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The nitrosated bile acid DNA lesion O^6-carboxymethylguanine is a substrate for the human DNA repair protein O^6-methylguanine-DNA methyltransferase

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ABSTRACT

The consumption of red meat is a risk factor in human colorectal cancer (CRC). One hypothesis is that red meat facilitates the nitrosation of bile acid conjugates and amino acids, which rapidly convert to DNA-damaging carcinogens. Indeed, the toxic and mutagenic DNA adduct O^6-carboxymethylguanine (O^6-CMG) is frequently present in human DNA, increases in abundance in people with high levels of dietary red meat and may therefore be a causative factor in CRC. Previous reports suggested that O^6-CMG is not a substrate for the human version of the DNA damage reversal protein O^6-methylguanine-DNA methyltransferase (MGMT), which protects against the genotoxic effects of other O^6-alkylguanine lesions by removing alkyl groups from the O^6-position. We now show that synthetic oligodeoxyribonucleotides containing the known MGMT substrate O^6-methylguanine (O^6-MeG) or O^6-CMG effectively inactivate MGMT *in vitro* (IC_{50} 0.93 and 1.8 nM, respectively). Inactivation involves the removal of the O^6-alkyl group and its transfer to the active-site cysteine residue of MGMT. O^6-CMG is therefore an MGMT substrate, and hence MGMT is likely to be a protective factor in CRC under conditions where O^6-CMG is a potential causative agent.

INTRODUCTION

Most sporadic colorectal cancers (CRCs) arise through an adenoma–carcinoma sequence, and the molecular pathways have been well-characterized (1,2). Known risk factors for CRC include red and processed meat (3–5), but the mechanisms by which these dietary factors modify CRC risk remain to be fully elucidated (6,7). Cooked red meat, for example, may contain carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons (3). Furthermore, faeces contain potent mutagens and other DNA-reactive agents (8,9), and studies of DNA isolated from colorectal mucosa have shown damage arising from exposure to a variety of genotoxic agents, including alkylating agents (AAs) (10–13).

AAs are a diverse set of chemicals, which include *N*-nitroso compounds that can be potent mutagens, clastogens and carcinogens (14,15). Human exposure to

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such agents is unavoidable and may arise from ingestion of pre-formed AAs or through *in situ* formation, typically mediated by the bacterial or chemical nitrosation of compounds containing amino groups (15). Red and processed meat intake increases *N*-nitrosation within the colon, generating increased levels of *N*-nitroso compounds and also *S*-nitrosated compounds (16).

The toxicological effects of AAs can be attributed largely to their ability to alkylate DNA, and the biological properties of some of the alkyl DNA adducts formed, especially O^6-alkylguanine lesions, are well-characterized (17). O^6-methylguanine (O^6-MeG) is a known toxic, mutagenic and carcinogenic base modification in DNA that, in the absence of repair, can induce GC→AT transition mutations and recombinations in the form of sister chromatid exchanges (18). Human CR DNA is known to contain O^6-MeG, the levels of which vary 100-fold, with the highest occurring in the sigmoid colon and rectum, where most sporadic tumors occur (13,19). These levels are sufficiently high to cause adverse biological effects, particularly in cells that are deficient in DNA repair (20). In addition, exfoliated colon cell DNA contains O^6-carboxylmethylguanine (O^6-CMG), probably arising from *N*-nitrosation of the bile component glycocholic acid (21), which also generates O^6-MeG in DNA (22). That the levels of O^6-CMG in DNA from exfoliated colonic cells increase with a red meat diet strongly implies a role in CRC (21).

O^6-MeG is eliminated from DNA by the DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) in a stoichiometric process that results in the transfer of the methyl group to Cys145 in the active site of the protein (18,23,24). MGMT overexpression in cell and animal models thus protects against the mutagenic, carcinogenic and toxic effects of AAs (25). In human colorectal mucosa, MGMT activity is highly variable, in part due to exposure to AAs and dietary factors but also as a consequence of MGMT polymorphisms, which have been found to modify CRC risk, depending on the diet (26–28). Furthermore, colorectal tumors can occur in gastrointestinal regions expressing low MGMT activity, and low activity in normal colon tissue has been associated with the presence of K-*ras* GC→AT transition mutations in colorectal tumors (29,30). In addition, cytosine-methylation of CpG islands within the promoter region of the MGMT gene is associated both with reduced MGMT expression and with an increased frequency of GC→AT transition mutations in K-*ras* in CRCs (31–33). Indeed, adenomas containing a K-*ras* GC→AT mutation have lower MGMT levels (relative to adjacent normal tissue) than adenomas without this mutation (33). As MGMT removes O^6-alkylguanine lesions from DNA, these observations strongly support the hypothesis that AAs are involved in the aetiology of at least a proportion of CRC.

