Conformational Change in Human DNA Repair Enzyme O^6-Methylguanine-DNA Methyltransferase upon Alkylation of Its Active Site by SN1 (Indirect-Acting) and SN2 (Direct-Acting) Alkylating Agents: Breaking a "Salt-Link"?†

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Received February 14, 1996; Revised Manuscript Received June 13, 1996⊗

ABSTRACT: Human O^6-methylguanine-DNA methyltransferase (MGMT) repairs DNA by transferring alkyl (R-) adducts from O^6-alkylguanine (6RG) in DNA to its own cysteine residue at codon 145 (formation of R-MGMT). We show here that R-MGMT in cell extracts, which is sensitive to protease V8 cleavage at the glutamic acid residues at codons 30 (**E**30) and 172 (**E**172), can be specifically immunoprecipitated with an MGMT monoclonal antibody, Mab.3C7. This Mab recognizes an epitope of human MGMT including the lysine 107 (**K**107) which is within the most basic region that is highly conserved among mammalian MGMTs. Surprisingly, the **K**107**L** mutant protein is repair-deficient and readily cleaved by protease V8 similar to R-MGMT. We propose that R-MGMT adopted an altered conformation which exposed the Mab.3C7 epitope and rendered the protein sensitive to protease V8 attack. This proposal could be explained by the disruption of a structural "salt-link" within the molecule based on the available structural and biochemical data. The specific binding of Mab.3C7 to R-MGMT has been compared with the protease V8 method in the detection of R-MGMT in extracts of cells treated with low dosages of methyliodide (SN2) and O^6-benzylguanine. Their identical behaviors in producing protease V8 sensitive R-MGMT and Mab.3C7 immunoprecipitates suggest that probably methyl iodide (an ineffective agent in producing 6RG in DNA) can directly alkylate the active site of cellular MGMT similar to O^6-benzylguanine. The effectiveness of MeI in producing R-MGMT, i.e., inactivation of cellular MGMT, indicates that this agent can increase the effectiveness of environmental and endogenously produced alkylating carcinogens in producing the mutagenic O^6-alkylguanine residues in DNA *in vivo*.

Alkylation of the exocyclic oxygen of guanine in DNA, whether by environmental exposure or by cancer chemotherapeutic agents, is repaired by O^6-methylguanine-DNA methyltransferase [MGMT, reviewed by Yarosh (1985) and Pegg (1990)], which transfers the alkyl group from O^6-alkylguanine residues in DNA to its own cysteine centered in the active site. This active site alkylated protein, R-MGMT, is inactive and is not regenerated to MGMT. In humans the expression of MGMT is barely inducible, and therefore the repair capacity of a cell is determined by the amount of MGMT and its constitutive rate of synthesis.

MGMT rapidly repairs O^6-methyl- and ethylguanine residues in DNA, although longer chain or bulkier derivatives are repaired with lesser efficiency. It is generally believed that the observed loss of MGMT repair activities, or the formation of R-MGMT, in mammalian cells treated with alkylating agents are the result of MGMT repair of the O^6-alkylguanine residues produced in the DNA by alkylating agents, i.e., by an indirect pathway. However, the observation that O^6-benzylguanine (6BG), which does not damage DNA, can effectively inactivate MGMT in human cells at micromolar concentrations (Dolan *et al*., 1990) suggests that cellular MGMT can be directly alkylated at its active site without DNA damage. It is of particular interest to examine whether alkylating agents, apart from damaging DNA, can directly inactivate cellular MGMT as compared to the observation *in vitro* (Brent, 1986). This can be studied by comparing the effectiveness of low dosages of SN1 and SN2 alkylating agents in producing R-MGMT (or a loss of MGMT activity) in cells, since SN2 alkylating agents are inefficient in producing O^6-alkylguanine in DNA (Lawley, 1984). This study is also relevant to human cancer because SN2 agents such as methyl halides are potential human carcinogens (Foster *et al*., 1985) which are common in the environment (Harper, 1985; Gschwend *et al*., 1985). Such study, however, requires a sensitive method to distinguish inactive R-MGMT from active MGMT.

R-MGMT is stable in purified preparations and is not spontaneously degraded (Belanich *et al*., 1994; Pegg *et al*., 1991). The fate of R-MGMT *in vivo*, however, varies among cells. Immunoreactive R-MGMT is lost within the first few hours from HT29 colon adenocarcinoma cells (Belanich *et al*., 1994; Pegg *et al*., 1991) and human rhabdomyosarcoma xenografts grown in mice (Belanich *et al*., 1996), whereas

† This project is supported by the National University of Singapore and the National Science and Technology Board of Singapore.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: MGMT, O^6-methylguanine-DNA methyltransferase; R-MGMT, active-site alkylated O^6-methylguanine-DNA methyltransferase; GSTMGMT, glutathione-*S*-transferase fusion protein of MGMT; Mab, mouse monoclonal antibodies; NMU, *N*-methylnitrosourea; MeI, methyliodide; 6MG, O^6-methylguanine; 6RG, O^6-alkylguanine; 6BG, O^6-benzylguanine; ECL, enhanced chemiluminescence; wt, wild type.

