Human thymine DNA glycosylase (TDG) and methyl-CpG-binding protein 4 (MBD4) excise thymine glycol (Tg) from a Tg:G mispair

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ABSTRACT

The repair enzymes thymine DNA glycosylase (TDG) and methyl-CpG-binding protein 4 (MBD4) remove thymines from T:G mismatches resulting from de-amination of 5-methylcytosine. Thymine glycol, a common DNA lesion produced by oxidative stress, can arise from oxidation of thymine or from oxidative deamination of 5-methylcytosine, and is then present opposite adenine or opposite guanine, respectively. Here we have used oligonucleotides with thymine glycol incorporated into different sequence contexts and paired with adenine or guanine. We show that TDG and MBD4 can remove thymine glycol when present opposite guanine but not when paired with adenine. The efficiency of these enzymes for removal of thymine glycol is about half of that for removal of thymine in the same sequence context. The two proteins may have evolved to act specifically on DNA mismatches produced by deamination and by oxidation-coupled deamination of 5-methylcytosine. This repair pathway contributes to mutation avoidance at methylated CpG dinucleotides.

INTRODUCTION

DNA bases are susceptible to damage by reactive agents derived from endogenous or exogenous origins. A prominent form of endogenous damage arises through hydrolytic deamination of bases such as cytosine and 5-methylcytosine carrying exocyclic amino groups (1). The deamination of the exocyclic amino group in cytosine and 5-methylcytosine generates uracil and thymine, respectively. The reactions give rise to a guanine (G):uracil (U) mispair and a guanine (G):thymine (T) mispair, respectively, in double-stranded DNA. If unrepaired prior to DNA replication, these mispairs can induce C to T transition mutations in the replicated DNA. Mutagenic mechanisms involving 5-methylcytosine appear to be particularly common since methylated CpG dinucleotides are mutational hotspots in human genes, for example in the cancer-relevant p53 gene (2–5).

All organisms have evolved repair enzymes that remove these deaminated bases. Several DNA glycosylases, repairing these mispairs, have been identified. The accurate and efficient repair of a G:U mispair is mediated by uracil DNA glycosylases (6). Whereas the deamination of cytosine produces a G:U mispair, the deamination of 5-methylcytosine produces a G:T mispair. This G:T mispair can be repaired by thymine DNA glycosylase (TDG) (7). The G:T mispair-specific TDG activity was first discovered in HeLa cell extracts (8). Although having an activity towards a G:T mispair, TDG shows preference for a G:U mispair substrate (9–11). In addition, recent studies show that the human TDG protein efficiently processes a mutagenic exocyclic adduct of cytosine, 3,*N4*-ethenocytosine (12–14), which arises in DNA as a consequence of reaction with lipid peroxidation products or by exposure to chemical carcinogens such as vinyl chloride.

Several proteins binding to methylated CpG sites have been identified, MeCP2 and a group of four other proteins which contain a conserved methyl-CpG-binding domain (MBD) domain (15). One of these methyl-CpG-binding proteins, MBD4, has a G:T mispair-specific DNA glycosylase activity (16). Although it has a methyl-CpG-binding domain, MBD4 efficiently recognizes and removes thymine from a G:T mispair and uracil from a G:U mispair at unmethylated CpG sequences as well (16,17). Furthermore, MBD4 also removes the uracil analog, 5-fluorouracil, in the context of a G:5-fluorouracil mispair (18) and has a weak glycosylase activity towards 3,*N4*-ethenocytosine (17). It is interesting that the function of MBD4 is quite similar to that of TDG, despite complete lack of sequence homology.