Although MGMT is known to have broad substrate specificity, an earlier report using cell-free extracts from *Escherichia coli* overexpressing the *E. coli* O^6-alkylguanine-DNA alkyltransferase-encoding genes *ada* or *ogt* and a human cell line expressing endogenous MGMT suggested that alkyltransferases do not act on O^6-CMG in DNA (22). Subsequent studies suggested that O^6-CMG was processed by the nucleotide excision repair pathway (34). Given the compelling evidence for a role for MGMT in protecting against CRC, this would suggest that O^6-CMG is unlikely to be a significant factor in CRC risk, despite the observation that its abundance increases in high-risk diet situations.

To address this apparent inconsistency, we have prepared short oligodeoxyribonucleotides (ODNs) containing O^6-alkylguanines (35) and used these as substrates for purified *E. coli* Ogt and MGMT proteins. We found that an O^6-MeG-containing ODN inactivated both alkyltransferases with similar efficiency. However, although the O^6-CMG-containing ODN was a poor substrate for Ogt, it was an effective inactivator of MGMT. To confirm this observation, we used a combination of methods and show that, *in vitro*, the CM group is removed from O^6-CMG, that this regenerates guanine and that the CM group is transferred to the active-site cysteine residue in MGMT to generate *S*-carboxymethylcysteine (CM-cysteine). Thus O^6-CMG is a substrate for MGMT. These results are discussed in relation to the hypothesis that O^6-CMG is a human colorectal carcinogen and that MGMT is a major protection factor.

MATERIALS AND METHODS

Expression and purification of MGMT, Ogt and Atl1

Proteins were expressed as maltose-binding protein (MBP) fusion proteins from pMAL-2c expression vector constructs essentially as described by Pearson *et al.* (36), with minor modifications as follows. Single bacterial colonies were inoculated into 100 ml of rich medium with glucose [w/v: 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose, containing 100 μg/ml ampicillin (Sigma)] and incubated at 37°C overnight in an orbital shaking incubator. Twenty millilitres of this culture were then used to inoculate 4 l of the same medium. The culture was grown to an optical density of 0.6 (at 600 nM) and then isopropylthiogalactoside from a 1 M stock solution was added to a final concentration of 0.4 mM, to induce protein expression. Cells were incubated for a further 3 h at 37°C and then harvested by centrifugation at 2500×*g* for 10 min. Cell pellets were washed with 20 mM Tris-HCl (pH 8.3) and re-suspended in 20 ml of binding buffer [BB: 20 mM Tris-HCl (pH 8.3), 200 mM NaCl, 1 mM EDTA]. Extracts were prepared by sonication (four 20 s pulses), with cooling on ice for 1 min between pulses. The extracts were then centrifuged at 17 000×*g* for 20 min and the supernatants pooled. The protein concentration of the extract was determined by the Bradford assay.

Amylose resin (New England Biolabs (NEB), MBP binding capacity 3 mg/ml) was pre-equilibrated with BB. The bacterial extract was diluted to 2.5 mg/ml in a total volume of 50 ml of BB, applied, washed with BB and eluted in 1-ml fractions using BB containing 10 mM maltose. The protein concentration of the eluted fractions was then determined by Bradford assay.

MBP-Atl1 fusion protein (40 mg) was cleaved with 0.1%w/v of Factor Xa (1 mg/ml, NEB) at room temperature for 2 h. The efficiency of the reaction was assessed by

resolving the cleavage products on a 15% sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE). The cleavage reaction was then applied to a Superdex 200 column (10 × 300 mm; GE Heathcare) that was pre-equilibrated with 50 mM Tris-HCl (pH 8.3), 100 mM NaCl. The column was eluted at a flow rate of 0.8 ml/min, and 1.6-ml fractions were collected. Protein elution was monitored by absorption at 215 nm using a flow cell. Pooled MBP-Atl1-containing fractions were further purified through amylose columns to remove remaining uncleaved MBP fusion protein.