S0006-2960(96)00363-7 CCC: $12.00

R-MGMT in human peripheral lymphocytes and T-cell lymphoma cells can be detected in cells several hours after exposure to alkylating agents (Ayi *et al*., 1994). The reasons behind these observations remain unclear. However, the methylation of the active site cysteine residue at codon 145 of the human protein (an apparently subtle modification) enables the preferential cleavage of the alkylated protein by protease V8 at codons **E**30 (at the N-terminus) and **E**172 (at the C-terminus), see Ayi *et al*. (1994). These observations suggest that a conformational change exposes new sites for or otherwise facilitates proteolysis: a possible model for the fate of R-MGMT *in vivo*. However, further studies are required to show whether there are further changes in the R-MGMT. Understanding the biological fate of R-MGMT is important because R-MGMT in peripheral blood cells with low turnover reflects the exposure to alkylating agents, such as volatile *N*-nitroso compounds from occupational exposure (Oesch & Klein, 1992) and chloroethylnitrosourea drugs used in chemotherapy (Pegg, 1990).

We report here the identification of a unique epitope exposed in R-MGMT, which enables an MGMT monoclonal antibody (Mab.3C7) to selectively immunoprecipitate R-MGMT from cell extracts in the presence of active MGMT. This observation supports further the concept of conformational changes in the repair protein upon alkylation of its active site. We discussed the possible mechanism behind these conformational changes on the basis of the available structural and biochemical data. By combining the protease V8 and Mab.3C7 immunoprecipitation techniques in the detection of R-MGMT, it is possible to show that R-MGMT can be readily produced in human cells by SN2 alkylating agents that are ineffective in producing 6RG residues in DNA, suggesting that these agents might alkylate directly the active site of human MGMT *in vivo*.

MATERIALS AND METHODS

Antibodies, Cell Culture, and Treatment with Alkylating Agents.

Polyclonal antibodies to MGMT (Pab.MGMT) and monoclonal antibodies Mab.5H7 and Mab.2G1 were produced as previously described (Ayi *et al*., 1992, 1994). Mab.3C7 was purified from tissue culture supernatant of clone 3 (from a limiting-dilution) using Protein-G Sepharose (Pharmacia, Sweden). Mab.3C7 preferentially recognizes the active-site methylated recombinant human MGMT (Ayi *et al*., 1992) as compared to the native MGMT and GST−MGMT fusion protein in ELISA assays. The procedures for cell cultures, drug treatments, total cell extract preparations, and protease V8/Western-blotting analyses of R-MGMT were as described previously (Ayi *et al*., 1992, 1994). Alkylating agents were prepared as 1 M stock solutions in DMSO, whereas 6BG was 80 mM.

Immunoprecipitation/Western-Blotting Analysis

Total cell extracts (200 μg), in duplicate, were made to 100 μL with immunoprecipitation buffer (IB, 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5 mM DDT). The mixtures were incubated with 0.5 μg Mab.3C7 at 4 °C overnight. Anti-mouse IgG linked to agarose (20 μL of 50% beads, Sigma) were then added to the reaction mixtures followed by 400 μL IB. The suspensions were shaken for 1 h at 4 °C and followed by centrifugation at 1000*g*. The recovered agarose beads containing the immunoprecipitates were washed three times with 500 μL of cold IB. After removal of the supernatants from the beads, 20 μL of Laemmli loading buffer was added. The suspensions were boiled for 10 min, and the contents of the duplicated samples were then mixed and resolved on a 13.5% SDS−PAGE. Protein-G Sepharose purified rabbit Pab.MGMT, which does not discriminate between the active MGMT and R-MGMT in Western analysis, was used to visualize the immunoprecipitated MGMT.

Epitope-Mapping of Mab.3C7

The rat MGMT protein was from the first ssDNA affinity chromatography (Liem *et al*., 1994). The human MGMT N-terminus deletion mutants were produced as GST fusion proteins by cloning the PCR products, using *Bam*H1-containing sense and *Eco*R1-containing antisense primers corresponding to 15 base residues in the 5′ and 3′ regions of the required inserts respectively (Ayi *et al*., 1992), into the pGEX2T vector. For example, the sense: (5′-CGACGTGGATCC**ATGGACAAGGATTGT**) and antisense (5′-CCTGACGAATTC**TCAGTTTCGGCCAGC)** (restriction sites underlined, MGMT codons bolded) primers were used for cloning the full-length GST-MGMT as previously described (Ayi *et al*., 1992). **K**101**L**, **K**104**L**, and **K**107**L** point mutation mutants of human GST−MGMT were created by oligonucleotide-directed mutagenesis (kit from Amersham, U.K.) using 5′ phosphorylated oligonucleotides **K**101**L** (5′pGGTGTTATGG**CTG**CTGCTGAAGGTTGTGAAA), **K**104**L** (5′pCTGCTG**CTG**GGTTGTGAAATTCGGAGAA), **K**107**L** (5′pCTGCTGAAGGTTGTG**CTG**TTCGGAGAAGTG), and a single-stranded M13 template containing the human MGMT cDNA [identical to Tano *et al*. (1990), as a *BamH*1−*Eco*RI fragment in BlueScript SK]. The manufacturer's protocols were followed. Mutant plasmids were transformed into JM109 cells and screened by DNA sequencing (Sanger *et al*., 1977). After release from the SK plasmid by *Bam*HI and *Eco*RI digestion, the mutant cDNAs were cloned into the *Bam*HI- and *Eco*RI-digested pGEX-2T for expression as GST fusion proteins (Ayi *et al*., 1992). The GST−MGMT fusion proteins were purified by GSH-Sepharose (Pharmacia, Sweden) following the manufacturer's protocols.

Assay of MGMT Repair Activity

The procedures for assaying the repair activities of the wild type and mutant GSTMGMT fusion proteins using the 32pCGCUCG labeled oligonucleotide were described previously (Liem *et al*., 1994).

