Inhibition of O^6-methylguanine-DNA methyltransferase by an alkyltransferase-like protein from *Escherichia coli*

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ABSTRACT

The alkyltransferase-like (ATL) proteins contain primary sequence motifs resembling those found in DNA repair O^6-alkylguanine-DNA alkyltransferase proteins. However, in the putative active site of ATL proteins, a tryptophan (W[83]) residue replaces the cysteine at the known active site of alkyltransferases. The *Escherichia coli atl* gene was expressed as a fusion protein and purified. Neither ATL nor C[83] or A[83] mutants transferred [3H] from [3H]-methylated DNA to themselves, and the levels of O^6-methylguanine (O^6-meG) in substrate DNA were not affected by ATL. However, ATL inhibited the transfer of methyl groups to human alkyltransferase (MGMT). Inhibition was reduced by prolonged incubation in the presence of MGMT, again suggesting that O^6-meG in the substrate is not changed by ATL. Gel-shift assays show that ATL binds to short single- or double-stranded oligonucleotides containing O^6-meG, but not to oligonucleotides containing 8-oxoguanine, ethenoadenine, 5-hydroxycytosine or O^4-methylthymine. There was no evidence of demethylation of O^6-meG or of glycosylase or endonuclease activity. Overexpression of ATL in *E.coli* increased, or did not affect, the toxicity of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in an alkyltransferase-proficient and -deficient strain, respectively. These results suggest that ATL may act as a damage sensor that flags O^6-meG and possibly other O^6-alkylation lesions for processing by other repair pathways.

INTRODUCTION

O^6-methylguanine (O^6-meG) is a potentially mutagenic and toxic lesion in DNA that is one of the many products of damage by certain classes of carcinogenic and chemotherapeutic alkylating agents. The processing of this lesion is thus of considerable interest in both the etiology and treatment of cancer (1,2). It is well established that the biological effects of such agents can be extensively prevented by the highly conserved DNA repair protein O^6-alkylguanine-DNA alkyltransferase, which reverses O^6-meG damage in DNA by the stoichiometric and auto-inactivating transfer of the methyl group to a cysteine residue in its active site (PCHRV) (3–5). Some prokaryotes, including *Escherichia coli*, contain two alkyltransferase genes: the inducible *ada* (6,7) and the constitutively expressed *ogt* (8), and other prokaryotes contain only one of these genes. Human cells express a single O^6-alkylguanine-DNA-alkyltransferase [referred to as MGMT (9)].

In silico analysis has revealed a family of genes we have called the alkyltransferase-like (ATL) genes because of their extensive amino acid sequence similarity to the alkyltransferase proteins (5). The *E.coli* ATL is 21 and 19% identical and 34 and 30% similar to OGT and MGMT, respectively. Alignment of ATL and other alkyltransferase sequences (Table 1) shows that in ATL, the alkyltransferase active site cysteine residue (C[145]) has been replaced with a conserved tryptophan (W[83] in the *E.coli* ATL). ATL proteins are common among prokaryotes, with *Deinococcus radiodurans R1* being an example of a prokaryotic organism containing ATL that does not have a recognizable alkyltransferase gene (i.e. one encoding the characteristic PCHRV). Within the eukaryotes, *Schizosaccharomyces pombe* contains a sequence homolog of ATL. Interestingly, this organism also does not possess an alkyltransferase protein. The method by

Table 1. Amino acid sequence alignments of the homologous regions of some alkyltransferase and ATL proteins

Human MGMT (P_002403)	113	SYQQLAALAGNPKAARAVGGAMRGNPVPIL--IPCHRVVCSSGAVGNYSGG--LAVKEWLLAHEG
S.cerevisiae MGMT (AAA34780)	137	TYGDIAKRIGKPTAARSVGRACGSNNLALL--VPCHRIVGSNRKLTGYKWS--CKLKEQLLNNEK
E.coli Ogt (CAA68548)	107	HYGQLAEQLGRPGAARAVGGAANGSNPISIV--VPCHRVIGRNGTMTGYAGG--VQRKEWLLRHEG
E.coli Ada (AAA23412)	289	SYQQLANAIGKPKAVRAVASACAANKLAII--IPCHRVVRGDGTLSGYRWG--VSRKAQLLRREA
M.tuberculosis ATL (AAK47642)	26	TYGDIAALAGLS-SPRIVGWIMRTD--SSD--LPWHRVIRASGRPAQHLAT----RQLELLRAEG
Pseudomonas ATL (AAL19421)	51	SYGQLAELAGLGRAARWVGRTLSQLPVDTR--LPWHRVLGAGGRISLPADSPGGREQRARLREEG
E.coli ATL (AAC73557)	51	TYGDVAKLAGSPRAARQVGGVLKRLPEGST--LPWHRVVNRHGTISLTGPDL--QRQRQALLAEG
S.typhimurium ATL (AAL19421)	51	TYGDVARLAGSPRAARQVGGVLKRLPEGST--LPWHRVVNRHGAISLTGPDL--QRQRQALLAEG
Y.pestis ATL (CAC92374)	102	TYGDIAQLIGSPRAARQVGGVLKRLPEGSA--LPWHRVINRYGEISLTGDDY--LRQKKALVAEG
V.cholerae ATL (AAF94224)	22	TYGAIAKMAGYPGYARHGKALGHLPEGSQ--LPWFRVINSQGKISLQGEDF--VRQRQLLLAEG
Deinococcus ATL (AAF10005)	49	TYGQLALLAGGPGAARQAGAVMHSLKD-SD--LPWQRVINAQGRVSTYKVGLG-EVQEGLLRAEG
S.pombe ATL (CAB54827)	24	TYGEIARYVGMPSYARQVGGQAMKHLHPETH--VPWHRVINSRGTISKRDISAGEQRQKDRLEEEG
S.coelicolor ATL (CAC01312)	87	TYGDVAEYLEEG-GPRQVGRVMSLY--GGG--VPWWRVVRADGVLLAGHEL----EALDRYREEG
Lactococcus ATL (AAK05902)	25	SYRDVGALAGLPNGARQVSRILHSMSKKYE--LPWWRVVRSDRTIGLPEPAKS--EQMELLKKEG
Myxococcus ATL (AAB60209)	58	SYAQVALYAGRPGAARGVGREMATLPQQPELPLPWWRVTRSDGTL-APQVAQ---EQARRLRAEG

The highly conserved residues are highlighted. Accession numbers are given in parentheses. Numbers indicate residue numbers.

which such organisms process O^6-meG in DNA, if any, is not known.