The other MBP fusion proteins were cleaved using factor Xa (NEB, 1 μg) with 5 μl of 10× concentrated buffer (NEB), 50 μg of MBP-MGMT and MBP-Ogt proteins and 1 mM dithiothreitol (DTT) in a total volume of 50 μl for 6 h at room temperature. The efficiency of the digest was analysed on 12% SDS–PAGE with Coomassie staining. The digestion mixtures were applied to DEAE-Sepharose (Sigma) columns (2 ml bed volume) that were pre-equilibrated with 10 mM Tris-HCl containing 25 mM NaCl (pH 8.0). The column was then washed with the same buffer for five column volumes and then eluted with five 1-ml fractions of 10 mM Tris-HCl containing 50 mM NaCl and then 75 mM NaCl. MGMT- and Ogt-containing fractions were pooled and applied to amylase columns to remove MBP and any remaining uncleaved MBP fusion protein. The protein concentration of the eluted fractions was determined by measuring the absorption at 280 nm using a Nanodrop ND-100 spectrophotometer, and those containing protein were analysed on 12% SDS–PAGE with Coomassie staining.

Determination of specific activity of MGMT and Ogt

The activities of the MGMT and Ogt proteins were determined by quantifying the amount of radioactivity transferred to protein from [^{3}H]-methylated substrate DNA (100 μl containing 100 fmoles O^6-MeG) at 37°C for 1 h. After incubation, 100 μl of 10 mg/ml bovine serum albumin (BSA) was added, and the DNA was hydrolysed in a total volume of 2.5 ml of 1 M perchloric acid (PCA) by heating at 77°C for 50 min. Samples were centrifuged at 2800 r.p.m. for 10 min at 20°C and the supernatant aspirated. The precipitate was washed with 4 ml of 1 M PCA and then 300 μl of double-distilled water and 2 ml of scintillation fluid (Ecoscint: Mensura Technology) were added. Radioactivity was quantified by scintillation counting.

Synthesis and purification of modified ODNs

ODNs of sequence 5′-GCCATGG*CTAGT, 5′-SIMA-G CCATGG*CTAGT or 5′-GAACTG*CAGCTCCGTGC TGGCCC where G* was G, O^6-MeG or O^6-CMG were synthesized using commercially available phosphoramidites and sulphone phosphoramidite (35) on an Applied Biosystems 394 automated synthesizer. For incorporation of O^6-CMG, base-labile phosphoramidites were used, and ODNs containing 2-amino-6-methylsulphonylpurine at the G* position were initially synthesized and then converted to O^6-CMG-containing ODNs using methylglycolate, as described (35,37). The ODNs were purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by electrospray ionisation mass spectrometry. Complementary ODNs containing 3′-biotin or 3′-HEX were synthesized by Sigma.

Inactivation of MGMT by modified ODNs

Purified MGMT or Ogt (100 fmoles) were incubated with varying concentrations of ODN (0–24.4 nM) in a total volume of 200 μl of buffer I (50 mM Tris-HCl pH 8.3 containing 1 mM EDTA and 3 mM DTT) for 1 h at 37°C, followed by the addition of excess [^{3}H]-methylated substrate DNA and processing, as described above. Results were used to calculate IC_{50}, i.e. the amount of ODN required to reduce MGMT activity to 50%, and were the means (± SD) of triplicate measurements.

Mass spectral analysis of alkyl group transfer to MGMT

Control (G) and O^6-alkG-containing ODNs (5′-SIMA-G CCA TGG* CTA GTA-3′, where G* is G or O^6-MeG or O^6-CMG) were annealed to the complementary oligonucleotide (5′-TAC TAG CCA TGG C-3′) in a volume of 20 μl of 50 μM NaCl by heating to 80°C for 5 min and cooling to room temperature (>2 h) and then transferred to ice. ODNs (130 pmole) were incubated with MGMT (130 pmole by activity) in 28 μl of buffer I for 1 h at 37°C. Trypsin (1 μg; ratio of MGMT:trypsin, 50:1) was added and the reaction was incubated overnight at 37°C. The digestion was terminated by the addition of 100% formic acid to 0.1% (v/v). MALDI-TOF analyses were performed on a Bruker Ultraflex™ (Bruker Daltonics, Bremen, Germany). Full scans of the peptide mixture from 800 to 4000 m/z and tandem mass spectral data of selected ions were collected with α-cyano-4-hydroxycinnamic acid as the matrix. External calibration was performed with QCAL standard (38).

Alkyl group removal assessed by ELISA

ODNs were annealed to 3′-biotinylated complement, as described previously, and immobilized on streptavidin-coated 96-well plates (80 fmoles/well in PBS/5 mg/ml BSA). Increasing amounts of MGMT (0–210 fmoles) in 100 μl buffer I were added to the wells and incubated at 37°C for 1 h. The plates were washed three times with 100 μl PBS/3 mg/ml BSA. ODNs were detected using Atl1 as a reagent or antisera to O^6-CMG. In the former case, plates were incubated with 1 pmole of Atl1 protein in 100 μl PBS/3 mg/ml BSA for 1 h at room temperature, followed by sequential 1-h room temperature incubations with anti-Atl1 antisera (1/500 in PBS/3 mg/mL BSA, the generation and characterization of which will be reported elsewhere) and then goat anti-rabbit HRP (1/1000 in PBS/3 mg/ml). In the latter case, rabbit polyclonal antisera to O^6-CMG were obtained by immunization with O^6-CMG coupled to keyhole limpet haemocyanin (Biogenes). Western Lightning reagent (Perkin Elmer) was added and binding quantified by measurement of chemiluminescence on a Tecan GENios plate-reader. Binding was expressed as percentage of control values obtained without

added MGMT. Results were the means (±SD) of triplicate measurements.