5R-Thymine glycol (Tg) is formed as a product of exposure to ionizing radiation and other oxidative stresses such as H_2O_2 (19). Tg is a major replication-interfering lesion generated by reactive oxygen species *in vivo* (20). Tg is recognized and excised from DNA by endonuclease III (endo III) and endonuclease VIII (endo VIII) encoded by the *nth* and *nei* genes in *Escherichia coli* (21), respectively. Mammalian homologs of these genes have been characterized (22–24). Recently, studies using Nth1-knockout mice suggested that there might be several back-up glycosylases for repairing Tg (25–27). Mammalian Nei-like genes, encoding proteins with sequence similarity to the bacterial Fpg/Nei family proteins, were identified, and recombinant NEIL1, but not NEIL2,

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showed efficient removal of Tg in double-stranded substrates (25,28–32).

Tg is produced in DNA by oxidation of thymine. However, it can also arise from oxidative deamination of 5-methyl-cytosine (33) and is then present as a Tg:G mispair. In this study, we have purified recombinant human MBD4 and TDG proteins and analyzed the activity of these enzymes for the repair of Tg:G mispairs.

MATERIALS AND METHODS

Reagents and oligonucleotides

Oligonucleotides for PCR primers were purchased from IDT (Coralville, IA). Oligonucleotides containing a *5R*-Tg residue were synthesized according to published procedures (34). DNase I, RNase A and restriction enzymes (BamHI, SacI and XhoI) were purchased from New England Biolabs (Beverly, MA). T4 DNA ligase and T4 polynucleotide kinase were purchased from Promega (Madison, WI). All other chemicals and reagents were purchased from Sigma (St Louis, MO). Fpg, Nth, Nfo and hAPE1 proteins were from laboratory stocks and were purified as previously described (35,36).

Construction of plasmids containing human TDG and MBD4 genes

PCR fragments of full-length cDNA of human TDG and MBD4 were prepared with Pfu turbo DNA polymerase (Stratagene, La Jolla, CA) and two primers: for human TDG, forward primer 5′-AG<u>GGATCC</u>ATGGAAGCGGA-GAACGCG-3′ (BamHI cutting site underlined) and reverse primer 5′-TG<u>GAGCTC</u>AGCATGGCTTTCTTCTTCC-3′ (SacI site underlined), and for human MBD4, forward primer 5′-AG<u>GGATCC</u>ATGGGCACGACTGGGCTG-3′ (BamHI site underlined) and reverse primer 5′-TC<u>CTCGAG</u>AGATA-GACTTAATTTTTC-3′ (XhoI site underlined). PCR products were subcloned into plasmid pET-28b(+) (Novagen, Madison, WI) by cutting with restriction enzymes, i.e. BamHI and SacI for human TDG, and BamHI and XhoI for human MBD4, respectively, and ligation with restricted PCR products and T4 DNA ligase. The ligated plasmids were transformed into bacterial competent BL21 (DE3) cells (Stratagene) for human MBD4 and into BL21 codonplus (DE3) cells (Stratagene) for human TDG. The plasmids were isolated and sequenced to confirm the cDNA sequence. These expression vectors generate a His$_6$-tagged TDG–MBD4 open reading frame. The His$_6$ tag is located at the C-terminal region of the proteins.

Purification of recombinant human TDG and MBD4 proteins

A single colony was inoculated into 100 ml of LB medium containing 30 µg/ml kanamycin. The overnight culture was added into 10 l of LB medium with kanamycin and grown at 37°C with vigorous shaking until the OD$_{600}$ was 0.5–0.7. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and growing the cultures for an additional 10 h at room temperature.

The cell pellet was resuspended in 30 ml of lysis buffer (50 mM sodium phosphate pH 8.0, 0.2% Triton X-100, 30 mM imidazole and 300 mM NaCl).

Lysozyme (1 mg/ml), DNase I (30 µl of a 10 mg/ml solution) and RNase A (30 µl of a 10 mg/ml solution) was added and incubated at room temperature for 20 min with mixing. The cell lysate was centrifuged at 12 000 *g* for 20 min and mixed with 5 ml of a 50% Ni-NTA slurry at room temperature. The lysate and Ni-NTA mixture were loaded onto a column. The column was washed with 30 ml of washing buffer (50 mM sodium phosphate pH 8.0, 0.2% Triton X-100, 80 mM imidazole and 500 mM NaCl). The bound proteins were eluted with elution buffer (50 mM sodium phosphate pH 8.0, 0.1% Triton X-100, 500 mM NaCl and 100, 150 and 250 mM imidazole, respectively).

