A novel DNA damage recognition protein in *Schizosaccharomyces pombe*

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ABSTRACT

Toxic and mutagenic O⁶-alkylguanine adducts in DNA are repaired by O⁶-alkylguanine-DNA alkyltransferases (MGMT) by transfer of the alkyl group to a cysteine residue in the active site. Comparisons in silico of prokaryotes and lower eukaryotes reveal the presence of a group of proteins [alkyltransferase-like (ATL) proteins] showing amino acid sequence similarity to MGMT, but where the cysteine at the putative active site is replaced by tryptophan. To examine whether ATL proteins play a role in the biological effects of alkylating agents, we inactivated the gene, referred to as atl1, in *Schizosaccharomyces pombe*, an organism that does not possess a functional MGMT homologue. The mutants are substantially more susceptible to the toxic effects of the methylating agents, N-methyl-N-nitrosourea, N-methyl-N′-nitro-N-nitrosoguanidine and methyl methanesulfonate and longer chain alkylating agents including N-ethyl-N-nitrosourea, ethyl methanesulfonate, N-propyl-N-nitrosourea and N-butylnitrosourea. Purified Atl1 protein does not transfer methyl groups from O⁶-methylguanine in [³H]-methylated DNA but reversibly inhibits methyl transfer by human MGMT. Atl1 binds to short single-stranded oligonucleotides containing O⁶-methyl, -benzyl, -4-bromothenyl or -hydroxyethylguanine but does not cleave the oligonucleotide in the region of the lesion. This suggests that Atl1 acts by binding to O⁶-alkylguanine lesions and signalling them for processing by other DNA repair pathways. This is the first report describing an activity that protects against the toxic effects of O⁶-alkylguanine adducts and the biological function of a family of proteins that is widely found in prokaryotes and lower eukaryotes.

INTRODUCTION

A wide variety of DNA repair mechanisms have evolved to protect cells and organisms against the adverse biological effects of diverse environmental genotoxic agents (1). Alkylating agents generate a number of different base modifications in DNA, including O⁶-alkylguanine, which is both toxic and mutagenic (2–5). Repair of this lesion involves the removal of the alkyl group from the O⁶ position of the affected guanine residue and its transfer to a cysteine residue at the active site of O⁶-alkylguanine-DNA alkyltransferase [the human version of which is MGMT (6–8)]. This reaction, which does not require any cofactors, reverses the damage and inactivates the protein, leading to its degradation. The alkyl acceptor cysteine is part of the motif PCHR1/V that is found in all functional alkyltransferase homologues in which the cysteine residue in the putative active site is replaced. Some organisms, such as *Escherichia coli* and *Caenorhabditis elegans* contain two functional genes.

In silico analysis shows the existence of a group of alkyltransferase homologues in which the cysteine residue in the putative active site has been replaced. These proteins have previously been designated as alkyltransferase-like (ATL) proteins (9,10). In the Conserved Domain Database (11) these proteins share a domain designated as COG 3695 (predicted methylated DNA-protein cysteine methyltransferase), although COG 3695 also covers sequences with cysteine at the active site. In the majority of ATL proteins, the active site cysteine residue has been substituted by tryptophan (9). Some organisms, such as *Schizosaccharomyces pombe*, contain only an ATL and no discernible alkyltransferase gene, whilst

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others, such as *Saccharomyces cerevisiae* and most higher eukaryotes, contain an alkyltransferase gene, but no recognizable ATL gene. *E.coli*, however, contains only two alkyltransferase proteins, ada (12) and ogt (13) but also an ATL protein, which is the product of the ybaZ open reading frame.