Alkyl group removal assessed by restriction endonuclease site deprotection

Control (G) and O^6-alkG-containing ODNs were annealed to the complementary ODN (terminating with 5′-HEX or biotin as appropriate), as previously described. The resulting double-stranded ODNs (7 pmoles) were incubated with 21 pmoles MGMT in a total volume of 17 µl of IBSA (Buffer I containing 0.1% BSA) at room temperature for 3 h and then subjected to digestion with NlaIII, CviKI, StyI, BsaJI or PstI in the buffer provided by the manufacturer (New England Biolabs) in a total volume of 10 µl at room temperature for 1 h. Non-denaturing PAGE gel-loading buffer (30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue: 2 µl) was added to each sample, which was then applied to a 20% PAGE gel and subjected to electrophoresis at 100 volts for 1 h. The gels were scanned on a Pharos phosphorimager (BioRad) using the fluorescent gel scanner settings.

RESULTS

Inhibition of MGMT by O^6-alkG-containing ODNs

Duplex ODNs containing O^6-MeG and O^6-CMG, but not the control ODN (which contained G in place of the alkylated base), potently inhibited the action of MGMT on [^{3}H]-methylated substrate DNA. Under the assay conditions used, the IC_{50} (50% inhibition) values for O^6-MeG- and O^6-CMG-containing ODNs were 0.9 and 1.7 nM, respectively (Figure 1). In contrast, the *E. coli* Ogt protein that was assessed in previous studies (22), while having a similar IC_{50} for the O^6-MeG-containing ODN (IC_{50} 1.7 nM), was substantially more resistant to inhibition by the O^6-CMG-containing ODN (IC_{50} 38 nM: Figure 1).

Mechanism of MGMT inhibition

The inhibition of MGMT activity by O^6-CMG-containing ODN may have been either competitive inhibition, as a consequence of the ODN binding tightly to MGMT, or inactivation, due to alkyl group transfer from the O^6-CMG to MGMT. In the former case, the lesion would still be present in the ODN after incubation with MGMT. We therefore used ELISA methodology to examine the effect of increasing amounts of MGMT on the levels of O^6-CMG remaining in the ODN after incubation and we compared this with O^6-MeG-containing ODN. We exploited the ability of the *Schizosaccharomyces pombe* Atl1 protein to bind to both of these lesions and detected this binding using an anti-Atl1 antiserum. We also used an anti-O^6-CMG antiserum that we generated and showed to be suitable for this purpose (Supplementary Figure S1). Increasing amounts of MGMT produced an almost linear decrease in the enhanced chemiluminescence signals generated by both the Atl1-based (Figure 2A) and anti-O^6-CMG antibody-based (Figure 2B) detection methods, strongly suggesting that alkyl groups are removed from the ODN.

There is ample literature to support this conclusion for DNA containing O^6-MeG. However, for O^6-CMG, about which considerably less is known, we could not exclude the possibility that strong binding of MGMT to this lesion was blocking access of Atl1 or the anti-O^6-CMG antibody or *in situ* modification of the lesion had ocurred so that it was no longer detected by these reagents.

Alkyl group removal assessed by restriction endonuclease site restoration

We reasoned that if MGMT repairs O^6-CMG by alkyl group transfer to the active-site cysteine residue, as it does for O^6-MeG, this would result in the generation of