RESULTS

Comparison of the Formation of R-MGMT in Human CEM Cells after Exposure to Alkylating Agents (SN1 and SN2) and O^6-Benzylguanine

We have previously shown that incubation of human cell extracts with an O^6-methylguanine (6MG) containing oligonucleotide (TATAC6MGTATA), which can specifically inactivate MGMT by methylating its active site, produces R-MGMT that is sensitive to protease V8. Subsequent experiments show that treatment of cells with SN1 alkylating

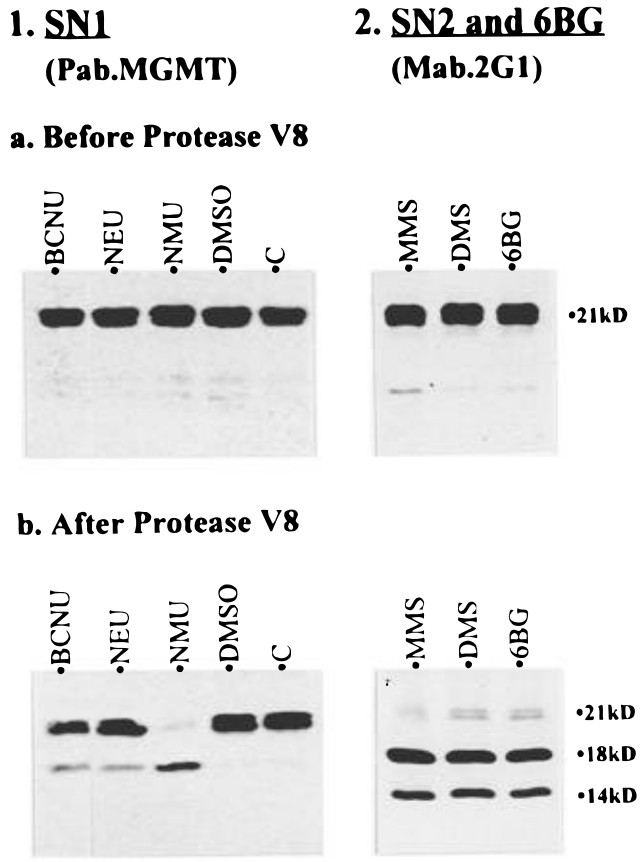


FIGURE 1: Detection of R-MGMT by protease V8/Western analysis in extracts of CEM cells treated with alkylating agents and O^6-benzylguanine. Cell extracts were obtained from CEM cells treated with (1) 1.5 mM SN1 drugs (NMU, *N*-methylnitrosourea; NEU, *N*-ethylnitrosourea; and BCNU, bischloroethylnitrosourea) and (2) 1.5 mM SN2 drugs (MMS, methyl methanesulfonate, and DMS, dimethyl sulfate) and 50 μM 6BG for 30 min. Panel a: Western analysis before protease V8 treatment. Cell extract proteins (200 μg/sample) were visualized with Pab.MGMT (3 μg/mL) for SN1 drugs (ECL, 2 min) and Mab.2G1 (6μg/ml) for SN2 and 6BG (ECL for 5min). Panel b: Western analysis after protease V8 as in panel a but samples were treated with protease V8. Cells were treated with SN1 and SN2 drugs without serum, 6BG was with serum, and DMSO represents the highest amount used as vehicle.

agents, which can effectively produce O^6-alkylguanine (6RG) lesions in DNA, also produces this protease V8 sensitive R-MGMT, see Figure 1.1b and Ayi *et al.* (1994). These agents produce R-MGMT indirectly by alkylating DNA, which is subsequently repaired by MGMT. To determine if the R-MGMT can be produced in cells without DNA damage, we compared the protease V8 sensitivity of R-MGMT produced in cells by direct alkylation with 6BG and SN2 alkylating agents (that are ineffective in producing 6RG in DNA). As shown in Figure 1, at equimolar concentrations SN2 alkylating agents such as MMS (methyl methanesulfonate) and DMS (dimethyl suphate) produced as much or more R-MGMT as detected by protease V8 cleavage as did SN1 agents such as bischloroethylnitrosourea (BCNU), *N*-ethylnitrosourea (NEU), and *N*-methylnitrosourea (NMU); compare the levels of the 21 kDa MGMT bands before and after protease V8 cleavage in panels 1a and 1b for SN1 and panels 2a and 2b for SN2 alkylating agents. Furthermore, the more sensitive Pab.MGMT (see legend in Figure 1) used in panel 1 for the reactive SN1 alkylating agents, especially the bifunctional alkylating agent BCNU, did not reveal any

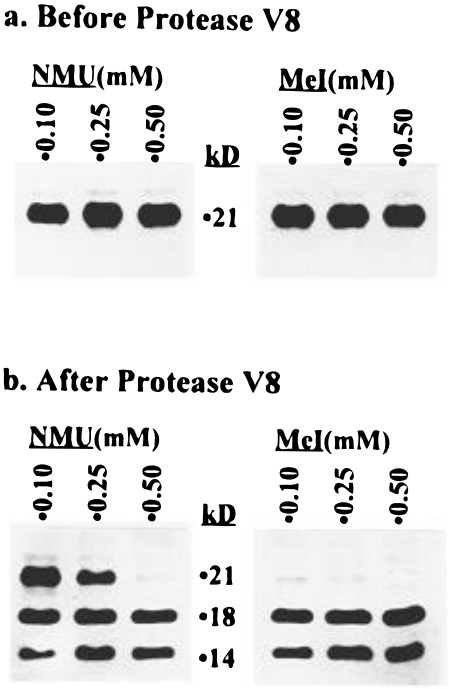


FIGURE 2: Comparison of R-MGMT in extracts of CEM cells treated with *N*-methylnitrosourea (NMU) and methyl iodide (MeI). Panel a: Western analysis before protease V8. Cell extract proteins (200 μg/lane) were visualized with Mab.2G1 (6 μg/ml) and ECL for 6min. Panel b: Western analysis after protease V8, as in panel a but samples were treated with protease V8. CEM cells were treated with 0.1, 0.25, or 0.5 mM NMU or MeI for 30 min without serum.

