Degradation of the alkylated form of the DNA repair protein, \(O^6\)-alkylguanine-DNA alkyltransferase

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\(O^6\)-Alkylguanine-DNA alkyltransferase (AGT) is a DNA repair protein that removes alkyl groups from DNA by transferring them to an internal Cys-145 residue. As the S-alkylcysteine is not converted back to cysteine, the protein can only act once and the resulting alkylated AGT molecule is rapidly degraded. The mechanism underlying the disappearance of the alkylated AGT has been studied in vivo in CHO cells and in vitro in reticulocyte lysates by using the pseudosubstrate \(O^6\)-benzylguanine (BG) and mutant forms of AGT. The wild-type AGT was stable but was ubiquitinated and degraded rapidly by the proteasome after treatment with BG or with an oligodeoxyribonucleotide, which contained \(O^6\)-methylguanine. Mutants C145F (and other mutants with bulky substituents at position 145), which have alterations that cause a steric alteration at the active site and also prevent hydrogen bonding involving Cys-145 resembled the alkylated AGT and were ubiquitinated and degraded rapidly irrespective of treatment with BG. Mutant M134F, which causes a steric alteration without interfering directly with the hydrophobic network involving Cys-145, partially destabilized AGT and its degradation was increased further by reaction with BG. Mutant C145S, which maintains the hydrophobic network and causes no distortion, was not rapidly degraded. The results indicate that the conformational change resulting in the opening of the asparagine hinge region in the structure, which is brought about by formation of an S-alkyl adduct, leads to an increased recognition by a ubiquitin ligase targeting the protein for degradation. This is a novel type of post-translational modification causing ubiquitination.

Introduction

DNA damage by carcinogenic alkylating agents including methylating agents such as \(N\)-methyl-\(N\'-\)nitro-\(N\)-nitrosoguanidine, dimethylantroamine and \(N\)-methyl-\(N\)-nitrosourea results in many adducts (1–4). Of these, \(O^6\)-methylguanine (m\(^6\)G) may be of particular importance in genotoxicity as it is very highly mutagenic causing G:C to A:T transitions (5,6). The presence of m\(^6\)G in DNA also induces apoptotic cell death probably by inducing repeated aberrant correction by the mismatch repair system (7–9).

A unique repair pathway that rapidly repairs a small number of m\(^6\)G lesions is very widespread. This repair is accomplished by a single protein, \(O^6\)-alkylguanine-DNA alkyltransferase (AGT) (4,10,11). AGT transfers the alkyl group from \(O^6\)-alkylguanine in DNA to a cysteine acceptor site located within the AGT protein sequence. This cysteine is located in the conserved sequence -PCHR- and is residue 145 in the human AGT sequence. The cysteine is not regenerated and each AGT molecule can therefore act only once.

As expected from its activity, AGT binds to DNA (12–14) and plausible models of this binding have been made based on the crystal structure of the protein (15–17). Such binding serves no useful purpose after the S-alkylcysteine is formed in the AGT as the alkyl-AGT is inactive, and there is evidence that it may actually retard the removal of additional m\(^6\)G lesions by either active AGT molecules or by the nucleotide excision repair system which does recognize such damage although inefficiently (18–20). One way in which this potential problem may be dealt with is for the inactive, alkylated form of AGT (alkyl-AGT) to be degraded. An enhanced degradation rate of AGT after exposure to methylating agents or to the inactivator \(O^6\)-benzylguanine (BG), which is a pseudosubstrate and reacts with the AGT protein forming S-benzylcysteine at the active site of the protein (21,22), has been reported (23). Evidence has also been presented for the ubiquitination of AGT and for an increased formation of ubiquitinated AGT after exposure of cells to alkylating agents or BG (24,25). This is consistent with degradation of AGT via the ubiquitin–proteasomal pathway but the molecular basis for the enhanced breakdown of alkyl-AGT degradation is unknown. Residues 9–17 and 96–104 in human AGT have some similarity to destruction box motifs required for cell cycle–specific ubiquitin-mediated degradation of mitotic cyclins (25,26) and AGT may be a target for phosphorylation (27–29) but no evidence has yet been provided showing directly the importance of these observations in AGT degradation in vivo.