The function of ATL proteins is unknown and it is also not clear whether they all have the same function. While the sequence similarity to alkyltransferase proteins suggests a role in the repair of potentially lethal alkylation damage, no reduced toxicity of the alkylating agent, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), was seen in *E.coli* overexpressing the ybaZ gene product (eATL) (10). Purified eATL was able to bind to single- and double-stranded oligonucleotides containing O\(^6\)-methylguanine (O\(^6\)-meG) but not other lesions such as S-oxoguanine, ethenoadenine, 5-hydroxymethylcytosine or O\(^2\)-methylthymine. Besides binding to O\(^6\)-meG containing DNA, no other activity of eATL was found in that study. This prompted us to investigate the function of a potential ATL protein in *S.pombe* identified through sequence homology (9,10), and the product of the open reading frame, ORF SPAC1250.04c, now named *atl1*\(^{+}\). It was considered that the absence of a functional alkyltransferase gene may result in functional differences between ATL proteins in *E.coli* and *S.pombe*. Previous studies of the mechanisms involved in the protection of *S.pombe* against the toxic effects of alkylating agents has concentrated on the response to methyl methanesulfonate (MMS), a bimolecular nucleophilic substitution (S\(N_2\)) alkylating agent whose toxicity is mediated by agents has. In order to avoid chemical decomposition of the agents that may have occurred if they were added directly to molten agar, aliquots (50 \(\mu\)l) of the diluted compounds were added to the plates and spread evenly. Within 10 min, 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\) and 10\(^{-4}\) dilutions of *S.pombe* were made from a culture containing 1.2 \(\times\) 10\(^7\) cells/ml and 5 \(\mu\)l aliquots were spotted immediately onto YES agar plates.

**MATERIALS AND METHODS**

**Deletion of *atl1*\(^{+}\) in *S.pombe***

Oligonucleotides were designed to PCR amplify 5\(^{\prime}\) and 3\(^{\prime}\) internal regions of *atl1*\(^{+}\) and the ura4 cassette. Oligonucleotides PRI43S 5\(^{\prime}\)-CGCTCGAGCGATATCTCAGGGATC-CAATGGC and PRI44AS 5\(^{\prime}\)-GCCGCTAGCGCTCCACACAGTTGACAAAGCCTTAATCAGATTCTTTATTGGGC and PRI46AS 5\(^{\prime}\)-GCCCATATGACCCAGCATCAAACTGACGTTAGTTTGACTCTGAAGG which bind to the 6-meG residue (see below).

A liquid culture assay was used to examine the effect of a series of nitrosoureas and methanesulfonates of increasing carbon chain length on the growth of wild type and *atl1*\(^{+}\) strains. To the outermost wells of a 96-well plate were added 200 \(\mu\)l of water and to one lane was added 200 \(\mu\)l of YES (blank). An aliquot (150 \(\mu\)l) of an overnight culture (2 \(\times\) 10\(^{5}\) cells/ml) of each of WT (h\(^{+}\), leu1-32, ura4-D18, his7-366, ade6-M210) and *atl1*::ura4\(^{+}\) (h\(^{+}\), leu1-32, ura4-D18, his7-366, ade6-M210, *atl1*::ura4\(^{+}\)) was then added to 10 ml of YES and 100 \(\mu\)l aliquots of this was added to the empty wells. For each agent to be tested, stock solutions in dry DMSO (40 mg/mL) were serially diluted (1:10) into YES and 100 \(\mu\)l aliquots added to the wells in triplicate. After mixing gently by rocking, the plates were left, covered, at 30°C for 24 h. The contents of the wells were then resuspended using a multi-channel pipette and the OD\(_{595}\) was measured using a plate reader (Tecan, Genios). Results were expressed as a percentage of the growth of the YES only control. From these results, a narrower range of concentrations of drugs were used for more accurate determination of the concentration required for 50% growth inhibition (IC\(_{50}\)) values.

**All *atl1*\(^{+}\) cloning, expression and purification**

The *atl1*\(^{+}\) gene was isolated from *S.pombe* genomic DNA, using the primers: 5\(^{\prime}\)-GGAATTCCATGGTATGGGACTAATTTATACAAAG and 5\(^{\prime}\)-CGGATCTCTTAAAGCCTTCCACATGTATTCTGG, cloned into pMAL2c, overexpressed
in *E. coli* and the protein purified essentially as described previously (10).