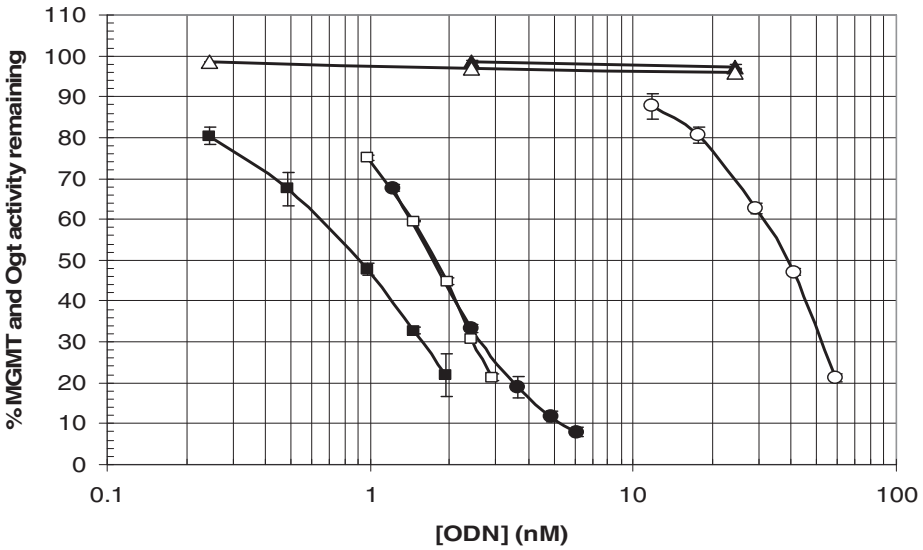

Figure 1. Inactivation of MGMT (closed symbols) and Ogt (open symbols) by pre-incubation with increasing concentrations of ODN (5′-GCCATG**G***CTAGTA) containing O^6-MeG (squares) or O^6-CMG (circles) but not G (triangles) for 1 h; values shown are means ± SD. of triplicate determinations. See Materials and Methods for details.

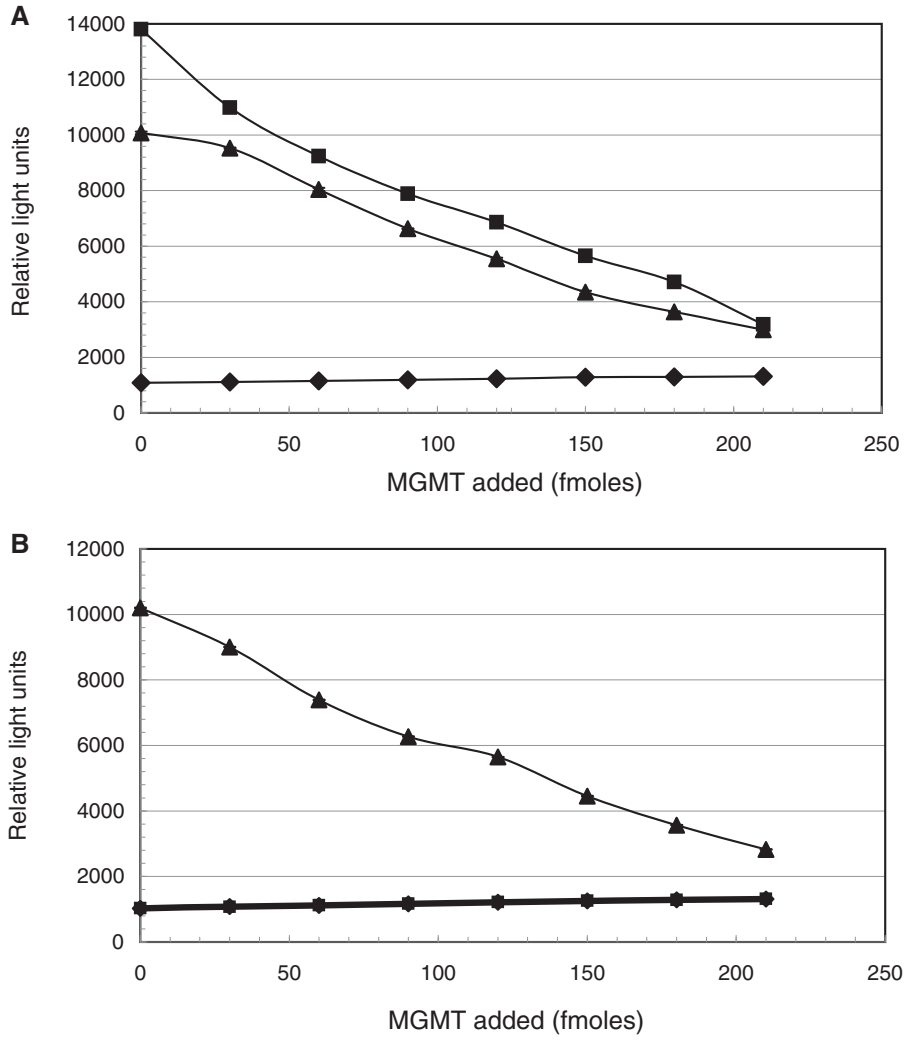

Figure 2. Incubation of duplex ODNs containing O^6-MeG (squares) or O^6-CMG (triangles) with MGMT decreases the binding of Atl1 (**A**) and anti-CMG antibody (**B**). No binding to control (G-containing) ODN (diamonds) was seen. To prepare the substrates, ODNs (5′-GCCATG**G***CTAGTA, where G* is G, O^6-MeG or O^6-CMG) were annealed to complementary ODN containing 5'-terminal biotin and then bound to streptavidin-coated microtitre plates. After incubation with MGMT, binding of Atl1 or anti-O^6-CMG to the ODN was monitored, as described in Materials and Methods.