In the present experiments, we have studied the mechanism of the degradation of alkyl-AGT in cultured cells and in reticulocyte lysates using both wild-type (WT) AGT and mutants in which the cysteine acceptor site and other residues in the active site pocket have been converted to other amino acids. Our results show that a conformational change in the AGT protein brought about by alkylation of the active site Cys-145 increases the ability of the protein to serve as substrate for ubiquitin ligase(s), which target it for degradation via the 26S proteasome. This conformational change involves the disruption of the hydrophobic bonding network involving Cys-145 and opening of the asparagine hinge in AGT described by Daniels et al. (17). The results therefore demonstrate a novel means by which a post-translational modification of a protein targets it for ubiquitination.

Abbreviations: AGT, \(O^6\)-alkylguanine-DNA alkyltransferase; alkyl-AGT; AGT with S-alkylcysteine at position 145; BG, \(O^6\)-benzylguanine; m\(^6\)G, \(O^6\)-methylguanine; MG132, \(N\)-carbobenzoxy-L-leucinyl-L-leucinyl-L-leucinal; NEM, \(N\)-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; WT, wild-type.

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Materials and methods

Materials

Oligodeoxynucleotides were purchased from Life Technologies (Gaithersburg, MD). t-I[^35]S)methionine was obtained from DuPont NEN (Boston, MA). TNT T7-coupled reticulocyte lysate translation system and RNasin were obtained from Promega (Madison, WI). All plasmid purification materials were from Qiagen (Chatsworth, CA). Ubiquitin aldehyde was purchased from Boston Biochemicals (Cambridge, MA). Rabbit reticulocyte lysate prepared from phenylhydrazine-treated New Zealand White rabbits for use in degradation assays was obtained from Cocalico Biologicals (Reamstown, PA). BG was synthesized (21) and generously provided by Dr R.C.Moschel (National Cancer Institute-Frederick Cancer Research Development Center, Frederick, MD). N-Carbobenzoyl-L-leucinyl-L-leucinyl-L-leucinal (MG132) was purchased from Calbiochem (LaJolla, CA) and dissolved in dimethyl sulfoxide as a 10 mM stock solution. N-Carbobenzoxyl-t-leucinyl-t-leucinyl-t-leucinal (MG132) was purchased from Calbiochem (LaJolla, CA) and dissolved in dimethyl sulfoxide as a 10 mM stock solution. ATP, creatine phosphokinase, ubiquitin, phospho-chased from Calbiochem (LaJolla, CA) and dissolved in dimethyl sulfoxide (PMSF) and cycloheximide were purchased from Sigma (St Louis, MO). PMSF was dissolved as a 0.1 M solution in ethanol and a stock solution of 6 mM cycloheximide was prepared in water. N-Ethylmaleimide (NEM) was purchased from Pierce (Rockford, IL). Protein A immobilized on Sepharose CL-4B was purchased from Sigma and prepared as 50% protein A as described (30) in 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.5% NP-40, 5 mM EDTA. Geneticin was and all other cell culture reagents were obtained from Gibco-BRL (Gaithersburg, MD). The oligodeoxynucleotide, 5'-GCTCCTCCCTGAAGCTTCACA-GGAG-GAGTGTCCTCTGCTCTCCCC-3' (45mer m6G), was obtained from NBI (Plymouth, MN).

The AGT-specific MAP-1 antibody (31) or the AGT monoclonal antibody (clone MT 1.1) (from NeoMarkers, Fremont, CA) was used for western blot analysis. The rabbit anti-ubiquitin polyclonal antibody was purchased from StressGen (Victoria, BC, Canada) and used according to the manufacturer’s recommendations.