In order to define the possible role of ATL in the processing of alkylation damage in DNA, we isolated and over-expressed the gene from *E.coli* and purified the protein. We characterized the protein, and two site-directed mutants in a series of *in vitro* assays using, as putative substrates, *N*-[^{3}H]-methyl-*N*-nitrosourea-methylated DNA and short synthetic oligonucleotides containing O^6-meG or other modified bases. We also assessed the effect of ATL overexpression in *E.coli*.

MATERIALS AND METHODS

Materials

All oligonucleotides containing DNA lesions were purchased from DNA technology A/S. PCR primers were synthesized by MWG. *E.coli* XL-1-blue bacterial strain was purchased from Novagen. Vent polymerase, restriction enzymes, factor Xa and amylose-resin were purchased from New England Biolabs. Ampicillin, isopropyl β-D-thiogalactopyranoside (IPTG), calf thymus DNA, VIVAspin (10 000 Da molecular weight cut-off) spin columns and most other biochemical reagents were purchased from Sigma. T4 polynucleotide kinase was purchased from Roche Applied Science. Molecular biology kits were purchased from Qiagen.

Generation of pMAL-2c constructs for the expression of maltose-binding protein (MBP) fusion proteins

Based on the predicted DNA sequence of an O^6-meG-DNA methyltransferase-like protein (GenBank accession no. AE000151), specific primers were synthesized and used for the amplification of ATL from *E.coli* genomic DNA. The primers used contained restriction enzyme sites for EcoRI (sense) and BamHI (antisense), which are indicated in boldface as follows: (i) 5′-CG**GAATTC**ATGCGTATGGAC-GAATTTTATACAAAG-3′ (sense) and (ii) 5′-CG**GGATCC**-TTAAGGCTTCCACATGTATTCTGG-3′ (antisense) (ATG start codon underlined). PCR amplifications were carried out

using Vent polymerase with 50 ng of *E.coli* genomic DNA as a template (genomic DNA was purified from AB1157 *E.coli* using a Qiagen genomic DNA purification kit) under the following conditions: initial denaturation for 1 min at 95°C followed by 25 cycles of denaturation (at 95°C for 45 s), annealing (at 55°C for 45 s) and extension (at 72°C for 1 min). The PCR product (409 bp) was digested with EcoRI and BamHI restriction enzymes and the band purified by electrophoresis on 1% agarose gel. The DNA was isolated from the gel using a Qiagen gel extraction kit and ligated into the pMAL-2c vector digested with the same enzymes to form pMAL-2c-ATL. The coding region of the construct was sequenced to ensure that no mutations were introduced during the production. The cDNA for human MGMT (GenBank accession no. M29971) was PCR amplified as above using the following primers: 5′-CG**GAATTC**ATGGACAAG-GATTGTGAAATGAAACG-3′ (sense) and 5′-CG**GGACC**-TCAGTTTCGGCCAGCAGGCGG-3′ (antisense) (ATG start codon underlined). The pMAL-2c-hMGMT construct was then generated and the sequence fidelity verified using the protocols described above.

Generation of C[83] and A[83] ATL mutants

To generate the C[83] and W[83] ATL mutant genes, site-directed mutagenesis was performed using a two-step PCR strategy. For the C[83] ATL mutant, primers (i) (ATL 5′ primer described above) and (iii) 5′-GGCAGCACCTTACCCGTGCCACCG-GGTGGTTAATCG-3′ (antisense) (underlined bases indicate W[83] to C[83] codon substitution) were used to generate a 5′ ATL fragment (265 bp) encoding the base change. The primers (ii) (3′ ATL primer described above) and (iv) 5′-CGATTAAC-CACCCGGTGGCAGGGTAAGGTGCTGCC-3′ (sense) were then used to generate the 3′ fragment (129 bp) encoding the base change. Both PCR products were purified by electrophoresis on a 1% agaorse gel and the DNA isolated using a Qiagen gel extraction kit. Equal amounts (25 ng) of the 5′ and 3′ overlapping PCR fragments were then mixed and PCR amplified with primers (i) and (ii) to generate the full-length C[83] ATL cDNA. To generate the A[83] variant, primers (iii) and (iv) were substituted for primers (v) 5′-GGCAGCACCTTACC-CGCGGCACCGGGTGGTTAATCG-3′; and primers (vi) and

(iv) 5′-CGATTAACCACCCGGTGCCGGGGTAAGGTGC-TGCC-3′ (underlined bases indicate $\overline{W^{83}}$ to A^{83} codon substitution). pMAL-2c constructs were generated as described above.