guanine in the ODN. The ODN sequence used in these studies (5′-GCCATG**G***CTAGTA, where G* is G, O^6-MeG or O^6-CMG) contained several restriction endonuclease sites (Supplementary Figure S2). Of these, we firstly demonstrated that *Bsa*JI was able to differentiate between G and O^6-alkylG (Supplementary Figure S3). We then showed that pre-incubation of the ODN with MGMT almost completely restored the blocked *Bsa*JI sites in both the O^6-MeG- and O^6-CMG-containing ODNs (Figure 3A). The same effect was seen with a different ODN sequence (5′-GAACT**G***CAGCTCCGTGCT GGCCC) in which the O^6-alkylG was part of the *Pst*I recognition sequence (Figure 3B). Thus the action of MGMT on ODN containing either O^6-MeG or O^6-CMG in two different sequence contexts results in the removal of both the methyl and the carboxymethyl group, resulting in the formation of guanine within the ODN.

Mass spectrometric analysis of alkyl group transfer to MGMT

To demonstrate that the action of MGMT resulted in the transfer of the alkyl group to the active-site cysteine residue in the protein, we undertook MALDI-TOF mass spectrometry. Tryptic digests of MGMT generated multiple peptides, the tryptic fragment (Tp20) containing the active cysteine (GNPVPILIP***C***HR) producing an $[M+H]^+$ ion with m/z 1315.7 (Figure 4A), the identity of which was confirmed by tandem mass spectrometry. The expected mass changes of this tryptic fragment if an alkyl group had been transferred are 14 (methyl) and 57 (carboxymethyl *i.e.* $CH_2CO_2^-$) producing $[M+H]^+$ ions at m/z 1329.7 and 1372.7, respectively (Figure 4B and C). The results of these analyses indicate that, as with the methyl group of O^6-MeG, the carboxymethyl group of O^6-CMG is indeed transferred to the active-site cysteine residue in MGMT, forming *S*-carboxymethylcysteine.