The procedure for human TDG purification was the same as that for human MBD4 except that the lysis buffer was 50 mM sodium phosphate pH 8.0, 0.2% Triton X-100, 10 mM imidazole and 500 mM NaCl. The column was washed with 30 ml of washing buffer (50 mM sodium phosphate pH 8.0, 0.2% Triton X-100, 20 mM imidazole and 500 mM NaCl). Proteins were eluted with buffer containing 50 mM sodium phosphate pH 8.0, 0.1% Triton X-100, 500 mM NaCl and 40, 80 and 100 mM imidazole, respectively.

Ion exchange chromatography was used for additional purification to remove contaminants. For human MBD4, an SP-Sepharose chromatography column (Amersham Pharmacia Biotech) was used. The 250 mM imidazole fractions of human MBD4 were dialyzed overnight at 4°C against loading buffer (20 mM Tris–HCl pH 7.2, 0.5 mM EDTA, 5 mM β-mercaptoethanol and 5% glycerol). After loading the dialyzed fraction containing 1 mg of protein onto a 1 ml SP-Sepharose column and washing with loading buffer, bound proteins were eluted with a linear gradient of 0–500 mM NaCl in 20 ml dialysis buffer. Human MBD4 was eluted in fractions at ~300 mM NaCl. The purified human MBD4 was dialyzed against storage buffer (20 mM Tris–HCl pH 7.5, 100 mM KCl, 0.5 mM EDTA, 5 mM β-mercaptoethanol and 30% glycerol) and stored at –80°C.

The 80 mM imidazole fraction of human TDG was dialyzed overnight at 4°C against dialysis buffer (20 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 5 mM β-mercaptoethanol and 5% glycerol). To separate it from low molecular weight contaminants, eluted human TDG protein was further purified by gel filtration (ACA 54). The gel filtration buffer was 10 mM Tris–HCl pH 7.5, 100 mM KCl, 0.1 mM EDTA, 5 mM mercaptoethanol and 5% glycerol. An SP-Sepharose column was used for additional purification of human TDG. The procedure of washing and elution was the same as that for human MBD4 as described above. Both MBD4 and TDG were judged to be >98% pure after analysis by SDS–PAGE.