Expression and purification of recombinant MBP fusion proteins

To express and purify the MBP fusion proteins, the pMAL-2c constructs were transformed into competent XL-1 blue *E.coli*. Fusion proteins expressed from this plasmid contain an MBP tag at the N-terminus and can be purified using the affinity of MBP for amylose resin. To express the protein, a single colony was grown in Luria–Bertani broth (LB) (500 ml with 50 μg/ml ampicillin; Sigma) to an OD_{600} of 0.6. Protein expression was induced by the addition 0.4 mM IPTG and the cultures were incubated at 37°C for 3 h. The cells were cooled on ice then harvested by centrifugation at 2500 *g* Bacterial cell pellets were resuspended in 20 ml of binding buffer [20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 3 mM DTT, 10% glycerol and 300 μl protease inhibitor cocktail (Sigma)] and extracts were prepared by sonication (four 20 s pulses, setting 4.5; Sonicator XL; Heat Systems). The samples were then centrifuged at 2500 *g* at 4°C for 20 min. The soluble fraction was applied to 1 ml of amylose resin and incubated at 4°C for 2 h. The resin was centrifuged at 1500 *g* for 30 s then washed in binding buffer. This process was repeated three times. The fusion proteins were eluted in 10 ml of binding buffer supplemented with 10 mM maltose. A VIVAspin 20 ml centrifugal concentrator (10 000 Da molecular weight cut-off) was used to concentrate the proteins. The final protein concentration was determined using a standard Bradford (10) assay.

Factor Xa cleavage of MBP fusion proteins

Factor Xa digest reactions were carried out using 1 μg of factor Xa, 5 μl of 10× reaction buffer (200 mM Tris–HCl, pH 8.0, 1 M NaCl and 20 mM $CaCl_2$) and 25 μg of MBP fusion protein in a total volume of 50 μl. The reactions were incubated at 23°C for 18 h. The efficiency of the digest was assessed by resolving the cleavage products on a 14% SDS–polyacrylamide gel.

5′ End labelling of oligonucleotides with [γ-^{32}P]ATP

Substrate oligonucleotides containing DNA adducts were synthesized by DNA technologies (Table 2). Labelling reactions were carried out using 5 pM of oligonucleotide, 2 μl of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Biosciences), 0.5 μl of PNK (Roche), 2 μl of 10× reaction buffer (500 mM Tris–HCl, pH 8.2, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM DTT and

1 mM spermidine) in a total volume of 20 μl. Reactions were incubated at 37°C for 60 min, then cooled on ice. The volume was adjusted to 40 μl with TE (0.1 M Tris–HCl, pH 7.5 and 10 mM EDTA) and passed through a G25-microspin column (Amersham Biosciences). Labelling efficiency was measured by counting 1 μl of the labelled oligonucleotide for 1 min in a scintillation counter. The labelled oligonucleotide (5 pM) was annealed with the complimentary oligonucleotide (10 pM) in a volume of 50 μl supplemented with 500 mM NaCl. The reaction mixture was heated to 80°C for 2 min and allowed to slowly cool to room temperature (∼2–3 h). To confirm annealing, an aliquot equivalent to 20 000 c.p.m. was added to 3 μl of 3× non-denaturing gel loading buffer [30% (v/v) glycerol, 0.25% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue] in a total of volume of 10 μl and analysed on a 20% non-denaturing polyacrylamide gel.

Gel-shift and cleavage assay analysis

Reaction mixtures (20 μl) contained 2.5 pM of MBP fusion protein and 5 fmol of 5′-^{32}P-labelled oligonucleotide substrate in buffer I (50 mM Tris–HCl, pH 8.3, 3 mM DTT and 1 mM EDTA). Incubations were carried out at 37°C for 15 min. Reactions that were subjected to subsequent PstI digestion step were first purified by phenol/chloroform followed by ethanol precipitation. Samples were analysed by either denaturing (20%) or non-denaturing (lower half 20%/upper half 5%) PAGE using Mini-PROTEAN II apparatus (Bio-Rad).

Alkyl transfer assays

A standard assay procedure (10) was used to monitor the transfer of radioactivity from F-methylated DNA calf thymus DNA to protein and the inhibition of this reaction by ATL. Purified MBP–ATL, MBP–MGMT fusion proteins or mutants thereof and extracts of *E.coli* harbouring the overexpressing vector, pRBShAT (11) were used as indicated. Aliquots (100 μl) of substrate DNA containing ∼1000 c.p.m. O^6-meG in buffer I were incubated at 37°C for the times indicated with various volumes of proteins or extract in a total volume to 300 μl made up with buffer I containing BSA (1 mg/ml; IBSA). In assays containing both ATL and MGMT, the former was added to the substrate and incubated at 37°C for 10 min before the addition of MGMT. Following incubation, processing of the samples was as described previously (10).

High-performance liquid chromatography (HPLC) analyses

Aliquots of [^{3}H]-methylated substrate DNA (10 μl, 2 mg/ml containing ∼6000 c.p.m. in O^6-meG) were incubated for 6 h at 37°C with either 5 μl of the MGMT preparation, 10 μl of the ATL preparation or both (in which case, ATL was pre-incubated with substrate for 10 min at 37°C before the addition of MGMT) or IBSA alone (control) in a total volume of 45 μl IBSA in capped 500 μl tubes. DNA was then hydrolysed to purine bases by the addition of 5 μl of 1 M HCl and heating at 75°C for 30 min. Samples were centrifuged and supernatants mixed with the marker bases 3-meA, 7-meG and O^6-meG. Samples were applied to a strong cation exchange column (Waters Spherisorb 5 μ SCX, 5 mm × 160 mm) and eluted

Table 2. Sequences of DNA adduct containing oligonucleotides

O^6-meG	GGACTG^{6}CAGCTCCGTGCTGGCCC
O^4-meT	GGACT^{4}GCAGCTCCGTGCTGGCCC
8-oxoG	GGACTG^{8}CAGCTCCGTGGTGGCCCGAATTC
5-OHC	CTGGGAC^{5}TGCAGCTCCGTGGTGGCCCGAATTC
ethenoA	GGACTGCA^{e}GCTCCGTGGTGGCGAATTC

The complimentary oligonucleotide sequences are not shown.

with 0.2 M ammonium phosphate–acetate buffer, pH 2.85 at 1 ml/min. Fractions (1 min) were collected and radioactivity determined by scintillation counting. Total radioactivity corresponding to the region of phosphotriester elution and the marker peaks were calculated.