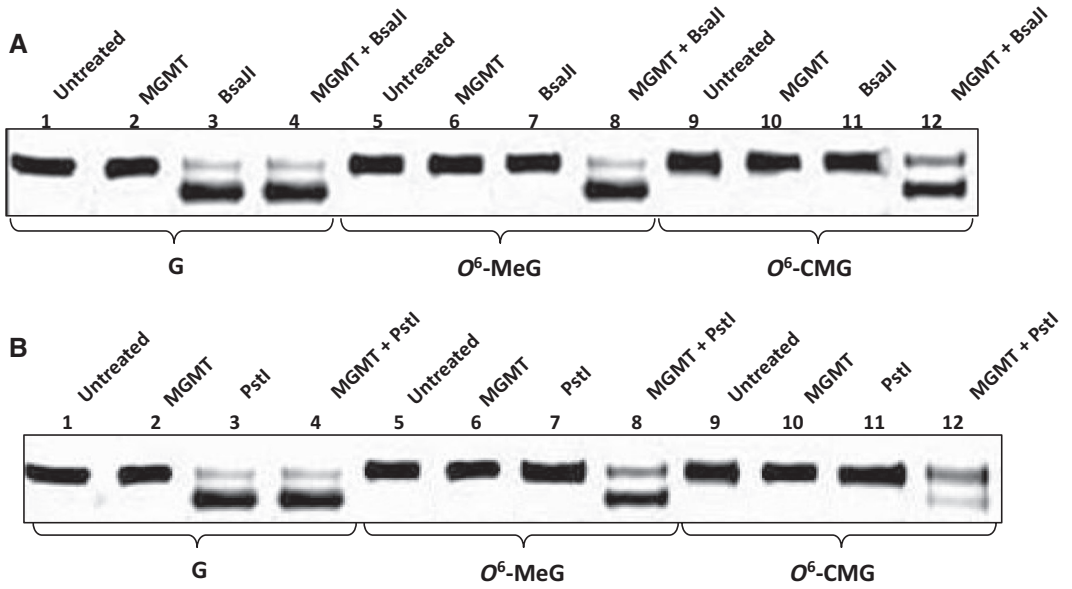

Figure 3. Pre-incubation with MGMT of duplex ODN containing O^6-MeG and O^6-CMG enables cleavage by (**A**) *Bsa*J1 (in duplexed 5′-GCCATGG*CTAGTA) and (**B**) *Pst*I (in duplexed 5′-GAACTG*CAGCTCCGTGCTGGCCC). Lanes 1-4, 5-8 and 9-12 show G-, O^6-MeG- and O^6-CMG-containing duplex ODNs, respectively; lanes 1, 5 and 9 show untreated ODN (7 pmoles); lanes 2, 6 and 10 show ODN incubated with MGMT (21 pmoles) only, and then digested with *Bsa*JI or *Pst*I; lanes 3, 7 and 11 show ODN incubated with *Bsa*JI or *Pst*I only; and lane 4, 8 and 12 show ODN incubated with MGMT and then digested with *Bsa*JI or *Pst*I. Non-denaturing PAGE gels were imaged as described in Materials and Methods.

DISCUSSION

There is considerable evidence to implicate O^6-CMG in the aetiology of human CRC and to suggest that lower levels of MGMT activity in more susceptible regions of the gut are a predisposing factor in this process. However, the limited previous data suggest that O^6-CMG is not a substrate for alkyltransferases (22,24), so it is not clear why decreased MGMT activity would result in predisposition to CRC if O^6-CMG is not a substrate for MGMT: thus there appears to be no correlation between these two factors. Our recent development of a generic method to generate ODNs containing specific O^6-alkG residues (35) has allowed us to readdress this question. MGMT substrates have mostly been assessed through the inactivation or inhibition of MGMT, and to a lesser extent the re-generation of G in the substrate or the transfer and covalent attachment of the alkyl group to the cysteine residue in the active site of MGMT. Such studies have shown that MGMT has a broad substrate range, acting on O^6-alkylguanines with a variety of alkyl substituents but at different rates. For the straight-chain alkyl series, the efficiency of alkyl transfer decreases with increasing chain length (23,39,40). However, this is not simply a question of alkyl group size, because O^6-benzylG and O^6-bromothenylG are better substrates than O^6-MeG when present in short ODNs (35,41).

Previously, DNA treated with the nitrosated bile acid *N*-nitrosoglycocholic acid (NOGC) was shown to inhibit the action of the two *E. coli* alkyltransferases encoded by the *ada* and *ogt* genes, and also MGMT (22). NOGC can both methylate and carboxymethylate DNA, and to determine whether repair of both lesions was taking place, HPLC was used to analyse enzymic hydrolysates of NOGC-treated DNA after incubation with alkyltransferases. It was thus found that although O^6-MeG was a substrate for the *E. coli* ada protein, it was unable to act on DNA containing O^6-CMG. From this observation it was concluded that O^6-CMG was not recognized by any alkyltransferases (22). Later studies in human cells treated with azaserine or potassium diazoacetate showed that MGMT did not provide measurable protection against the toxicity of these agents, whereas the nucleotide excision repair pathway did, again suggesting that O^6-CMG is not a substrate for MGMT (34).

In the present report, we have used a variety of methods to analyse ODN and MGMT protein and for the first time conclusively demonstrate that O^6-CMG is in fact a substrate for MGMT. Indeed ODNs containing O^6-CMG inactivated MGMT almost as effectively as those containing O^6-MeG; both of these lesions were converted to G; and both of the alkyl groups became covalently attached to the cysteine residue in the MGMT tryptic peptide encompassing the active site. It is reasonable to conclude that the previous report implying that O^6-CMG is not a substrate for alkyltransferases in general was an extrapolation from bacterial to mammalian MGMT that, under the conditions used, was experimentally unsupported. Indeed the present results show that the *E. coli* Ogt protein is >20 times more resistant than MGMT to inactivation by O^6-CMG-containing ODNs but similarly susceptible to inactivation by O^6-MeG-containing ODN and this probably explains the previous observation.