Construction of plasmids

Plasmid pCMV-AGT was made and described previously (32). Plasmid pCMZEO-AGT was prepared by inserting the AGT cDNA from pCMV-AGT into pcDNA3.1(–) vector (Invitrogen, Carlsbad, CA) using the multiple cloning site between BamHI and KpnI. The mutations at Cys-145 (C145A, C145F, C145G, C145S, C145E, C145W, C145Y and C145M) in pGEM vector were made using the Chameleon double-stranded mutagenesis kit (Stratagene, La Jolla, CA), with a random mutagenic primer 5’-CCATCTCCATCCGGGNCCAGAGGTGTCCTGC-3’ (N stands for A, T, G or C). The individual mutations were selected by sequencing analysis. The mutant cDNAs were subcloned into pcMZEO-AGT using the EcoNI (located at position 99) and AgeI (located at position 521) restriction sites. All lysine to arginine mutants in AGT were created using the Chameleon double-stranded mutagenesis kit according to manufacturers instructions using pcMZEO-C145F AGT as template and appropriate primers. The G177stop/c145F mutant was made by replacing the DNA fragment produced by digestion of the pcMZEO-C145F plasmid with AgeI and KpnI with an equivalent fragment containing G177stop mutation that had been excised from pQE-G177stop. The DNA fragment containing the mutation M134F was isolated from pUC-M134F (33) by digestion with EcoNI and AgeI and was used to replace the equivalent DNA fragment from pcMZEO-WT AGT to form pcMZEO-M134F. The entire coding sequence of all AGT mutants was checked by DNA sequencing analysis.

CHO cell lines expressing WT and mutant AGTs

All cells were maintained in α-MEM, supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. CHO cells were transfected with pCMV-AGT, pCMV-C145F or pCMV-C145A using LipofectAMINE™ (Gibco-BRL). Cells were selected with 1 mg/ml Geneticin and individual colonies were isolated and examined for mutant AGT expression by western blot analysis as described below. For all experiments, cells were plated at a density of 3.5×10^4 cells/100 mm tissue culture plate, allowed to grow to 16–18 h before drug applications and harvested at 75–80% confluency.

Western blot analysis of AGT expression

Crude cell extract was prepared as described below and separated on a 12.5% SDS–polyacrylamide gel at 45 mAmp/gel and transferred electrophoretically at 30 V overnight to PVDF-Plus transfer membrane (MSI, Frederick, MD). The blots were then analyzed by standard western blotting procedures (Amersham, Arlington Heights, IL). Visualization and quantification were carried out using Fluorimager 595 (Molecular Dynamics, Sunnyvale, CA) with the ImageQuant software.

Measurement of AGT half-life

Cells were treated with 0.2 mM cycloheximide to inhibit protein synthesis in the presence or absence of 80 µM BG to inactivate AGT. In some experiments 25 µM MG132 was also added. Cells were harvested in PBS at time points of 1–2 h following drug application. Cells were centrifuged at 3000 g, resuspended in 200 µl of ice-cold harvest buffer (50 mM Tris–HCl pH 7.5, 0.1 mM EDTA and 5 mM dithiothreitol) and sonicated on ice for 1 min with 10 s pulses on setting three, using a Sonicator Ultrasonic Processor (Msonix, Farmington, NY). Protein content was determined using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Fifty micrograms of extract from each time point was analyzed for AGT content by western blot analysis.

Detection of AGT–ubiquitin complexes in vivo

Cultures were treated for 4 h with 80 µM BG to inactivate AGT, and 25 µM MG132 to inhibit proteasomal degradation and to increase the amount of ubiquitin–AGT conjugates in the cells. The cells were then harvested in cold PBS, centrifuged at 3000 g for 5 min, resuspended in 1 ml cold harvest buffer (200 mM Tris–HCl pH 8.5, 50 mM NaCl, 1% v/v Triton X-100, 1 mM PMSF, 0.5 mM ATP, 10 mM phosphocreatine, 0.05 mg/ml creatine phosphokinase, 0.02 mg/ml ubiquitin aldehyde and 10 µg/ml of proteinase inhibitors). The lysates were centrifuged at 10 000 g for 15 min at 4°C. Supernatant was harvested and extract equivalent to 50 µg of protein was used for western blotting probes with MT 3.1 AGT monoclonal antibody.