DNA glycosylase activity assay

The DNA glycosylase activity of the purified human MBD4 and TDG proteins was tested by means of a cleavage assay with oligonucleotides containing a G:T or G:Tg mispair. For this, 30mer oligonucleotides (5′-CTCGTCAGCATCA-T or Tg-C or G-ATCATACAGTCAGTG; T is normal thymine and Tg indicates *5R*-thymine glycol) were 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The end-labeled
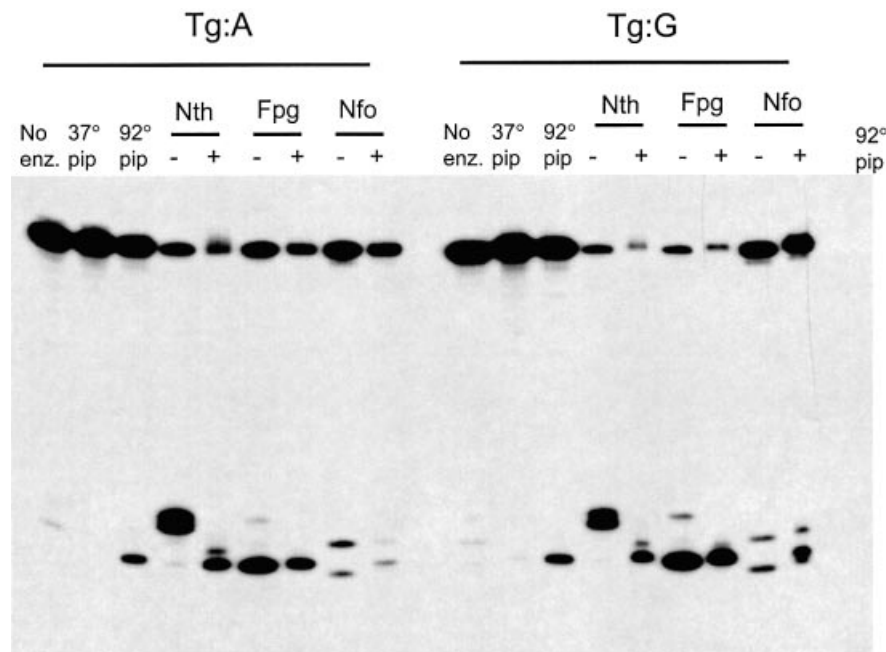
Figure 1. Cleavage of thymine glycol-containing oligonucleotides by *E.coli* DNA glycosylases. The left panel shows an oligonucleotide with thymine glycol opposite adenine, and the right panel shows an oligonucleotide with thymine glycol opposite guanine. The oligonucleotides were incubated with *E.coli* Nth, Fpg or Nfo proteins (100 ng each) at 37°C for 60 min. Strand cleavage by β–δ elimination was accomplished by heating the DNA in 1 M piperidine at 92°C for 20 min before gel loading.
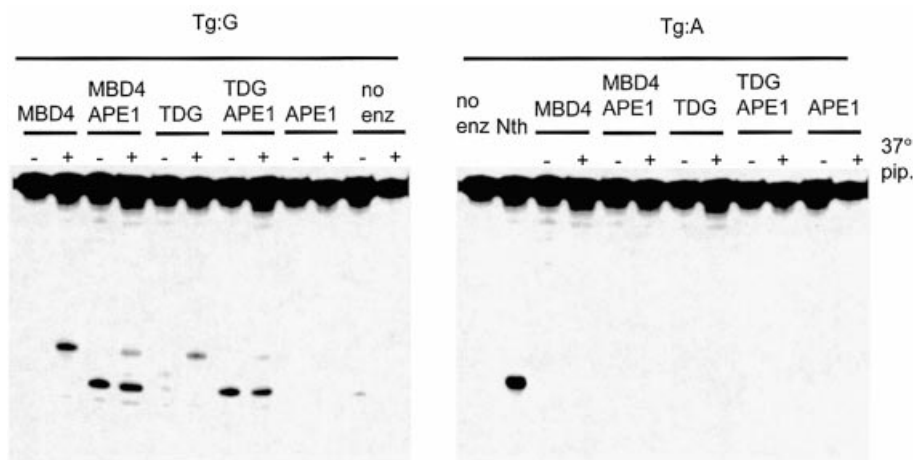
Figure 2. Cleavage of thymine glycol-containing oligonucleotides by human MBD4 and TDG DNA glycosylases. The left panel shows cleavage of a double-stranded oligonucleotide with thymine glycol opposite guanine, and the right panel is the same sequence with thymine glycol opposite adenine. The sequence context of Tg is 5′ ATgG with a 5-methylcytosine opposite the guanine. The oligonucleotides were incubated with 0.5 µg of human recombinant MBD4 or TDG proteins at 37°C for 60 min. Strand cleavage by β elimination was accomplished by heating the DNA in 1 M piperidine at 37°C for 20 min before gel loading. Human recombinant APE1 was included in the glycosylase reactions where indicated.

oligonucleotides were annealed to the bottom strands in annealing buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 30 mM NaCl and 40 mM KCl). The bottom strand contained 5-methylcytosine opposite the guanine when the ATG or ATgG sequence substrates were used. The standard cleavage assay was carried out in a 30 µl volume with 1× reaction buffer (10 mM HEPES pH 7.4, 100 mM KCl and 10 mM EDTA) and human MBD4 or human TDG (0.2–0.5 µg of protein). To enhance cleavage activities, 0.6 µg of human