unusual MGMT species present [see discussion in Ayi *et al.* (1994)] in the cell extracts under this conditions. However, Pab.MGMT could not detect the 14 kDa protease V8 cleavage product as compared to Mab.2G1 used in panel 2 (see further direct comparison between MeI and NMU using Mab.2G1 in Figure 2). Apparently, 6BG produced R-MGMT as detected by its sensitivity to protease V8 since in the control experiments either with no agent or DMSO alone did not result in production of protease V8 sensitive MGMT. This result suggests that 6BG produces protease V8 sensitive R-MGMT in cells by direct alkylating the active site of cellular MGMT, which is consistent with the previous data on the loss of MGMT repair activity by activity assay (Dolan *et al.*, 1990).

In Figure 2, we compared the dose−response of the indirect acting SN1 agent NMU with the direct acting SN2 agent methyl iodide (MeI) in producing R-MGMT sensitive to protease V8. In the range of 100−500 μM, MeI was also equally or more effective in producing R-MGMT sensitive to protease V8 than NMU, see the relative levels of the 21 kDa MGMT bands in lanes labeled 100 μM NMU and MeI in Figure 2b. Since at equimolar concentrations SN2 alkylating agents produce far less O^6-alkylguanine in DNA than do SN1 agents (Lawley, 1984), these results support the *in vitro* observation that R-MGMT can be produced by direct alkylation of MGMT (Brent, 1986) and suggest that the increased protease sensitivity of R-MGMT does not require repair of DNA *in vivo*. Additionally, 6BG also produced R-MGMT sensitive to protease V8 (Figure 1.2b), further supporting the conclusion that R-MGMT can be generated without repairing DNA.

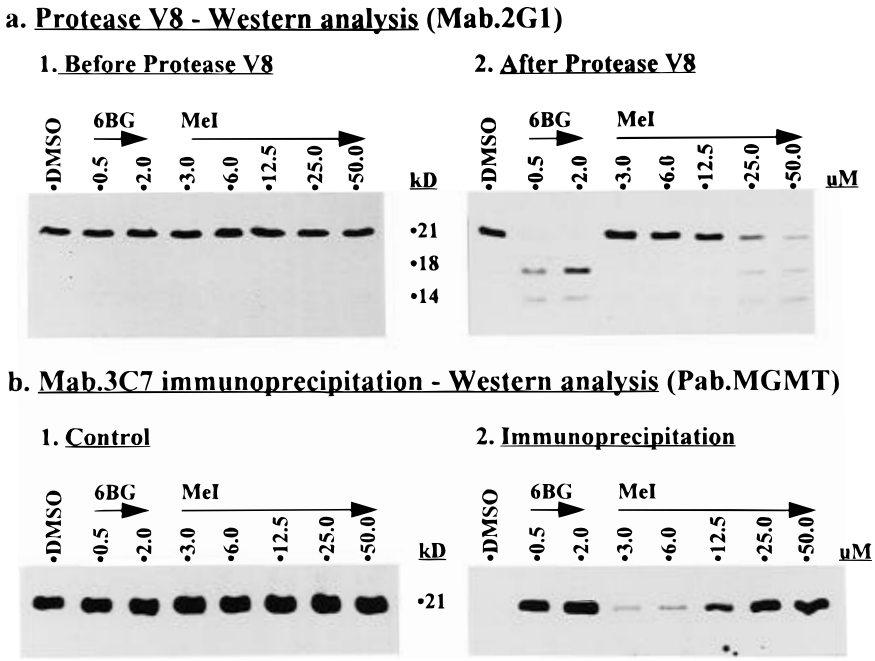


FIGURE 3: Comparison of protease V8/Western analysis and Mab.3C7 immunoprecipitation in the detection of R-MGMT in extracts of CEM cells treated with methyl iodide (MeI) and O^6-benzylguanine (6BG). CEM cells were treated with MeI without serum and 6BG with serum for 30 min. Panel a: protease V8/Western analysis. 1. Before protease V8: Cell extract proteins (200 μg/lane) were visualized with Mab.2G1 (6 μg/mL) and ECL for 3 min. 2. After protease V8: Samples were treated with protease V8. Panel b: Immunoprecipitation/ Western analysis. As in panel a, but cell extract proteins (200 μg) were mixed with Mab.3C7 (0.5 μg) and the immunoprecipitates were visualized with Pab.MGMT (3 μg/mL) and ECL for 3 min. 1. Control: Cell extract proteins without immunoprecipitation. 2. Immunoprecipitation: Protein precipitated by Mab.3C7.