Degradation of AGT in reticulocyte lysates

WT or mutant AGTs labeled with [^35]S)methionine were synthesized from 0.1 mM of the appropriate pCMZEO-AGT plasmid in the TNT-coupled transcription-translation system according to manufacturer’s instructions. A 4 µl aliquot was then used as a substrate for degradation by adding it to a standard degradation assay. The assay mix consisted of a total volume of 200 µl containing 40 mM Tris–HCl pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine, 0.05 mg/ml creatine phosphokinase, 0.02 mg/ml ubiquitin aldehyde and reticulocyte lysate prepared from phenylhydrazine-treated New Zealand White rabbits (50 µl unless specified otherwise). When ubiquitin was included, 141 µM was used for each reaction. In some experiments, 5 µM ubiquitin aldehyde and 100 µM MG132 were also present. The mixture was incubated at 37°C for various times as indicated. The rate of[^35]S)AGT degradation was measured using 40 µl aliquots of the degradation assay. These were taken at various time intervals, mixed with SDS sample buffer and boiled for 10 min prior to being separated by SDS–PAGE. The rate of[^35]S)labeled protein degradation was determined by quantifying the 22 kDa band on fixed and dried gels using a Molecular Dynamics PhosphorImager and ImageQuant application software. This method allowed the measurement of the disappearance of the AGT protein and detection and measurement of the polyclubiquitinated species formed from AGT.

In some experiments, a fractionated reticulocyte lysate degradation system was prepared as described (34) using DE52-cellulose chromatography (35). Fraction II from this material, which is devoid of free ubiquitin and is also missing some of the E2 and E3 proteins, was used instead of the total reticulocyte lysate to evaluate conjugation of AGT protein with ubiquitin, methylated ubiquitin and K48R-mutant ubiquitin molecules. The[^35]S)AGT protein used in these experiments was prepared as described below. A degradation system which was primed by the appearance of low molecular weight fragments from AGT were measured as follows. The[^35]S)AGT degradation was measured using 40 µl aliquots of the degradation mix was taken and precipitated by incubation with 5% trichloroacetic acid for 30 min. After centrifugation at 14 000 r.p.m. at 4°C for 15 min, the supernatant was combined with 10 mC Sscintillation fluid and the radioactivity, representing labelled AGT broken down to small fragments, was determined.

Measurement of AGT activity from protein synthesized in reticulocyte lysates

The TNT assay reaction was carried out as described above without unlabeled methionine. The extracts were then incubated with a [^3]H-methylated DNA substrate and the AGT activity was determined by measuring [^3]H-methylated protein formed, which was collected on nitrocellulose filters as described previously (33).

Results

Degradation of AGT and mutants C145F and C145A in CHO cells

Sequences corresponding to the cDNA for WT AGT and for mutants C145A and C145F were inserted into the pCMV-neo

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Degradation of alkyltransferase vector in which protein is expressed from the CMV promoter. CHO cells, which lack endogenous AGT, were transfected with these constructs and stable clones isolated and analyzed by western blot analysis for AGT protein expression. As reported previously (32), a single band with mobility corresponding to human AGT (apparent molecular weight of 22,000) was seen. Clones were selected in which the level of protein expression for WT, C145F and C145A were similar. The stability of the AGT protein in the presence and absence of BG was then determined by following the content of the AGT protein after inhibition of protein synthesis by cycloheximide (Figure 1A). WT AGT was stable over an 8 h period and so was the mutant C145A. In contrast, mutant C145F was degraded rapidly with a half-life of ~90 min (Figure 1A). When BG was added to the cell cultures in order to convert the AGT to the benzyl-AGT, the stability was substantially reduced and the benzyl-AGT was degraded with a half-life of ~160 min. BG had no effect on the stability of the C145A and C145F mutants (Figure 1A).