His6-tagged apurinic endonuclease (APE1) was added to the reaction mixture. The mixture was incubated at 37°C for 1 h followed by phenol/chloroform extraction. After ethanol precipitation, the mixture was incubated with 50 µl of 1 M piperidine at 37°C for 20 min. The solution was lyophilized and the DNA pellet dissolved in formamide loading dye. The cleavage of the oligomer was analyzed by denaturing polyacrylamide gel electrophoresis. The bands were detected and quantitated by phosphorimaging.
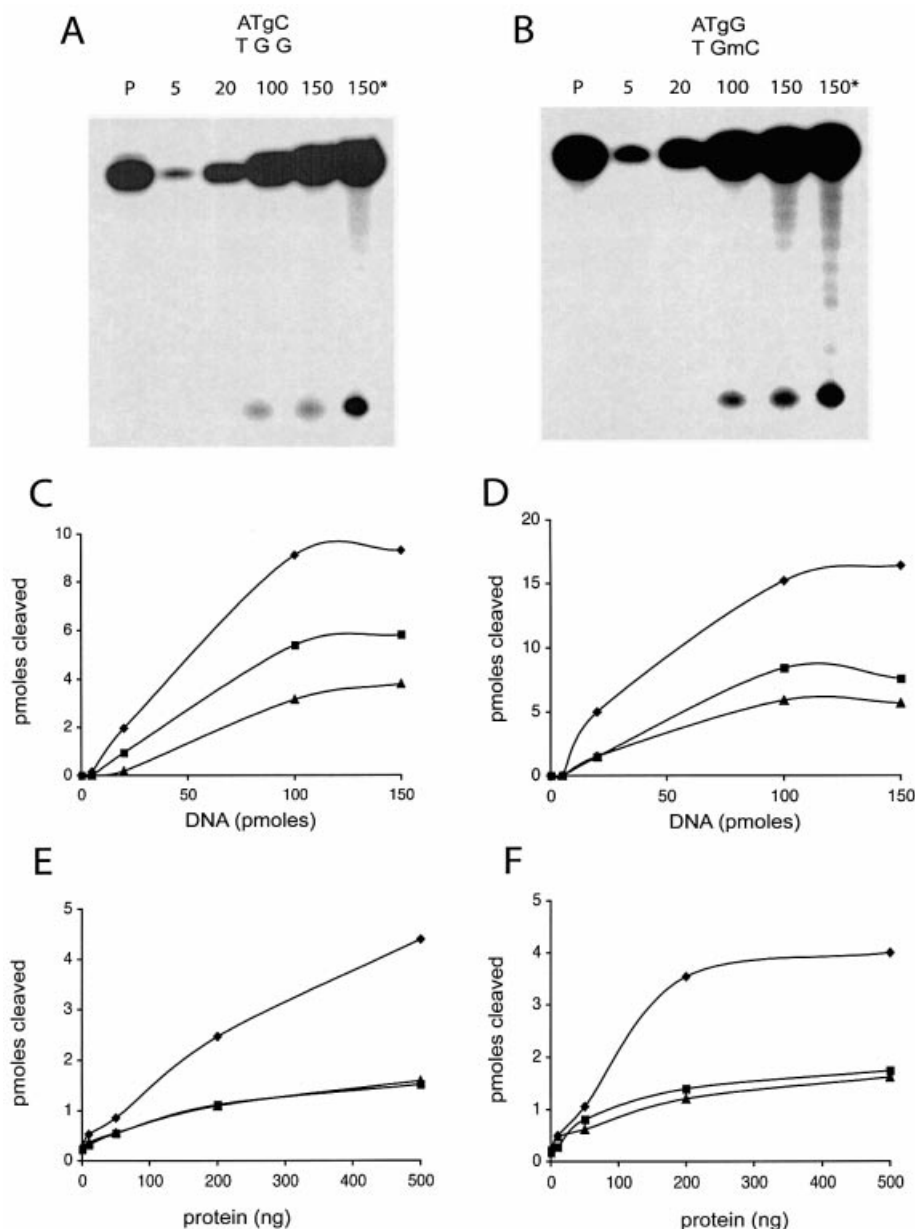
Figure 3. Enzyme substrate plots for TDG and MBD4 with two thymine glycol oligonucleotides. (**A**) A 200 ng aliquot of MBD4 was incubated for 30 min with different amounts of substrate (Tg:G mismatch in the ATgC sequence context). The lane labeled with 150* contains 150 pmol of Tg oligonucleotide but an additional 200 ng of MBD4 protein was added after 30 min. (**B**) Same as in (A) except that the oligonucleotide has Tg in the methylated CpG sequence context. (**C**) Quantification of the MBD4 reaction (200 ng of protein) with different substrates. (**D**) Quantification of the TDG reaction (200 ng of protein) with different substrates. (**E**) Different amounts of MBD4 were incubated for 30 min with 20 pmol of different oligonucleotide substrates. (**F**) Different amounts of TDG were incubated for 30 min with 20 pmol of different oligonucleotide substrates. All reactions contained 200 ng of hAPE1 protein. Diamonds, T:G mismatch in the ATG sequence context; squares, Tg:G mismatch in the ATgG sequence context; triangles, Tg:G mismatch in the ATgC sequence context.