Cell survival assay

AB1157 (wild type) or the isogenic GWR111 (ada^-/ogt^-) cells containing the plasmids pMAL-2c (control) or pMAL-2c-ATL or -MGMT were grown in 10 ml of LB supplemented with 50 µg/ml of ampicillin at 37°C with shaking at 220 r.p.m. (orbital shaker; Forma Scientific) to an OD_{600} of 0.5. Immediately prior to plating, round-bottomed 96-well plates were prepared by adding 100 µl of LB containing increasing concentrations of MNNG. The cultures were diluted 100-fold into fresh LB and 100 µl plated into each well of the plate. The plates were incubated at 37°C for 16 h. Each well was then resuspended and the OD_{600} measured using a Tecan plate reader. Results were expressed as a percentage of control (vehicle-treated) growth.

RESULTS

ATL is not an alkyltransferase protein

Using incubation and processing conditions under which human MGMT rapidly transferred [^{3}H]-methyl groups from [^{3}H]-methylated substrate DNA to protein, purified ATL displayed no ability to undertake this reaction (Figure 1A) even after an incubation period of 6 h (Figure 1B). Conversion of the ATL putative active site W^{83} to C^{83} (or, as a negative control, A^{83}) did not confer methyl transfer capacity on the ATL protein under the incubation conditions used. As expected, conversion of the active site C^{145} in human MGMT to W^{145} removed its methyl transfer capacity (Figure 1A).

The nature of the MGMT assay would not allow us to assess if [^{3}H]-methyl groups had been released from the O^6-meG in substrate DNA into solution by ATL or if the strong acid hydrolysis step of the assay may have degraded a putative methylated ATL protein to acid solubility. To determine this, we used mild acid hydrolysis and HPLC to determine the amounts of O^6-meG in substrate DNA. Incubation with ATL for 6 h had no detectable effect on O^6-meG levels, whereas methyl transfer to the human MGMT under protein limiting conditions substantially reduced the amounts of O^6-meG in the substrate. There were no detectable differences in the amounts of 7-methylguanine, 3-methyladenine or phosphotriesters in the [^{3}H]-methylated substrate DNA following incubation with either MGMT or ATL (Figure 2).

To further explore the resistance of O^6-meG to demethylation by ATL, oligonucleotides containing O^6-meG in a PstI restriction endonuclease site were 5′ end-labelled with [^{32}P], incubated with ATL, or MGMT, de-proteinized and subjected to PstI digestion and denaturing gel electrophoresis. While MGMT restored the blocked digest site and resulted in two fragments following PstI digestion, no such cleavage was seen following incubation with ATL again indicating that demethylation had probably not occurred (Figure 3).
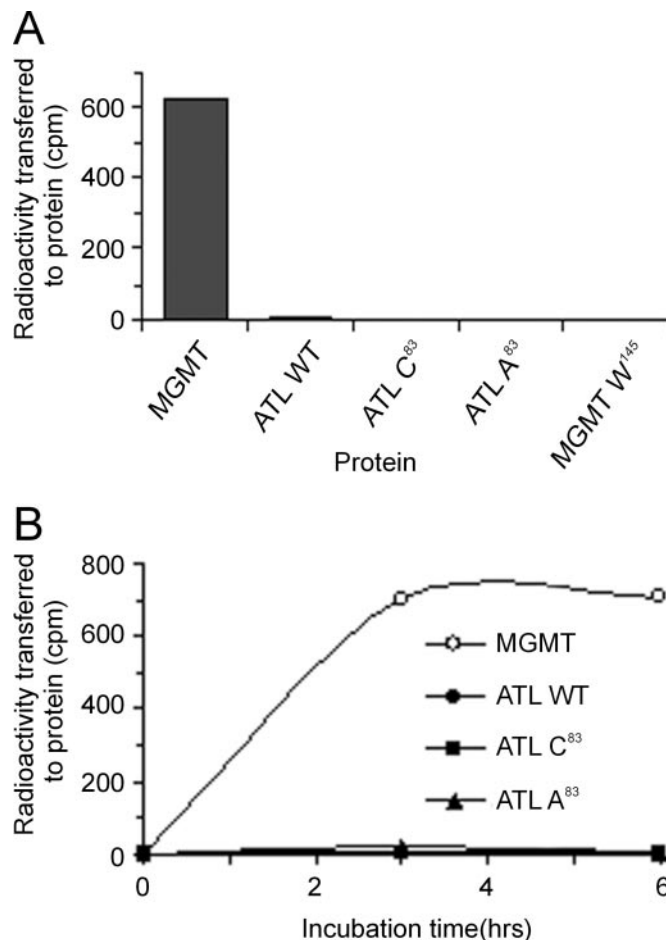

Figure 1. Transfer of [^{3}H]methyl groups from [^{3}H]-methylated substrate DNA to protein. (**A**) Substrate was incubated with the proteins indicated for 60 min at 37°C. (**B**) Substrate was incubated with MGMT, ATL wild type, ATL C^{83} and ATL A^{83} for up to 6 h at 37°C.

ATL inhibits the action of MGMT on O^6-meG in DNA

Although ATL displays no ability to release or transfer methyl groups from O^6-meG in substrate DNA, we found that the ability of MGMT to transfer methyl groups was extensively inhibited by the presence of ATL during the incubation. In initial assays, ATL was pre-incubated with substrate DNA for 10 min and then a protein-limiting amount of MGMT was added and incubation continued for a further 50 min. Under these conditions, inhibition of MGMT activity was proportional to the amount of ATL added. The C^{83} or A^{83} mutant proteins were both, and to a similar extent, rather less effective than wild-type protein (Figure 4A). The MGMT W^{145} mutant did not inhibit methyl transfer by wild-type MGMT under the assay conditions used (data not shown).