In order to test whether the rapid degradation of the benzylated WT AGT protein and the C145F mutant was brought about by degradation of ubiquitinated AGT by the 26S proteasome, the cell cultures were treated with the inhibitor MG132, a peptide aldehyde that reversibly blocks protein hydrolysis at the 26S proteasome. The cells were pre-treated with BG and with cycloheximide and the AGT protein remaining at various times determined (Figure 1B). In the absence of MG132, the AGT protein disappeared rapidly from cells expressing WT and C145F mutant AGT, whereas the C145A AGT protein was stable over the 8 h period tested. When MG132 was added, all three AGT proteins were stable. Lysates from CHO cells transfected with WT or C145F AGT and treated with BG and MG-132 to prevent protein degradation were examined for the presence of a ubiquitinated form of AGT by western blot analysis of extract prepared in the presence of NEM to prevent hydrolysis of ubiquitin conjugates during sample preparation. The samples were probed with an antibody specific to the human AGT protein. As shown in Figure 1C, a ladder of bands of higher molecular weight than AGT itself were seen and this ladder was equally intense with benzyl-AGT or C145F. Treatment with BG increased the fraction of WT AGT present as ubiquitin conjugates but had no effect on the conjugation of C145F (results not shown).

**Degradation of AGT and mutants in vitro**

Further studies to examine the mechanisms of alkyl-AGT degradation were therefore carried out *in vitro*. The substrate in these experiments was made in a coupled transcription–translation (TNT) system using the AGT cDNA contained in plasmid pCMZEO as template. When synthesis was carried out in the presence of [35S]methionine, a band of the correct molecular weight for AGT was produced (see Figure 3). In order to check that the AGT protein made was active, the [35S]methionine was replaced with 20 μM unlabeled methionine and the ability of the extracts to repair [3H]-methylated DHFR substrate was determined. Results were shown for WT AGT (circles), C145A AGT (squares) and C145F AGT (triangles). The effect of MG132 on the stability of WT, C145A and C145F AGT after treatment with BG. Cells were harvested at the indicated times following treatment with WT AGT (circles), C145A AGT (squares) and C145F AGT (triangles). (B) The effect of MG132 on the stability of WT, C145A and C145F AGT after treatment with BG. Cells were harvested at the indicated times after treatment with BMG132 (open symbols) or alone (closed symbols). AGT protein content was analyzed by western blot analysis and plotted as percent protein remaining against time. Results are shown for WT AGT (circles), C145A AGT (squares) and C145F AGT (triangles). (C) The ladder of ubiquitinated forms of AGT in transfected CHO cells treated with BG and MG132. Lane 1 shows results for the CHO cells expressing no protein (pCMV empty vector), lane 2 shows results for cells expressing WT AGT and lane 3 shows results for cells expressing mutant C145F. Cells were treated with 80 μM BG and 25 μM MG132 for 4 h before being harvested and sonicated in a buffer containing 1 mM PMSF and 5 mM NEM. Fifty micromolars of such crude cell extract were separated on a 12.5% acrylamide SDS–PAGE. The proteins were transferred overnight to a PVDF membrane, and blotted with monoclonal antibody to AGT. The bands of higher molecular weight than AGT itself were not seen in analysis of WT AGT from cells untreated with BG.
Table I. Production of AGT activity in the TNT system and its inactivation

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Pre-incubation</th>
<th>AGT activity (fmol/µl reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMcZEO-AGT</td>
<td>No addition</td>
<td>20</td>
</tr>
<tr>
<td>pCMcZEO-AGT</td>
<td>200 µM BG</td>
<td>3.6</td>
</tr>
<tr>
<td>pCMcZEO-AGT</td>
<td>9 µM 45mer m6G</td>
<td>0.4</td>
</tr>
</tbody>
</table>

DNA measured (Table I). After translation of pCMZEO-AGT for 30 min, an amount of AGT activity equivalent to 20 fmol of m6G in 3H-methylated DNA repaired per microliter of TNT reaction. This activity was reduced by >80% by incubation for 30 min with BG and by >95% by 45mer m6G showing that the AGT synthesized was converted to the alkylated form by these conditions. The more extensive conversion by the 45mer m6G represents the faster rate of reaction of AGT with such substrates (13,21,22).