RESULTS AND DISCUSSION

Oligonucleotides containing site-specific 5R-Tg residues were synthesized and used as substrates for DNA glycosylases. Tg is one of the major pyrimidine lesions produced by oxidative damage in DNA. Two *cis*-isomers (*5R,6S* and *5S,6R*) are produced in DNA in equal amounts by γ irradiation (37), but chemical oxidation of thymine produces predominantly the

5R,6S isomer (38). The *cis* isomers are in equilibrium with the *trans* isomers in solution (39).

We initially tested *5R,6S*-Tg-containing substrates using the *E.coli* DNA glycosylases Nth protein, Fpg protein and Nfo protein (Fig. 1). The Tg-containing double-stranded oligonucleotides were efficiently cleaved by the bacterial proteins, confirming the suitability of the DNA substrates for studies with the mammalian proteins. Cleavage occurred when Tg

was either paired with adenine or was opposite guanine. Cleavage of the Tg:G oligonucleotide was slightly more efficient (Fig. 1).

Recombinant human MBD4 and TDG were purified as C-terminal His-tagged proteins using Ni-NTA columns and SP-Sepharose (for MBD4) or Ni-NTA columns, SP-Sepharose and gel filtration columns (for TDG). We next tested the Tg-containing substrates with the recombinant human MBD4 and TDG proteins (Fig. 2). Both of these DNA glycosylases cleave thymine from T:G mismatches (7,16). Surprisingly, both TDG and MBD4 were able to cleave the substrates with the Tg:G base pairs. However, they did not react with substrates containing Tg opposite A (Fig. 2). Neither TDG nor MBD4 possess AP lyase activity to cleave the resulting abasic sites after removal of the base. Cleavage can be achieved by treatment of the glycosylase reaction products with alkali, such as 1 M piperidine at 37°C, which produces β elimination, or at 92°C, which results in β–δ elimination and the appearance of the shortest fragments. Alternatively, the sugar–phosphate backbone can be broken by incubation with an AP endonuclease such as hAPE1. Since several DNA glycosylases including TDG and MBD4 are prone to substrate inhibition by binding to abasic sites (17,40), the inclusion of APE1 is expected to increase the turnover rate of these enzymes as can be seen from the increased cleavage observed with TDG, and to a lesser extent MBD4, in the presence of human APE1 (Fig. 2).