The ATL-mediated inhibition of MGMT was reversible. Using amounts of the wild type, C^{83} or A^{83} proteins that were approximately equally effective in the inhibition of MGMT seen above (1.4, 5.7 and 5.7 µg, respectively), progressively more methyl transfer to MGMT occurred with time up to 6 h (Figure 4B). The wild-type and both mutant proteins showed almost identical kinetics of loss of inhibition.
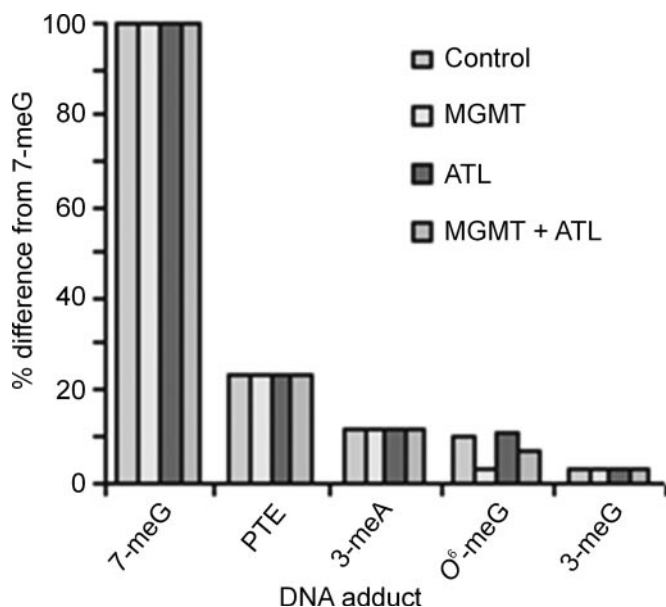
Figure 2. Amounts of methylphosphotriesters and methylated purines in HPLC fractionated mild acid hydrolysates of [^{3}H]-methylated substrate DNA following incubation for 6 h at 37°C with MGMT, ATL or a combination of MGMT and ATL (ATL pre-incubated with substrate for 10 min at 37°C). HPLC conditions are described in Materials and Methods. PTE, region of elution profile containing phosphotriesters and methylated pyrimidines.
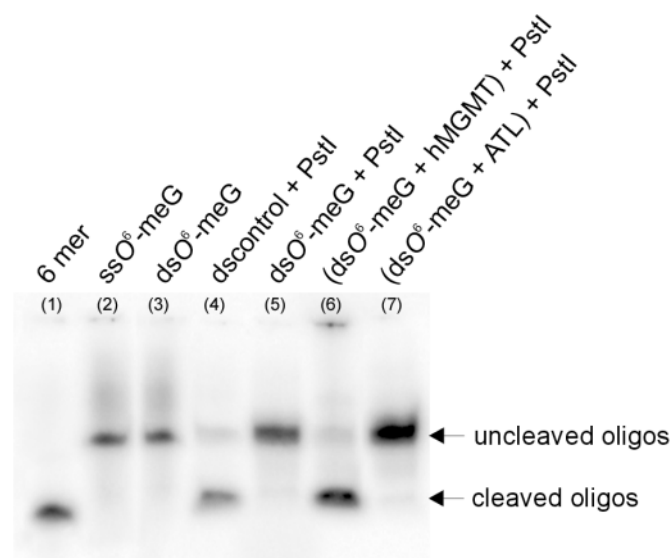

Figure 4. Effect of ATL wild-type, ATL C^{83} and ATL A^{83} on the transfer of radioactivity from [^{3}H]-methylated substrate DNA to MGMT. Substrate was incubated with the proteins for 10 min before the addition of MGMT and (**A**) incubated for a further 50 min at 37°C with incrementally increasing amounts of protein, or (**B**) incubated without ATL or with a fixed amount of ATL protein for up to 6 h.

Figure 3. Phosphorimage of 5′-^{32}P-labelled oligonucleotides after denaturing PAGE. Lane 1, marker 6mer (GGACTG); lanes 2 and 3, single-stranded (ss) and double-stranded (ds) O^6-meG-containing oligonucleotides, respectively; lanes 4 and 5, control (does not contain the O^6-meG) and O^6-meG double-stranded oligonucleotides, respectively, digested with PstI; lanes 6 and 7, dsO^6-meG oligonucleotides pre-incubated with MGMT or ATL, respectively, purified (Materials and Methods) and digested with PstI.

In agreement with the methyl transfer assays (see above), HPLC analysis also showed that MGMT could partially deplete the levels of O^6-meG in substrate DNA after 6 h incubation in the presence of ATL (Figure 2).
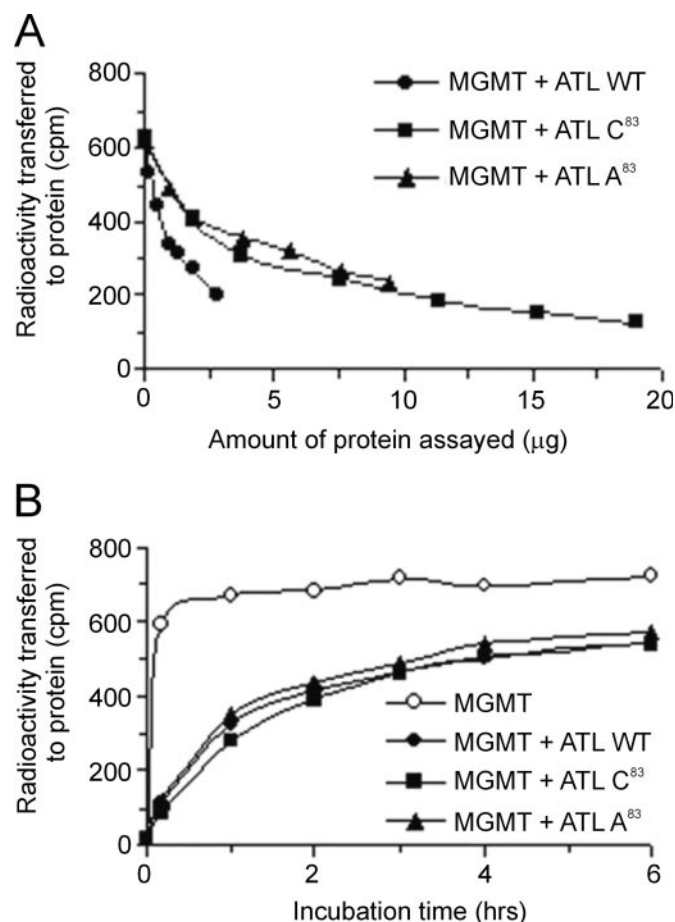
ATL binds specifically to O^6-meG in DNA