**MGMT Competition assays**

The effect of preincubation of [*3H*-methylated MGMT substrate DNA or short oligonucleotides with ATL proteins on the transfer of [*3H*-methyl groups to human MGMT were determined in a series of competitive inhibition assays. In all cases, following incubation with MGMT, excess substrate DNA was hydrolysed to acid solubility and radioactivity transferred to protein determined by liquid scintillation counting as described previously (20).

Methyl transfer to MGMT was determined after preincubation of the substrate with varying amounts of purified ATL proteins, then incubation with excess MGMT for 15 min at 37°C. Based on this, the kinetics of recovery from ATL inhibition was determined by incubation with MGMT for varying times up to 40 min. The highest extent of inhibition was observed at 2.5 min, and the effect of varying the amounts of ATL was also assessed using this time interval. The effect of varying the time of inhibition was determined by incubation with MGMT for varying times up to 40 min. The highest extent of inhibition was observed at 2.5 min, and the effect of varying the amounts of ATL was also assessed using this time interval. The effect of varying the time of preincubation with ~50% inhibiting amounts of Atl1 for up to 3 h on the inhibition of MGMT was also assessed.

Oligonucleotides of the sequence 5’-AACAGCCCATAT-XGCC (where X indicates the location of the alkylated bases: O°-methyl, hydroxyethyl, benzyl or (4-bromothenyl)-guanine) were synthesized as described by Williams and Shibata (2005) (21). Oligonucleotides containing O°-benzyl or (4-bromothenyl)guanine are potent inactivators of MGMT. To investigate the effect of Atl1 on the ability of these oligonucleotides to inactivate MGMT, increasing amounts of purified Atl1 were added to a fixed amount of oligonucleotide that almost completely inactivated a fixed amount of MGMT. After incubation at 37°C for 15 min MGMT was added and the incubation continued for 10 min after which substrate DNA was added and the incubation continued for 10 min.

To examine the thermal stability of the ATL proteins, dilutions of these and MGMT were placed in a heating block set at 95°C. The temperature of the samples, monitored in a parallel tube with a thermocouple thermometer, increased from room temperature to 90°C over 1 min and from 90 to 95°C over the next minute. The samples were held at 95°C for 1 min then removed from the block to room temperature. They cooled to 55°C over 2 min and were then placed in ice. Inhibition of MGMT by the heat-treated proteins was determined as above in parallel with non-heated samples.

**Oligonucleotide binding assays**

Gel shift assays using the above oligonucleotides or those containing 8-oxoguanine (5’-GGACTOCAGCTCCTGTGGTGCCCGAATTC; O° = modified base), 5-hydroxyethylcytosine (5’-CTGGGAHTGCACTCCGTGTTGGCCCGAATTC; H = modified base) or ethenoade- nine (5’-GAACCGCCTCCTGTGGTGGCCCGAATTC; E = modified base) were carried out as described previously (10). Briefly, oligonucleotides were 5’-end labeled using poly-nucleotide kinase (Roche) and [*32P*-ATP (6000 Ci/mmol; Amersham Biosciences) purified by microspin column chromatography and incubated with crude *S. pombe* extracts or purified ATL proteins, then subjected to polyacrylamide gel electrophoresis and phosphorimager analysis.

Oligonucleotides containing a PstI restriction endonuclease site that was blocked by the presence of O°-methyl (5’-GAAC-TXAGCTCCGTGCTGGCC, where X = O°-methyl) were used to investigate possible demethylation by Atl1. These were 5’ end labeled with [*32P*-P] as above, hybridized to complement oligonucleotide (C opposite O°-methyl), incubated with Atl1 or MGMT, deproteinized and subjected to PstI digestion and denaturing polyacrylamide gel electrophoresis as previously described (10).