The AGT protein synthesized in the TNT system in the presence of [35S]methionine was used as a substrate for degradation by a reticulocyte lysate system. Small aliquots of the synthesis reaction containing labeled AGT were incubated with the degradation system and the protein present analyzed by SDS–PAGE (Figure 2). The 22 kDa band corresponding to WT AGT was stable over a 160 min incubation period. However, if the WT AGT was inactivated by the use of either BG or an oligodeoxyribonucleotide containing m6G to convert it to the form containing S-benzylcysteine or S-methylcysteine at the active site, the AGT was rapidly degraded (Figure 2A and B). Similar studies of the in vitro degradation of AGT mutants C145A, C145F and M134F were carried out (Figure 2B and C). Mutant C145A was relatively stable but the C145F protein was degraded rapidly (Figure 2B and C). Treatment with BG had no effect on the degradation of these mutant AGT proteins. Mutant M134F was degraded quite rapidly but degradation was slightly accelerated after reaction with BG, giving a rate similar to that of C145F and benzyl-AGT (Figure 2B and C).

Degradation of [35S]methionine-labeled AGT was also measured by following the release of radioactivity into a form soluble in trichloroacetic acid. The labeled protein was precipitated from the synthesis reaction using ammonium sulfate, washed to remove free [35S]methionine and added to the degradation system. At various times, the amount of protein converted to products of low molecular weight was measured by determination of the radioactivity released into the supernatant fraction after precipitation with trichloroacetic acid (Figure 3). The results were in good agreement with the results based on the decline of the 22 kDa band described above. There was a progressive release of labeled low molecular weight material from benzyl-AGT and this was strongly inhibited by the addition of MG132 showing that it occurs by proteasomal-mediated degradation. The release was most rapid with C145F, benzyl-AGT and benzyl-M134F. Degradation of M134F was intermediate but was considerably faster than that from WT AGT.

A wider range of mutants altered at position Cys-145 was tested for susceptibility to degradation. The loss of the 22 kDa AGT band after incubation of the [35S]methionine-labeled AGT protein with the reticulocyte lysate system (Figure 4) indicated that these mutants can be placed in three classes. Mutants C145F, C145R, C145Y and C145W were the most susceptible to degradation. Mutants C145M, C145I, C145G and C145A were all degraded more rapidly than WT. Mutants C145S and C145E resembled WT in resistance to proteasomal degradation. Results in agreement with this classification were also obtained when the amount of [35S]methionine-labeled AGT protein converted to trichloroacetic acid soluble components in a proteasomal-mediated reaction was used (Table II). The mutants indicated were synthesized in the TNT system with [35S]methionine and the labeled protein incubated for the
Degradation of alkyltransferase

Fig. 3. Release of low molecular weight degradation products from WT AGT and mutants M134F and C145F in vitro. The [35S]methionine-labeled AGTs were incubated for 30 min with (filled symbols) or without (open symbols) 200 µM BG as shown, and then precipitated with ammonium sulfate, washed and added to the reticulocyte lysate degradation system. After incubation for the time shown, the amount of radioactivity converted to a form soluble in 5% trichloroacetic acid was measured. Results are shown for WT AGT (circles), M134F (triangles) and C145F (squares). Results are also shown for WT benzyl-AGT when 20 µM MG132 was included in the assay mix.