To examine the mechanism by which ATL inhibited the action of MGMT on O^6-meG in substrate DNA, we incubated ^{32}P-labelled synthetic oligonucleotides containing single O^6-meG residues with purified ATL and analysed the products by non-denaturing gel electrophoresis and phosphorimaging. ATL was able to bind to O^6-meG-containing, but not control (G replacing O^6-meG) oligos in either single- or double-stranded form (Figure 5). That the shifted band had the same electrophoretic mobility when either single- or double-stranded substrate was used may indicate that ATL had denatured the double-stranded oligonucleotide. Factor Xa cleaved fusion protein also produced a gel shift, while purified MBP did not, demonstrating that the shift was a function of ATL binding (Figure 5).

Similar gel shifts were seen using double-stranded oligonucleotides, in which the base complementary to O^6-meG was T, C, G or A (Figure 6A). However, oligonucleotides containing O^4-methylthymine, 8-oxoguanine, 5-hydroxycytosine or ethenoadenine (Figure 6B) did not bind to ATL in either
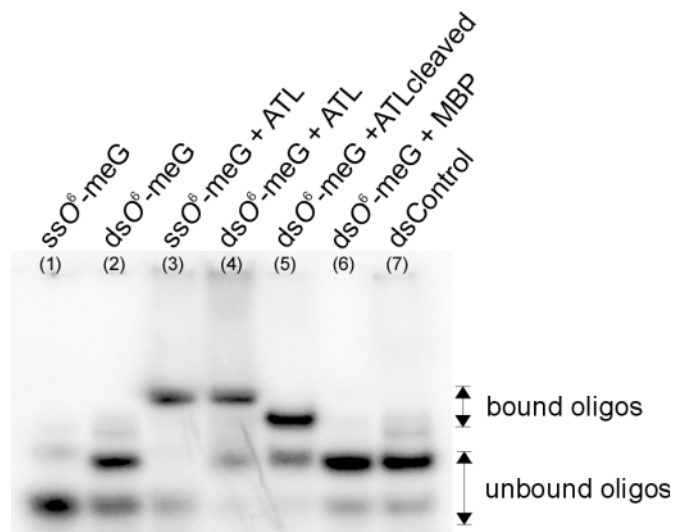
Figure 5. Phosphorimage of 5′-^{32}P-labelled oligonucleotides after non-denaturing PAGE. Lanes 1 and 2, ss- and dsO^6-meG oligonucleotides, respectively; lanes 3 and 4, as 1 and 2 but following incubation with ATL; lane 5, dsO^6-meG oligonucleotide incubated with factor Xa cleaved MBP–ATL fusion protein; lane 6, as lane 2 but following incubation with MBP alone; lane 7, dscontrol oligonucleotide after incubation with ATL.

single- or double-stranded form. This indicates that among the base modifications examined, ATL binding was specific for O^6-meG.

ATL has no apparent endonuclease or glycosylase activity

In order to assess if ATL had endonuclease activity, ATL-treated O^6-meG-containing oligonucleotides were heat denatured and subject to denaturing gel electrophoresis. There was no evidence of cleavage at or near the location of the O^6-meG residue (Figure 7, lanes 9 and 10). We also noted in this study that heat denaturation of the ATL-O^6-meG-containing oligonucleotide complex (at 95°C for 10 min) appeared not to completely dissociate the complex, or the complex was reformed on cooling (Figure 7, lane 9).

The possibility that ATL may be a glycosylase acting on O^6-meG in DNA was assessed by incubation of ATL-treated O^6-meG-containing oligos with formamidopyrimidine glycosylase (FPG), which has AP lyase activity. In contrast to the FPG-mediated cleavage seen using the 8-oxoguanine-containing oligonucleotide, the ATL-FPG combination did not result in cleavage of the O^6-meG-containing oligonucleotides (Figure 7, lanes 1–8).

Sensitization of *E.coli* to MNNG by ATL

Overexpression of ATL in the wild-type *E.coli* AB1157 increased sensitivity to the toxic effects of MNNG, in contrast to the protective effect provided by the overexpression of human MGMT (Figure 8A). In the isogenic ada^-/ogt^- strain (GWR111), which is MNNG-sensitive, there was a greater protection provided by human MGMT, but there was no effect of ATL overexpression (Figure 8B).
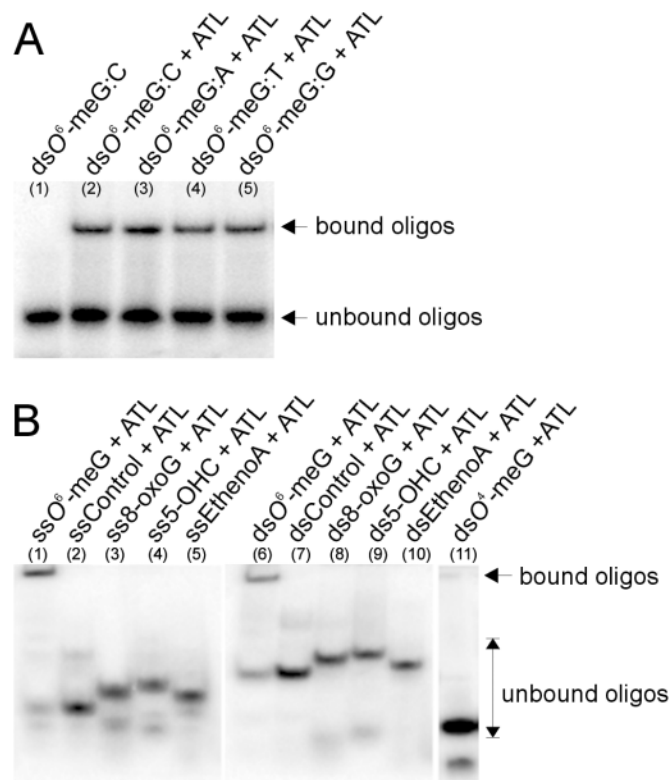