**RESULTS**

**Atl1+ deletion in *S. pombe* sensitizes to alkylating agent toxicity**

Insertional inactivation of the *S. pombe* *atl1*+ gene did not detectably affect morphology, or growth rate in YES, but did affect the phenotype as shown in the oligonucleotide binding experiments described below. Incorporation of methylating agents into the agar onto which aliquots of serial dilutions of WT or Δatl1 strains were spotted resulted in a dose dependent killing that was substantially greater in the *atl1* deletant. The difference was considerably more marked with MNU than MMS (Figure 1).

To quantify the effects of two series of alkylating agents on the growth of wild-type and Δatl1 strains, and to avoid the possible problems of incorporation of unstable compounds into molten agar or spreading them homogeneously onto agar plates, a microtitre plate liquid assay was used: the results are shown in Figure 2. Δatl1 cells were, to varying extents, more sensitive to the growth inhibitory effects of all the agents tested. For the nitrosourea series, the difference decreased in the order MNU ≈ ENU > PNU > BNU, and for the methanesulfonate series it increased, MMS < EMS < PMS. Sensitization to N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) was similar to that seen with MNU. Table 1 summarises the IC50 values for the agents shown in Figure 2.

![Figure 1](image-url)  
**Figure 1.** Sensitivity of WT and Δatl1 *S. pombe* strains to MMS and MNU in an agar plate assay. Undiluted (U) or serial 1:10 dilutions of the yeast were spotted onto agar plates containing the concentrations of MMS or MNU indicated.
**Atl1 inhibits the action of MGMT**

Preincubation of \([^{3}H]\)-methylated DNA with increasing amounts of \textit{S.pombe} Atl1 protein for 2.5 min (Figure 3A) at 37°C inhibited the transfer of \([^{3}H]\)-methyl groups to MGMT following its addition and further incubation at 37°C for 15 min. The ATL protein from \textit{E.coli} showed similar effects. These results indicated that the ATL proteins bind rapidly to substrate DNA and prevent the action of MGMT.

Using amounts of ATL proteins that caused \(~80\%\) inhibition of MGMT in the above assays, varying the length of the incubation at 37°C following addition of MGMT showed that this inhibition was reversible (Figure 3B). The most extensive
inhibition was seen at the shortest incubation times post MGMT addition and this progressively decreased during incubation with an initial half life of between 20 and 30 min (Figure 3C). Very similar effects were seen with both the E.coli and S.pombe ATL proteins. These results suggest that ATL proteins are not able to demethylate or depurinate O6-meG, although the possibility of cleavage outside the region of the lesion cannot be excluded.

Using an amount of Atl1 or eATL that caused ~50% inhibition of MGMT in these assays, varying the time of preincubation with the substrate up to 3 h at 37°C had no influence on inhibition (Figure 4A). Under these conditions, there was also no apparent transfer of radioactivity to either of the ATL proteins. These results suggest that the reversibility of MGMT inhibition by ATL was not due to any time-dependent decreased binding capacity of the ATL proteins to O6-meG in substrate DNA, such as might have occurred if the ATL proteins had undergone slow degradation. Furthermore, while heat treatment (see Materials and Methods) of MGMT resulted in complete loss of its methyl transfer activity it caused only ~40% reduction in the ability of both E.coli and S.pombe ATL proteins to inhibit MGMT (Figure 4B), again indicating a relatively high thermal stability of the ATL proteins.

Preincubation of Atl1 with short single-stranded oligonucleotides containing O6-benzyl or O6-[4-bromothenyl]-guanine reduced their ability to inactivate MGMT (Figure 4C). The amounts of oligonucleotides used were selected to almost completely inactivated MGMT. Increasing amounts of Atl1 progressively inhibited MGMT in the absence of oligonucleotides, but preincubation of the oligonucleotides with these amounts of Atl1 progressively reduced their ability to inactivate MGMT. As the amounts of Atl1 increased further, this effect was lost, presumably because there was sufficient Atl1 to bind to both the oligonucleotides and the MGMT substrate DNA.

