent cations for activity and is not inhibited in either the presence or the absence of 1 mM EDTA (data not shown).

Comparison of the specificity constant (k_{cat}/K_M) values of the viral enzymes under optimal reaction conditions indicates that the mpUNG protein is more efficient than vUNG (Table 1). Indeed, higher k_{cat} values obtained for mpUNG indicate better processivity compared to that of vUNG. Interestingly, under optimal reaction conditions, mpUNG prefers ssDNA ($K_M = 15$ nM) over dsDNA ($K_M = 30$ nM). However, under physiological conditions, mpUNG K_M values for ssDNA and dsDNA substrates are in the same micromolar range. In contrast, K_M values of vUNG for ssDNA were always lower than K_M values for dsDNA substrates, regardless of the reaction conditions. Under physiological reaction conditions, hUNG was 100-fold more efficient than the viral enzymes (Table 2). Despite dramatic differences among mpUNG, vUNG, and hUNG, all three proteins have a similar base pair specificity of uracil excision with the following relative order of excision: U·C > U·T > U·G ≫ U·A. It should be stressed that the base pair preference in the context of a whole genome cannot be determined since the literature data indicate that this preference is sequence-dependent, although, in the majority of contexts, U·G is preferred (*9*).

Alignment of protein sequences for family 1 of UNGs shows that vUNG is its most diverged member (*35*). Indeed, both mpUNG and vUNG excise uracil residues from DNA in the presence of the UGI peptide, indicating that structures of the active sites in poxvirus proteins differ significantly from the structures in other members of the UNG family, including the human enzyme. In agreement with this observation, we demonstrated that uracil analogues discriminate between viral and human UNGs (Figure 5). These results can provide a basis for the development of specific mpUNG inhibitors which will not inhibit the hUNG2 protein.

Biochemical characterization of the mpUNG protein allowed us to develop an in vitro assay based on the fluorescence quenching mechanism of a molecular beacon (*28*). Here, this assay was miniaturized to perform the reactions in 96-well microplates. We demonstrated that the two viral recombinant enzymes have been highly efficient toward molecular beacons under our experimental conditions (Figure 6). Taken together, these results provide a rationale

for undertaking a high-throughput in vitro screening assay to search specific viral enzyme inhibitors. This type of assay would have the advantage of avoiding in vivo experimentations with the monkeypox virus. Furthermore, the molecular beacons could be used to study the mode of action of viral inhibitors.

Recently determined crystal structures of the vUNG protein reveal dimeric assembly which may function as part of the processivity factor in the replication machinery of poxviruses (*36*). In fact, vUNG interacts directly with the A20 protein, forming a dimeric complex which can associate with one of the catalytic subunits of the viral DNA polymerase to combine the processive capacity of the polymerase holoenzyme with a robust DNA synthesis (*21*). Study of the vUNG·A20 complex coupled to the design of the molecules disrupting the vUNG·A20 complex would provide new antiviral compounds. In perspective, combining the search for inhibitors of the DNA glycosylase activity of mpUNG and the search for inhibitors of the mpUNG·A20 interaction might enlarge the search for antiviral inhibitors.

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