Figure 6. Phosphorimages of 5′-^{32}P-labelled oligonucleotides after incubation with ATL and non-denaturing PAGE. (**A**) Lane 1, dsO^6-meG oligonucleotide (no ATL control); lanes 2–5, dsO^6-meG oligonucleotides where the O^6-meG adduct was paired with C, A, T or G (lanes 2–5, respectively). (**B**) Single (lanes 1–5) and double (lanes 6–11) stranded oligonucleotides containing O^6-meG (lanes 1 and 6), control oligonucleotides (lanes 2 and 7), 8-oxoguanine (lanes 3 and 8), 5-hydroxycytosine (lanes 4 and 9), ethenoadenine (lanes 5 and 10) and dsO^4-methylthymine (lanes 11).

DISCUSSION

The primary sequence similarities between ATL and prokaryotic and eukaryotic alkyltransferase proteins led us to hypothesize that ATL may have a role in processing the type of DNA damage that is repaired by MGMT. The presence of a tryptophan residue in place of cysteine at the region of ATL that is homologous to the active site of MGMT suggested that the ATL protein would not be an alkyltransferase but might be an unusual demethylase, glycosylase or endonuclease. Whether or not conversion of the W^{83} to C^{83} would confer alkyltransferase activity was also considered.

Our initial approach was to assess the effect of ATL and the C^{83} and A^{83} mutants on [^{3}H]-methylated substrate DNA. Under assay conditions in which MGMT was highly effective, no transfer of radioactivity to protein was evident with any of these proteins. The absence of transfer activity in the C^{83} mutant, which has the putative active site sequence identical to that of MGMT, is intriguing, given that many of the other residues around the active site of MGMT that are required for alkyl transfer function (12,13) are also present in ATL. Presumably, other amino acid residues essential for this function are missing from ATL.

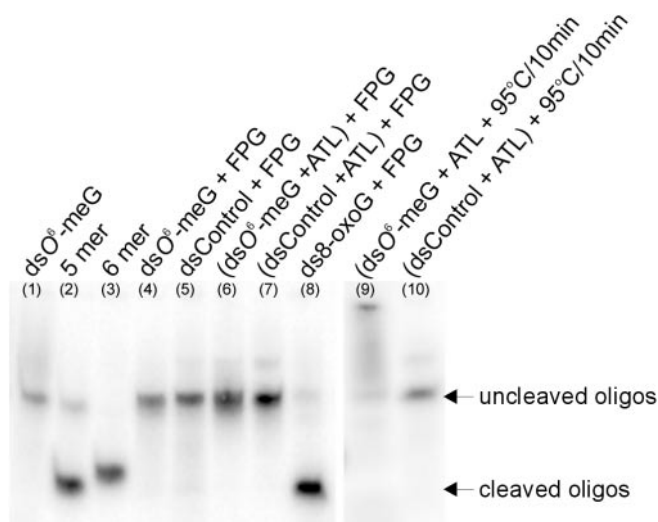Although no O^6-meG processing was seen (see below), it was very clear that preincubation of the substrate DNA

Figure 7. Phosphorimage of $5'$-^{32}P-labelled oligonucleotides after denaturing PAGE. Lane 1, dsO^6-meG oligonucleotide; lanes 2 and 3, marker 5 and 6mer oligonucleotides; lanes 4 and 5, O^6-meG (lane 4) and control (lane 5) ds-oligonucleotides incubated with FPG; lanes 6 and 7, O^6-meG (lane 6) and control (lane 7) ds-oligonucleotides pre-incubated with ATL, purified and incubated with FPG; lane 8, ds8-oxoG oligonucleotide incubated with FPG. Lanes 9 and 10 (no cleavage is seen in the absence of FPG; data not shown): O^6-meG and control ds-oligonucleotide, respectively, incubated with ATL then heated to 95°C for 10 min before electrophoresis.
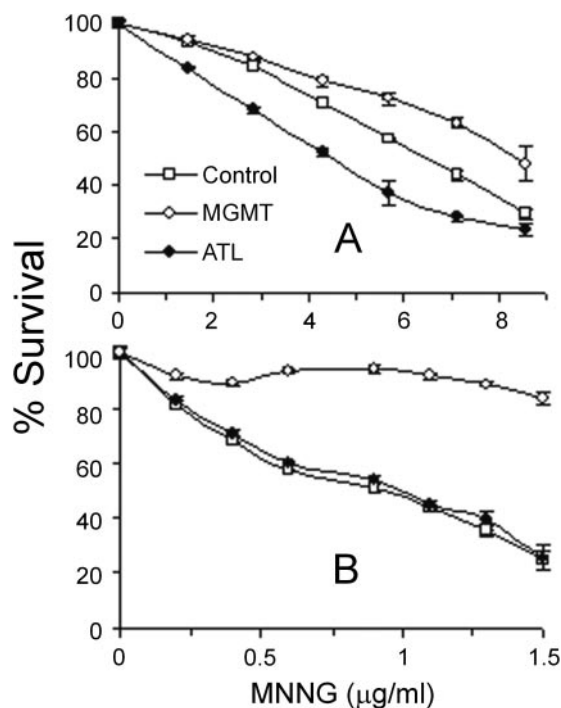