### Table 1. IC50 values of alkylating agents in WT and ∆atl1 S.pombe strains

| Agent                        | IC50 (µg/ml) | Ratio |
|------------------------------|--------------|-------|
| N-methyl-N-nitrosourea (MNU) | 48           | 4.0   |
| N-ethyl-N-nitrosourea (ENU)  | 98           | 1.7   |
| N-propyl-N-nitrosourea (PNU) | 200          | 1.5   |
| N-butyl-N-nitrosourea (BNU)  | 214          | 1.2   |
| N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) | 0.60 | 2.3 |
| Methyl methanesulfonate (MMS) | 70           | 1.3   |
| Ethyl methanesulfonate (ENS) | 2.3          | 1.8   |
| Propyl methanesulfonate (PMS) | 3.1          | 2.4   |

### Atl1 binds to short oligonucleotides containing O6-alkylguanines

Incubation of cell-free extracts of WT S.pombe with [32P]-labelled oligonucleotides containing a single O6-meG residue showed a shifted band that was not present with extracts of ∆atl1 (Figure 5A). This phenotypic demonstration of loss of Atl function correlated with the genotype described above.

The purified S.pombe Atl1 bound to 5'-[32P]-labeled short singlestranded oligonucleotides containing a guanine residue that was modified at the O6-position with methyl, benzyl, 4-bromothenyl or hydroxyethyl groups. Under the conditions used, no binding was evident to a non-modified oligonucleotide (Figure 5B).

Denaturing gel electrophoresis showed that [32P]-labelled oligonucleotides containing a Pst1 recognition site were extensively cleaved by Pst1 and this was prevented in oligonucleotides containing O6-meG in the recognition sequence. Incubation of such oligonucleotides with MGMT extensively demethylated the O6-meG and thus restored Pst1 digestability, but incubation with Atl1 had no effect (Figure 5C). This is consistent with the earlier suggestion that Atl1 is not able to remove methyl groups from O6-meG, although depurination of this base may also have resulted in lack of Pst1 cleavage in this assay.

There was no evidence of binding to oligonucleotides containing the modified bases, 8-oxoguanine, 5-hydroxymethylcytosine or ethenoadenine under conditions in which there was clear binding to an O6-meG-containing oligonucleotide (Figure 5D). This suggests that Atl1 does not bind to these other modified bases, although we cannot exclude the possibility that binding may occur under different incubation conditions, or was disrupted during electrophoresis.
DISCUSSION

To investigate whether or not Atl1 plays a role in the processing of alkylation damage in DNA in the intact organism, we generated a deletion mutant of \( \text{atl1}^{+} \) by insertional inactivation using a selectable marker. In comparison with wild type, the mutants showed increased sensitivity to the growth inhibitory effects of a number of agents that are known to alkylate the \( O^6 \)-position of guanine in DNA. The difference was most apparent with the \( S_N1 \) methylating agents, MNU and MNNG for which \( O^6 \)-meG represents \( \sim 6 \% \) of the total DNA methylation products (3). In the nitrosourea series, the difference tended to decrease with increasing length of the alkyl group, such that
smaller differences were observed for ENU, PNU and BNU. For the methanesulfonate series, the smallest difference was seen with MMS, probably reflecting its character as an 
6-substituted electrophile which results in relatively small amounts (<0.1%) of 6-meG being generated in DNA (3). The increasing differences seen with EMS and PMS may reflect the increasing 
6-substituted character of these agents and the generation of relatively larger amounts of 6-alkylguanine in DNA (2). It should be noted that both nitrosoureas and methanesulfonates generate widely differing amounts of up to 12 different DNA alkylation products, several of which are known to be toxic (4,22). This may explain the variable effect of atl1 deletion. Toxicity may also arise from alkylation of cellular constituents other than DNA. Nevertheless, these results demonstrate that Atl1 is an important factor in the protection of S.pombe against the toxic effects of the agents examined.