Immunoprecipitation (Mab.3C7)/Western Analysis of R-MGMT

Although several monoclonal antibodies have been raised against human MGMT (Ayi *et al*., 1992), none can distinguish between MGMT and R-MGMT in Western-blotting analysis, i.e., under denaturing condition. We have now found, however, that Mab.3C7 does distinguish between active and active-site methylated MGMT by immunoprecipitation (e.g., native condition). In these experiments, protein extracts from CEM cells treated to inactivate MGMT were mixed with Mab.3C7, the immunoprecipitates were collected and analyzed by Western-blotting using a second antibody (Pab.MGMT) which does not discriminate between MGMT and R-MGMT. In Figure 3, we compared the R-MGMT immunoprecipitated by Mab.3C7 with the protease V8 sensitive R-MGMT in these cell extracts. Panel a.2 shows that treatment with either 6BG or MeI at micromolar concentrations produced R-MGMT cleaved by protease V8. As shown in panel b.2, Mab.3C7 only precipitated R-MGMT following treatment with 0.5 and 2 μM 6BG or 3, 6, 12.5, 25, and 50 μM MeI. MGMT and/or R-MGMT was present in all the extracts (see controls in panel b.1), and treatment with DMSO alone did not produce an immunoprecipitate with Mab.3C7. Since the only effect of 6BG on MGMT is to alkylate the active site, the appearance of the immunoprecipitate after 6BG treatment strongly suggests that Mab.3C7 specifically recognizes active-site alkylated MGMT. Of special note is that the protease V8 assay only detected R-MGMT conclusively in 12.5 μM MeI treated samples (e.g., the decrease in the 21 kDa MGMT band and appearance of the 14 and 18 kDa polypeptides in panel a.2), while Mab.3C7 detected R-MGMT in 3 μM MeI treated samples (panel b.2). This indicates the increased sensitivity of the immunoprecipitation method in detecting R-MGMT.

Epitope Mapping of Mab.3C7

To identify the polypeptide which is revealed upon active-site alkylation of MGMT, bacterial expressed deletion mutants representing a progressive 30-amino acid deletion from the N-terminus of MGMT were probed with Mab.3C7 (Figure 4a and 4b). Figure 4c shows that in Western blots Mab.3C7 recognized only the full-length MGMT and mutants containing amino acids from codon 90−120, but did not recognize deletion mutants lacking these amino acids. We then compared the specificity of Mab.3C7 toward the human and the homologous rat MGMT in Western blots (Figure 4d). While Mab.5H7 binds only the human and not the rat MGMT, Mab.3C7 recognizes both proteins with equal efficiency. This might be expected given the highly conserved primary amino acid sequences between codons 90 and 120 in human (Tano *et al*., 1990) and rat (Sakumi *et al*., 1991) MGMTs. This region is also conserved in the mouse (Shiota *et al*., 1992) and hamster (Rafferty *et al*., 1992) MGMTs as shown in Figure 4e. We speculated that the basic motif, LW**K**LL**K**VV**K**, from codon 99 to 107 of human MGMT, which is centered in the region we mapped, might be the Mab.3C7 recognition epitope. To test this hypothesis, we constructed three mutant GST−MGMT fusion proteins, each with a single lysine (**K**) to leucine (**L**) amino acid substitution, at codons 101, 104, and 107 (**K**101**L**, **K**104**L**, and **K**107**L**). In Western blots, Mab.5H7 recognized the wild type (wt) and all three mutant fusion proteins, but Mab.3C7 failed to bind to the mutant protein with the **K**107**L** substitution (Figure 4f). This suggests that the lysine (**K**) at position 107 is a key component of the Mab.3C7 epitope.