Figure 8. Effects of ATL and MGMT overexpression, in comparison with control, on the toxic effects of MNNG in WT (**A**) and isogenic *ada-ogt-* double mutants (**B**).

with ATL could almost completely inhibit the transfer of methyl groups from O^6-meG to human MGMT. This was also the case but to a lesser extent, with the mutant ATL proteins, whereas no inhibition was seen using similar quantities of the

W^{145} mutant of MGMT. For all three ATL proteins, continuing the incubation after the addition of MGMT resulted in progressively more methyl transfer to MGMT, suggesting that the binding was competitive with that of MGMT. The mutations we had introduced did not appear to have a major impact on such binding. We cannot exclude the possibility that the reversal of inhibition represents a slow denaturation of the ATL proteins, although the gel-shift assay results that involved thermal denaturation suggest that this might not be the case. However, it seems more likely that the bound ATL protein is in equilibrium with the free protein and that MGMT competes with ATL for the O^6-meG in substrate DNA. These data are difficult to interpret since, given that the assay for ATL activity is indirect, we cannot determine the number of functionally active ATL molecules, so the stoichiometry of the inhibition cannot be established. Inhibition of MGMT by ATL resembles the action of the A^{145} mutant of MGMT, which binds to O^6-meG and is reported to mask it from nucleotide excision repair (14). Masking and protection of O^4-methylthymine in DNA against nucleotide excision repair has also been observed with wild-type (15) and mutant (14) MGMT.

The slow reversal of MGMT inhibition by ATL provides further evidence that the O^6-meG in the substrate DNA was not affected by ATL. We confirmed this by HPLC analysis of the substrate following incubation with amounts of ATL that inhibited MGMT. This showed no change in the amounts of O^6-meG that had been structurally modified by ATL, as had originally been reported for the action of the *ada* alkyltransferase on O^6-meG in DNA (16), but that the products co-eluted with the marker O^6-meG seems improbable but was made even less likely by the use of reverse-phase HPLC. This also demonstrated no ATL-mediated decrease in the size of the O^6-meG peak (data not shown).

Binding of ATL to O^6-meG was further confirmed in non-denaturing gel-shift assays using either single- or double-stranded synthetic oligonucleotides containing single O^6-meG residues, paired in the latter case with T, C, G or A. We also showed that this binding is specific insofar as oligonucleotides containing 8-oxoguanine, ethenoadenine, 5-hydroxycytosine or O^4-methylthymine did not bind to ATL under the same conditions. The ability of ATL to bind to O^6-meG in the oligonucleotides appears to be preserved after heat denaturation, suggesting that the protein is thermostable, or rapidly renatures upon cooling.

MGMT did not result in any visible gel shift using O^6-meG-containing oligonucleotides under the conditions we used, indicating that ATL binding may have been much stronger than that of MGMT. However, it has been shown that modification of the active site cysteine residue alters the affinity of MGMT for DNA (17) and this may be the reason that we did not see a gel shift with this protein.

In support of the HPLC analyses, restriction endonuclease digestion of ATL-treated O^6-meG-containing oligonucleotides did not restore the PstI site therein and thus gave no evidence of a demethylase activity; we did not see evidence of glycosylase or endonuclease activity on the O^6-meG-containing oligonucleotides. It is important to note that in most of the assays we have used one set of experimental conditions and we cannot exclude the possibility that different conditions may have led us to other conclusions. Nevertheless, it seems

reasonable to conclude that ATL binds specifically to O^6-meG in methylated DNA and one consequence of this is that access of MGMT to the damage is restricted. The ability of ATL to bind but not to modify O^6-meG in DNA suggests that it may act as a damage sensing protein that flags such damage for processing by other repair pathways. However, in most of the organisms we have examined *in silico* (the exceptions being *S.pombe* and *Deinococcus*), ATL encoding genes are present in addition to recognizable alkyltransferase genes. Our *in vitro* results indicate that ATL may mask O^6-meG from efficient repair by such alkyltransferases.

This possibility was examined by the overexpression of ATL in isogenic *E.coli* strains differing in endogenous alkyltransferase activity. Thus, in the wild-type AB1157 strain, and in support of the *in vitro* results, ATL increased the toxic effects of the methylating agent MNNG, presumably by attenuating the repair of O^6-meG by the endogenous alkyltransferases. In non-adapted *E.coli*, this would predominantly be by OGT and to a lesser extent by ADA (18). Overexpression of human MGMT in this strain complemented the effects of the endogenous proteins and reduced the toxicity of MNNG. In the MNNG-sensitive *ada⁻/ogt⁻* strain, overexpression of human MGMT had a very substantial protective effect, but ATL did not affect the toxicity of MNNG. This suggests that ATL is not the rate-limiting step in O^6-meG processing in the absence of OGT and ADA, again in support of the *in vitro* data.

In conclusion, we have shown that the ATL protein from *E.coli* has the ability to bind specifically to O^6-meG in oligonucleotides and DNA without methyl transfer or removal or glycosylase or endonuclease activity. The binding strongly, but reversibly, inhibits the action of MGMT on O^6-meG in substrate DNA. Overexpression of ATL in *E.coli* increases the toxicity of MNNG probably via binding to O^6-meG and inhibition of endogenous alkyltransferase. It seems reasonable to speculate that the function of ATL is not the inhibition of alkyltransferase activity. It is more likely that it act as a damage sensor that flags O^6-alkylguanine lesions for processing by other pathways. It would thus complement alkyltransferase activity in situations where the number or type of lesions present are not effectively repaired by alkytransferase.

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