Table II. In vitro proteasomal release of low molecular weight degradation products from AGT mutants at Cys-145

<table>
<thead>
<tr>
<th>Amino acid present at position 145</th>
<th>Percent of radioactivity released in</th>
<th>44 min</th>
<th>88 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys (WT)</td>
<td></td>
<td>3.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Glu</td>
<td></td>
<td>3.6</td>
<td>4.8</td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td>3.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Ala</td>
<td></td>
<td>5.6</td>
<td>7.4</td>
</tr>
<tr>
<td>Gly</td>
<td></td>
<td>5.1</td>
<td>7.6</td>
</tr>
<tr>
<td>Ile</td>
<td></td>
<td>5.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Met</td>
<td></td>
<td>5.4</td>
<td>9.1</td>
</tr>
<tr>
<td>Phe</td>
<td></td>
<td>7.3</td>
<td>11.2</td>
</tr>
<tr>
<td>Trp</td>
<td></td>
<td>8.1</td>
<td>12.4</td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td>8.1</td>
<td>12.6</td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td>10.8</td>
<td>13.8</td>
</tr>
</tbody>
</table>

The [35S]-labeled mutant AGT was precipitated with ammonium sulfate, washed and added to the reticulocyte lysate degradation system. After incubation for the first time, shown in the presence or absence of 20 µM MG132, the amount of radioactivity converted to a form soluble in 5% trichloroacetic acid was measured and the difference is plotted.

Fig. 4. Loss of the 22 kDa AGT band when AGT mutants at Cys-145 were incubated with reticulocyte lysate degradation system.

Fig. 5. Accumulation of ubiquitinated products from benzyl-AGT and mutants C145F and M134F in the presence of ubiquitin aldehyde and MG132. (A) SDS–PAGE analysis of [35S]-labeled C145F AGT after incubation in the reticulocyte lysate degradation system in the presence of 141 µM ubiquitin in the absence (left lanes) or presence (right lanes) of 5 µM ubiquitin aldehyde (ubal) and 100 µM MG132. (B) WT AGT, M134F AGT and C145F AGT before and after incubation for 45 min in the presence of 5 µM ubiquitin aldehyde and 100 µM MG132. The proteins were incubated with 200 µM BG for 30 min prior to the start of the degradation reaction.

Ubiquitination of AGT and mutants in vitro

A faint ladder of ubiquitinated AGT bands was seen when mutant C145F was incubated in the reticulocyte lysate system. The bands were much more prominent when MG132 was added to prevent their degradation and ubiquitin aldehyde was added to block their hydrolysis (36) (Figure 5A). Under these conditions, ~40% of the C145F AGT was converted to bands of higher molecular weight and a significant proportion accumulated as highly polyubiquitinated material, which migrated very slowly. Similar bands were seen with WT benzyl-AGT and mutant M134F (Figure 5B).

In an attempt to find the site for ubiquitination, individual mutations to arginine of all 12 lysine residues in AGT were made. However, mutations at K3, K8, K18, K32, K36, K101, K104, K107, K125, K165 and the truncation G177stop (which removes both K178 and K193) did not alter the ubiquitination pattern of mutant C145F (Figure 6A–C) or change the rate time shown with the reticulocyte lysate degradation prior to separation by SDS–PAGE.
of conversion of \[^{35}\text{S}\]methionine-labeled AGT protein to trichloroacetic acid soluble components (not shown). The triple mutants in which the double mutant K101R/K104R was combined with either K107R or K125R also did not show an altered ubiquitin pattern (Figure 6C). This suggests that more than one lysine can be used for the first ubiquitin addition. However, there is likely to be only one primary site of ubiquitination in WT AGT as when a partially fractionated degradation system supplemented with either methylated ubiquitin or the K48R ubiquitin mutant was used in the reaction with C145F (Figure 6D) or benzyl-AGT (not shown), only a single monoubiquitinated band was formed. This result shows that (i) only one lysine in the AGT is modified and that (ii) the linkage forming polyubiquitinated chains is through Lys-48 of the ubiquitin. Thus, it appears that when the lysine normally used for ubiquitination is not available another can be used instead.