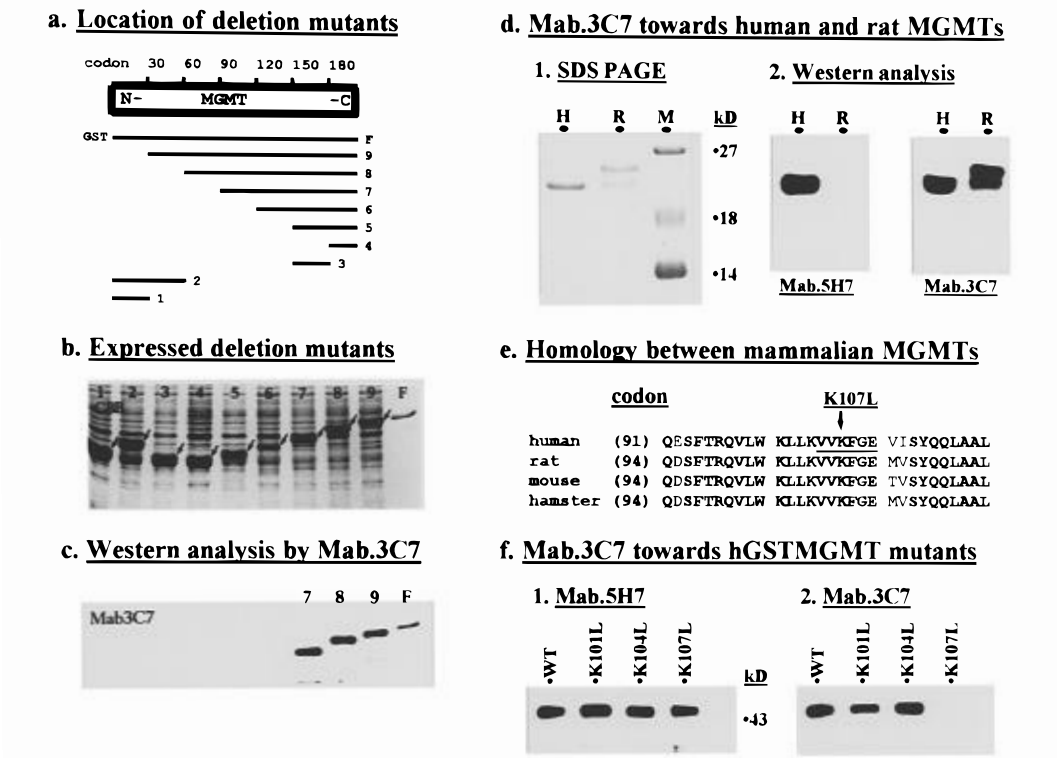


FIGURE 4: Epitope-mapping of Mab.3C7. Panel a: Summary of the locations of deletion mutant as GST fusion proteins. F is the full-length human GST−MGMT, and fragments labeled 1−9 are deletion mutant proteins. Panel b: Coomassie Blue staining of 13.5% SDS−PAGE gel for analysis of the soluble GST−MGMT deletion mutant proteins. Lanes are numbered corresponding their locations in panel a, and arrows indicate appropriate deletion mutant protein bands. Panel c: Western blots analysis by Mab.3C7. A filter from the gel in panel b was probed with Mab.3C7 (6 μg/mL, 5 min ECL). The protein bands recognized by Mab.3C7 are labeled 7, 8, 9, and F corresponds to the expressed proteins in panel b and their locations in panel a. Panel d: Western analysis of human (H) and rat (R) MGMT by Mab.3C7 and Mab.5H7. 1. SDS−PAGE: Coomassie Blue staining of the gel used to resolve the recombinant MGMTs. 2. Western analysis: A filter from SDS−PAGE gel in 1, was visualized with Mab.5H7 and Mab.3C7 (6 μg/mL and 6 min ECL for both Mabs). Panel e: Homology among mammalian MGMTs in the analogous Mab.3C7 epitope. The numbers represent codons and K107L is negative mutant for Mab.3C7 as determined in panel f. Panel f: Epitope mapping by point mutation analysis of human GST−MGMT at codons 101−107 (KLLKVVK), mutants K101L, K104L, and K107L have the replacement of lysine (K) with leucine (L) at the indicated codon. Western analysis: 13.5% SDS−PAGE with 100 ηg of GSH-Sepharose purified proteins/lane, (1) Mab.5H7 and (2) Mab.3C7 (6 μg/mL and 3 min ECL for both Mabs).

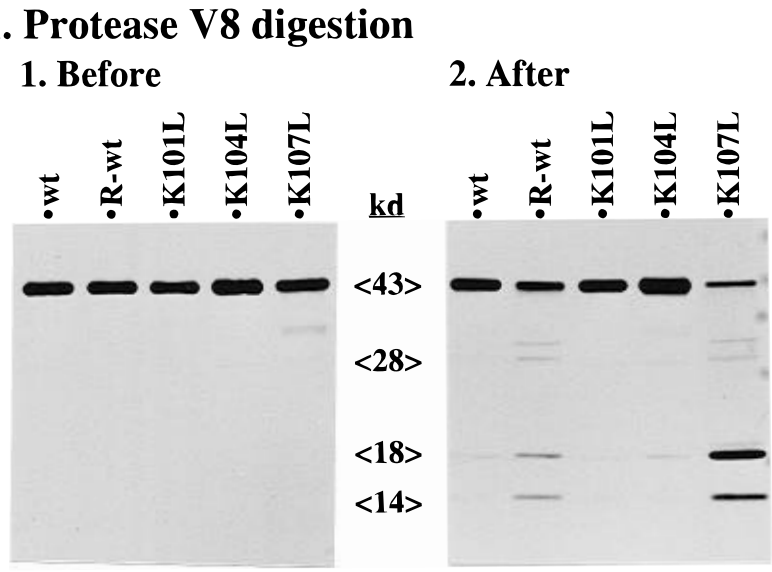
Properties of GSTMGMT Proteins Carrying Point Mutations Around the Mab.3C7 Epitope (the KLLKVVK Region)

As the above experiment shows that the Mab.3C7 epitope is around codon **K**107, which is some distance from the active site (centered around the cysteine 145), this result suggests that upon alkylation of the active site, the protein undergoes a conformational change which then unmasks this portion of the MGMT protein and allows Mab.3C7 binding. To understand whether the Mab.3C7 epitope did play a role in the conformation changes between the active and active-site alkylated protein, we attempted to investigate the possible function(s) of the Mab.3C7 epitope in the holoprotein by characterizing the point mutation mutant GSTMGMTs, i.e., **K**101**L**, **K**104**L** and **K**107**L** (in Figure 4f). These bacterial fusion proteins were subjected to (1) protease V8 digestion and (2) 6MG repair activity measurement by incubating the proteins with a known concentration labeled substrate 32pCGCUCG (Liem *et al*., 1994).

Figure 5 shows some interesting observations from these experiments. First, only the **K**107**L** mutant was cleaved by protease V8 into a polypeptide pattern identical to the active-site alkylated GSTMGMT (R-wt or R-GSTMGMT), see Figure 5A. Although two extra polypeptides were observed in the protease V8 cleavage of the R-GSTMGMT and **K**107**L** proteins (see the bands above 28 kDa in panel A.2) as compared to R-MGMT (see Figure 3a), this might be expected because of the GST fused to the N-terminus of MGMT. Second, despite their distance from the active site, the **K**107**L** mutant had no detectable repair activity, except some repair activities were observed in the **K**101**L** and **K**104**L** mutants, see Figure 5B. These results indicated the specificity of the protease V8 cleavage in that only the **K**107**L** mutant protein could be cleaved by the protease but not the **K**101**L** and **K**104**L** mutants, despite they were all repair-deficient. Although we could not study the immunoprecipitability of the **K**107**L** mutant protein by Mab.3C7, since this antibody could not recognize this mutant protein (see Figure 4f), its resemblance to R-GSTMGMT toward cleavage by protease V8 might suggest that the **K**107**L** mutant could be structurally similar to the active-site alkylated protein.