Promoter hypermethylation of MGMT, which results in the down-regulation of MGMT expression, is associated with the presence of G:C to A:T transition mutations in

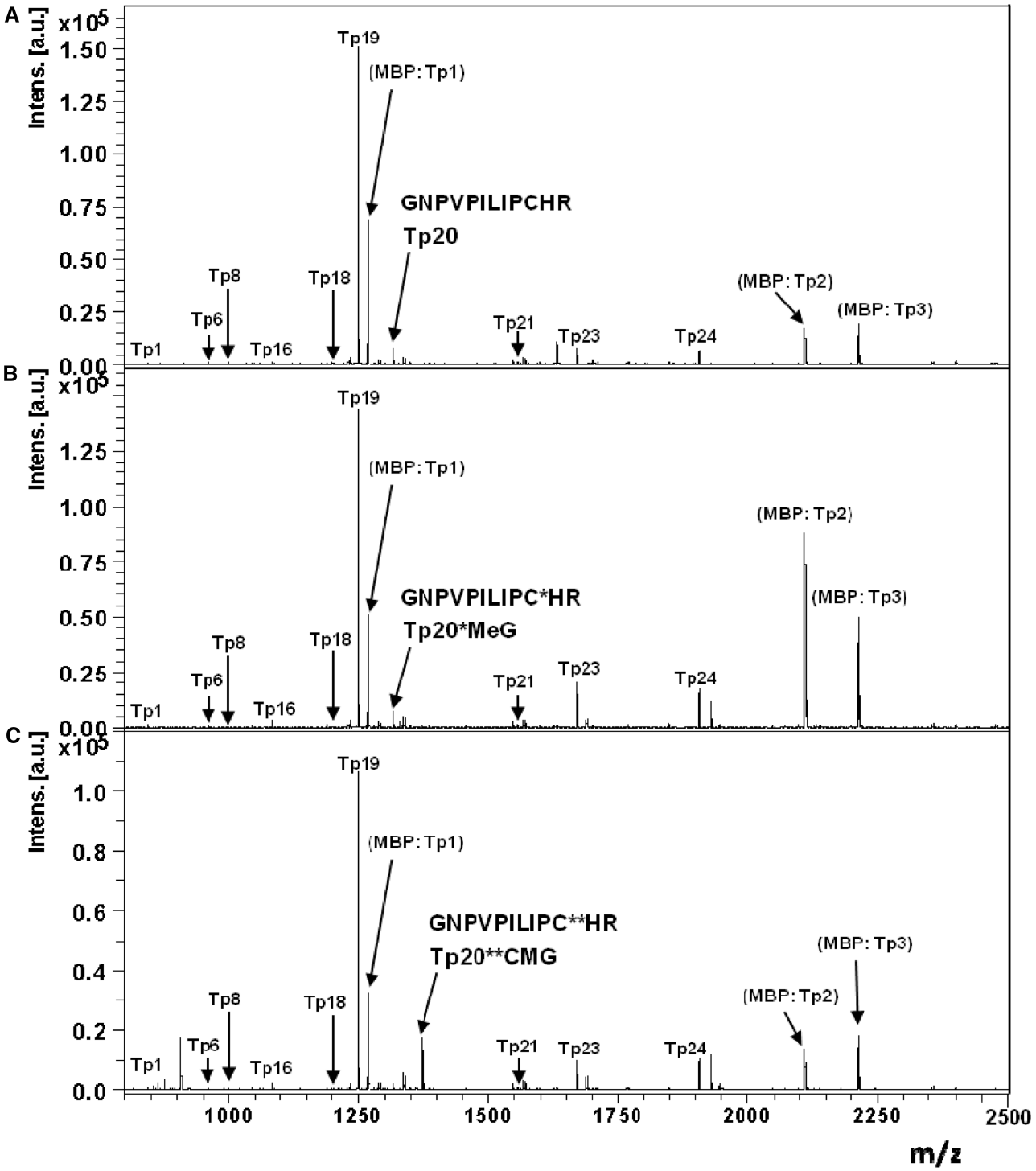

Figure 4. MALDI-TOF mass spectral analysis of tryptic (Tp) peptides of unmodified MGMT (**A**; control) or MGMT incubated with ODNs containing O^6-MeG (**B**) or O^6-CMG (**C**). Modified peptides (Tp20* MeG and Tp20** CMG) are observed at *m/z* 1329.7 and 1372.7 in (B) and (C), respectively. MBP describes tryptic peptides derived from the MBP affinity tag on MGMT.

p53 in human colorectal tumorigenesis and hence considered an important risk factor in this disease (42). Another risk factor is dietary red meat (4,6), which has been shown to increase the formation of O^6-CMG in DNA (21). These two risk factors seem at variance with the earlier reports that O^6-CMG is not a substrate for MGMT. However, our present results clearly demonstrate that O^6-CMG is almost as effective a substrate for MGMT as is O^6-MeG. Thus, decreased levels of MGMT would be expected to increase the risk of malignant transformation initiated by the presumed promutagenic lesion (43) O^6-CMG. These observations

therefore support the concept that MGMT may play a key role in protection against human CRC by repairing not only O^6-MeG but also O^6-CMG.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3 and Supplementary references [44, 45].

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