To investigate the possible mechanism by which Atl1 protects the host against the toxic effects of alkylating agents we overexpressed the protein in E.coli and investigated its properties in vitro after purification. After incubation of purified Atl1 with [3H]-MNU methylated DNA, no detectable radioactivity was transferred to the protein This indicated that, using experimental conditions developed for the assay of MGMT activity, the S.pombe Atl1 protein does not exhibit methyltransferase activity. However, Atl1 strongly inhibited the action of MGMT on substrate DNA, suggesting that Atl1 binds to DNA containing 6-meG. Given the active site homology, it seems likely that this binding is directly to the 6-meG residues. Inhibition of MGMT was rapid, but reversible, demonstrating that Atl1 was not capable of removing the 6-methyl groups or changing the substrate in a way that completely prevented the action of MGMT. This precludes extensive removal of the base by a glycosylase activity. Prolonged preincubation of Atl1 with substrate DNA showed that the interaction was stable at 37°C. Indeed, heat treatment of Atl1 resulted in only partial loss of the MGMT inhibitory activity, indicating that the protein is quite resistant to thermal inactivation.

Very similar results were obtained with the E.coli homologue with which Atl1 has 33% sequence similarity (9,10). Further support of the suggestion that Atl1 does not demethylate 6-meG was provided by the observation that while Atl1 bound to double-stranded oligonucleotides containing this base in a Pst1 recognition sequence, this did not result in the restoration of the ability of Pst1 to cleave at this site. In these experiments there was also no evidence of cleavage at the position of the 6-meG residue in the oligonucleotide, suggesting that no glycosylase/AP lyase or endonuclease activity, operating in the vicinity of the alkylated residue was associated with Atl1.

We exploited the ability of Atl1 to bind to 6-meG and inhibit MGMT to investigate the range of lesions recognized by the protein. The competitive inhibition and gel-shift assays show that Atl1 can bind to oligonucleotides containing guanine residues modified with a range of 6-substituents. The overlap between the lesions recognized by Atl1 and MGMT may explain why some organisms are able to dispense with one of these genes. Of note is that Atl1 bound effectively to oligonucleotides containing hydroxyethylguanine, a lesion that is not effectively processed by human MGMT (23). This may explain the presence of an ATL alongside two alkyltransferase genes in some organisms such as E.coli. Given the differences between the Human and E.coli MGMT proteins in processing guanine residues with large 6-alkyl adducts and that ATL has a tryptophan residue in place of a cysteine residue, examination of the MGMT structures may allow speculation on the critical residues in the binding pocket.

Our results suggest that Atl1 is a damage recognition factor that licenses a range of 6-alkylguanine lesions in DNA for processing and elimination. Atl1 might thus, for example, act in a similar fashion to the damage sensing heterodimer in higher eukaryotes, XPC-HHR23B (24). In this case it might be expected to signal to the downstream components of nucleotide excision repair. However, alternative possibilities not involving lesion removal, such as damage tolerance or lesion replicative by-pass (25) cannot be excluded.

There is evidence that nucleotide excision repair is involved in the processing of longer chain alkylguanines in DNA in mammalian cells (26–28) however, the proteins that recognize these lesions have not been identified. S.pombe Atl1 would be a candidate for such a protein, but we have been unable to find ATL homologues in higher eukaryotes by searching for sequence similarity. This does not exclude the possibility of functional homologues and given the broad substrate specificity of the S.pombe Atl1 protein, such a homologue in man would have important implications in studies of human diseases including cancer and its treatment.

In conclusion, we have identified a protein that protects S.pombe against the toxic effects of a wide range of 6-alkylguanine adducts in DNA. It is the first report of such an activity in S.pombe as well as the first report on the biological activity of a family of proteins that is widely found in prokaryotes and lower eukaryotes.

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