DISCUSSION

The results obtained from this study support the previous data (Ayi *et al*., 1994) that formation of R-MGMT from human MGMT induces conformational changes in the protein, which result in the exposure of novel epitopes that can be detected by an increased sensitivity to protease V8 (**E**30 and **E**172, see Figures 1 and 2) and by binding to Mab.3C7 (Figure 3). We have demonstrated that active-site methylation required for this conformational change can



B. Repair Activity

MGMT status	32pCGCGCG cpm (repaired)	32pCGC6MGCG cpm (unrepaired)	% Repair	% Efficiency
wt	53192 ±2150	31539 ±1981	62.7	100
K101L	23722 ±1782	61787 ±2611	27.8	44
K104L	12151 ±1511	73039 ±2705	14.3	22
K107L	N.D.	84511 ±3010	0	0

FIGURE 5: Properties of K101L, K104L, and K107 point mutaion mutant GSTMGMTs. A. Protease V8. 50 μg of bacterial cell extracts containing the wild type (wt), K101L, K104L, and K107L mutant GST−MGMTs were digested with protease V8 (100 ηg) in digestion buffer (100 μL containing 50 mM Tris, pH 8, 0.3 M NaCl, 1 mM EDTA, and 1 mM DTT) at 37 °C for 20 min. The reaction mixtures were then quenched with an equal volume of Laemmli loading buffer (2×) and boiled for 10 min. The samples (approximately 200 ηg of the GSTMGMT proteins/lane) were then resolved on a 13.5% SDS−PAGE. After transfer to nitrocellulose membrane, the proteins were visualised with Mab.2G1 using ECL reagents for 2 min. Panel 1: Before (i.e., control) and after (panel 2) protease V8 digested samples. The lane labeled R-wt is the active-site alkylated GST−MGMT which was obtained by treating the above wt (wild type) bacterial extract with an oligonucleotide CGCUCG (0.005 OD at 260 nm) for 10 min at 37 °C before adjustment to the protease V8 digestion condition (i.e., 0.3 M NaCl). B. Repair activity. Duplicated samples of GSH-Sepharose purified bacterial extracts with the amount of GST−MGMT similar to the Western blot in panel A.1 were incubated with the substrate 5′^{32}pCGCUCG (approximately 85 000 cpm/10 pM) in assay buffer (100 μL, 50 mM Tris, pH 8, 1 mM EDTA, and 1 mM DDT) at 37 °C for 10 min. The reaction mixtures were then quenched with NaCl (final concentration 1 M) and analyzed for the product ^{32}pCGCGCG and the substrate ^{32}pCGCUCG. Four HPLC runs (two injections per sample in duplicate) were carried out for each protein. The average cpm of the product ^{32}pCGCGCG and the unrepaired residual substrate ^{32}pCGCUCG are summarized in columns 2 and 3, respectively, and ND represents "not detectable". Column 4 is the percentage of repair calculated from the ratio of product cpm to the sum of product and residual substrate cpm detected. Column 5 is the percentage efficiency of repair with respect to the wt protein.

be produced either by indirect alkylation (SN1 agent alkylation of DNA which is then repaired by MGMT) or by direct alkylation (6BG and SN2 agents direct alkylation of MGMT). Although we have not formally excluded the possibility that SN2 agents are alkylating sites on the protein other than the active site, the identical behavior of 6BG (which can only alkylate the active site of MGMT) in our assays using protease V8 and Mab.3C7 argues against other "nonspecific" alkylation as a cause of the conformational change. In relation to human exposure to alkylating agents, this study provides some parameters for further studies. First, MeI at the micromolar level is clearly effective in producing R-MGMT (inactivation of cellular MGMT) in human cells (see Figure 3b.2). This observation suggests that we should not underestimate the effect of our exposure to SN2 alkylating agents since the levels of active MGMT can determine cellular susceptibility toward other alkylating agents that produce substantial O^6-alkylguanine and 0^4-alkylthymine residues in DNA. Second, since the Mab.3C7 recognition epitope is in a region of MGMT which is absolutely conserved among mammalian MGMTs (Figure 4f and 4e), and if this epitope is unmasked in all mammalian R-MGMTs, this agent can be applied to the study of R-MGMT in many animal models.

We have identified three unique sites [at the Mab.3C7 epitope (around **K**107), **E**30, and **E**172 (by protease V8)] in human R-MGMT that could represent the consequence of conformational changes upon active-site alkylation of MGMT. To understand their possible roles in this process, however, requires the knowledge of MGMT structure.

Therefore, we have attempted to use the invaluable crystal structure of Ada-C as a model [Ada-C is the *Escherichia coli* Ada c-Terminus 6MG repair domain from codon **M**175−**R**354; **T**176 was renamed as codon 1 in Ada-C by Moore *et al.* (1994)]. On the basis of the alignment of the 22 highly conserved amino acids within the amino acid residues from **F**96 to **E**173 of the Ada-C and from **F**94 to **E**172 of human MGMT, the analogous Mab.3C7 epitope of Ada-C can be positioned at the early part of the coil region C_3 (N-terminal side of **G**110) between helices 3 and 4, see Figure 1A and B in Moore *et al.* (1994). The analogous Mab.3C7 epitope in Ada-C, therefore, faces the active-site motif **PCHR** located at the front region of the long coil C_5 between helix 5 and the ending helix 6. Crucially, the region of C_5 (after **N**138) carrying the active-site motif runs into the molecule, i.e., the active site becomes buried, and emerges upward onto the surface of the molecule before entering helix 6. This arrangement of C_5 covers C_3, which makes the front region of C_3 containing the analogous Mab.3C7 epitope rather unaccessible [see Figure 1B in Moore *et al*. (1994)]. It appears that the front and back regions of C_5, the analogous Mab.3C7 epitope of Ada-C, and helix 6 encompass the active site.