In order to determine the relative activities of TDG and MBD4 towards substrates containing T:G mismatches and Tg:G base pairs, we carried out enzyme reactions using different substrates and sequences (Fig. 3). When Tg was present in place of T opposite guanine but within the same sequence context, the reaction was about half of that of the T cleavage reaction, for both MBD4 and TDG (Fig. 3C and D). The reactions of TDG and MBD4 on Tg:G substrates did not strongly depend on whether this base pair was present in the sequence context of a methylated CpG, but was slightly more efficient in the mCpG sequence (Fig. 3).

Data from the literature on T:G mismatch cleavage by TDG and MBD4 (17,40) indicate that the enzymes do not turn over due to inhibitory binding to abasic sites. Experiments in which addition of enzyme protein after an initial incubation of 30 min resulted in additional cleavage (Fig. 3A and B) suggest that both MBD4 and TDG do not turn over on Tg substrates either.

We suggest that two of the back-up DNA glycosylases for repairing Tg that become apparent in an Nth1-deficient background (25–27) are TDG and MBD4. In addition, mammalian Nei/Fpg homologs which function as a back-up for Tg:A repair may also act on Tg:G mismatches, although this has not yet been specifically tested.

Our results indicate that both TDG and MBD4 not only can remove thymine from T:G mismatches but can also repair Tgs when formed by an oxidation–deamination process operating on 5-methylcytosine (33). In analogy with thymidine oxidation, 5,6-dihydroxy-5,6-dihydro-5-methyl-2′-deoxycytidine is expected to be a major oxidation product of 5-methyl-2′-deoxycytidine (41). Saturation of the 5,6 double bond of cytosine or 5-methylcytosine promotes the deamination reaction (42,43). In fact, it was found that oxidative attack on 5-methylcytosine results almost exclusively in the formation of Tg (33). Although Tg was shown to be a
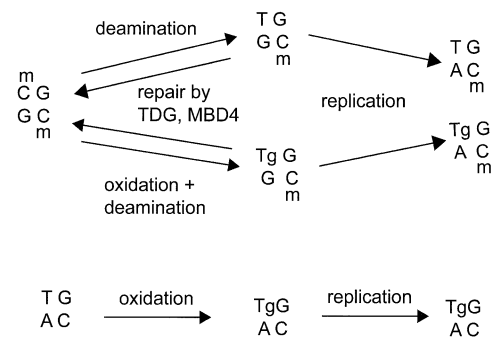
Figure 4. Schematic model for the generation of mutations induced by oxidation and deamination of 5-methylcytosine. The hydrolytic deamination of 5-methylcytosine produces a G:T mispair in duplex DNA. If deamination of 5-methylcytosine occurs through an oxidative process, thymine glycol (Tg) is formed opposite guanine. If not repaired by TDG or MBD4, these mispairs may induce C to T transition mutations by polymerase bypass. In contrast, the production of thymine glycol by oxidation of thymine may be mostly a non-mutagenic event assuming that a DNA damage-tolerant DNA polymerase bypasses this lesion correctly during replication.

replication-blocking lesion in assays using replicative DNA polymerases, it can nonetheless be correctly bypassed by DNA damage-tolerant polymerases such as DNA polymerase η, ζ and κ (44–46). If Tg arises from oxidation of thymine, the outcome would be mutation avoidance. However, if Tg arises through oxidation and deamination of 5-methylcytosine, the outcome is a mC to T transition mutation (Fig. 4). Thus, the mutagenic pathway leading from 5-methylcytosine to Tg followed by error-free bypass with incorporation of adenine across this lesion is likely to be biologically relevant. Mutations involving 5-methylcytosine at CpG dinucleotides are particularly common, and it is possible that at least a fraction of these may be produced through an oxidative damage pathway.

From our data, it is apparent that the specificity of the TDG and MBD4 enzymes is not limited to repairing deaminated 5-methylcytosine bases (and 3,N4-ethenocytosine in the case of TDG) but also extends to Tg damage produced by oxidative deamination of 5-methylcytosine.

ACKNOWLEDGEMENTS

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