One characteristic of this structure is the "salt-link" between **H**147 of the active site **PCHR** and the acidic residue **E**173 of helix 6 [see Figure 3A in Moore *et al*. (1994)]. The **H**147 and **E**173 residues of Ada-C are conserved among all MGMTs, which are **H**146 and **E**172 of human MGMT. Therefore, one would envisage that such a "salt-link" might exist in all MGMTs. It appeared to serve a crucial function by maintaining MGMT in a repair active conformation because mutations in either **H**146 or **E**172 of human MGMT severely impaired the repair activity (Chueh *et al*., 1992; Rafferty *et al*., 1994) and **E**172 (and **E**30) was the specific residue cleaved by protease V8 in the active-site alkylated human protein (Ayi *et al*., 1994). This observation could explain some of our experimental observations in that the physical interaction between **H**146 and **E**172 residues within the "salt-link" in the active human MGMT could, therefore, protect the **E**172 residue from protease V8 attack [see DMSO and control lanes in Figures 1b and 3a.2 and Ayi *et al*. (1994)]. As the Mab.3C7 epitope remained unaccessible (e.g., analogous to the early region of C_3 in Ada-C) within the active human MGMT molecule, it would not be recognized (immunoprecipitated) by Mab.3C7 (see DMSO lane in Figure 3**b**.2). Conversely, alkylation of the active site of MGMT induced a conformation change that disrupted the "salt-link" between **H**146 and **E**172: this might be feasible because **H**146 was next to **C**145 (the alkyl acceptor of the active site) and the hydrophobic alkyl group of the alkylated **C**145 could prevent the reformation of the two crucial water molecules that bridged the active-site motif with the rest of the molecule. For example, **Y**159 (near the end of coil C_5 in Ada-C) shares a water molecule with **H**147 (in the active site) which is linked to **E**173 (in helix 6) via the "salt-link", see Figure 3A of Moore *et al*. (1994). Therefore, alkylation of the active site should induce significant structural alterations in the molecule that could allow the exposure of the Mab.3C7 epitope (e.g., at least the displacement of helix 6 since **E**173 is involved in the "salt-link") for the binding by Mab.3C7, i.e., R-MGMT can be immunoprecipitated by Mab.3C7 (see MeI and 6BG lanes in Figure 3b.2). The breakage of the "salt-link"should enable protease V8 to cleave the unprotected **E**172 (see alkylating agents and 6BG lanes in Figures 2b and 3a.2).

The lack of homology between the N-terminus of human MGMT and Ada-C has precluded us from making a similar conclusion for **E**30, although it behaves similarly to **E**172 toward protease V8 when human MGMT is alkylated at its active site. However, the above explanations for the experimental observations are entirely dependent on whether the Ada-C and human MGMT do share a similar structure, which might be questionable due to the following observations. First, the C-termini of mammalian MGMTs have additional 32−35 amino acid residues [see Figure 1**A** in Moore *et al*. (1994)]. Second, the Mab.3C7 epitope is within the most positively charged (basic) motif **K**LL**K**VV**K** (codons 101−107 of human MGMT) which is unique to mammalian MGMTs [see the region between **W**101 and **G**110 of Ada-C in Figure 1A of Moore *et al*. (1994)]. Third, human MGMT repairs O^6-benzylguanine but not the Ada protein (Pegg *et al*., 1993), a significant biochemical difference. Fourth, a proper structure is required for mammalian MGMTs, not Ada-C, to enter the nucleus to repair the DNA.

In the case where human MGMT and Ada-C only shared some structural homology, there might be an alternative explanation for our results. It could be possible that the positively charged (basic) Mab.3C7 epitope (**K**107) and the negatively charged (acidic) **E**172 (and **E**30) residue might be related to each other either directly or indirectly, since it was unusual to observe two oppositely charged entities being perturbed simultaneously within the R-MGMT molecule. Mab.3C7 epitope (including **K**107) was located within the most positively charged (basic) region of human MGMT, i.e., the **K**LL**K**VV**K** motif at codon 101−107 as shown in Figure 4e and 4f. This motif in the active human MGMT could potentially serve as a direct or indirect positively charged (basic) acceptor for the negatively charged (acidic) **E**172 residue in the formation of a possible "salt-link" alternative to the previous "salt-link" between **H**146 and **E**172. Following the same arguments as above, one could explain the experimental observations: the formation of the "salt-link" between the **K**LL**K**VV**K** motif (within the Mab.3C7 epitope) and **E**172 in the active human MGMT protected **E**172 from protease V8 cleavage and the Mab.3C7 epitope from binding by Mab.3C7. Upon alkylation of the active site, the protein underwent a conformational change which disrupted the "salt-link" and rendered **E**172 toward protease V8 cleavage and the Mab.3C7 epitope binding by Mab.3C7. This could be extended for **E**30 if the three lysine (**K**) residues in the **K**LL**K**VV**K** motif could accommodate **E**30 and **E**172 simultaneously. In this situation, an indirect interaction between this basic motif and the acidic residues (**E**30 and **E**172) would be preferred, i.e., by the possible sharing a water molecule (or a histidine) between them. These suggestions seem to explain our experimental results according to the properties of the Mab.3C7 epitope associated with the basic **K**LL**K**VV**K** motif and the acidic residues (**E**30 and **E**172), but they remain as speculations. However, the observations in Figure 5, which shows the similarity between the R-GSTMGMT and the **K**107**L** mutant toward protease V8 cleavage, may be indicative of such a "salt-link".

ACKNOWLEDGMENT

We are grateful to Dr. R. C. Moschel for the O^6-benzylguanine and to Dr. P. F. Swann and Dr. E. Manser for their critical readings of the manuscript.

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BI9603635