**Discussion**

The enhanced degradation of the AGT protein after it has been converted to its inactive alkylated form would remove any interference with DNA repair of additional O\(^6\)-alkylguanine lesions by both active AGT molecules and other repair pathways, would remove an ineffective protein from the nuclear environment, and could conceivably trigger the synthesis of additional AGT proteins that would be needed to maintain the capacity to repair such DNA damage. Such enhanced synthesis is known to occur in tissues and cells treated with alkylating agents (3,37–40).

Our results outline a plausible model for the disposal of the alkyl-AGT that is produced in the repair of DNA lesions by this protein. The WT AGT protein is normally stable as indicated by the lack of decrease in its content in CHO cells when protein synthesis is inhibited by cycloheximide and is only a poor substrate for ubiquitination. The recognition of proteins by enzymes adding ubiquitin is the key step in targeting proteins for degradation by the 26S proteasomal system (41,42). After conversion of the AGT protein to the alkylated form by exposure to the substrate/inhibitor BG, the protein becomes unstable in CHO cells and is degraded. This degradation occurs via the proteasome as it is prevented completely by exposure to MG132, an inhibitor of proteasomal proteolysis. Ubiquitin conjugates of benzyl-AGT occurred in CHO cells [as reported previously for other mammalian cells (24) and human tissue extracts (25)] and as shown in Figure 5 were accumulated in reticulocyte lysates in vitro, provided that MG132 and ubiquitin aldehyde were added to block proteasomal degradation and ubiquitin hydrolases (36,41). It is well established that ubiquitin conjugates are in a dynamic state and subject to such degradation and that proteasomal degradation requires the synthesis of a multi-ubiquitinated form with more than four ubiquitin additions involving linkage through Lys-48 of ubiquitin (43,44).

Thus, the formation of S-alkylcysteine at the active site of the AGT provides a signal for ubiquitination. It is probable that this is caused by a conformational change in the AGT protein. Recent studies by X-ray crystallography (17) provide strong evidence that such a change occurs and confirm earlier studies that have suggested it, based on physiochemical techniques (45,46) or sensitivity to proteases (47–49). The crystal structure of WT AGT shows that Cys-145 is part of an extensive hydrogen-bonded network in which a water molecule hydrogen bonds to the side chains of Cys-145 and Tyr-158 as well as the N\(_6\) of His-146 and the backbone nitrogen of Ile-141 (16,17). Comparison of the structures of the native human AGT with its S-methyl- and S-benzyl-derivatives show that not only is this hydrogen-bonded network disrupted by alkylation but there is also a sterically driven helix displacement (see figure 5 in ref. 17). The adducts are in close van der Waals contact with the carbonyl oxygen of Met-134 and the helix in which this residue is located moves by 0.5–1.5 Å away from the N-terminal domain of the protein in order to accommodate this close contact (17). This results in an opening of the asparagine hinge formed by residues from Asn-137 to Pro-144 (17).

We suggest that this alteration in structure not only facilitates the dissociation of the AGT from DNA as postulated (17), but also increases the recognition of the protein by an E3 ubiquitin ligase with its associated E2 ubiquitin-conjugating enzyme. There are many well-known examples in which such recognition occurs by virtue of structural changes caused by post-translational modification such as phosphorylation (41,50,51). Recently, it has been observed that AGT is a substrate for phosphorylation at multiple sites (27,28). At present, we cannot rule out the possibility that such phosphorylation is enhanced after the conformational changes described above and then mediates the interaction with the relevant E2/E3, but a simpler explanation would be that the alkylation-induced conformational change itself is sufficient to bring about this recognition.

The replacement of Cys-145 with any bulky residue might be expected to mimic the formation of an S-alkyl adduct at this cysteine and our studies confirm that such an alteration renders AGT subject to ubiquitin-mediated proteolysis. The C145F mutant is a reasonable facsimile of the benzylated AGT and should be a useful tool with which to identify and
characterize the components of the system recognizing alkylated AGT. As would be expected, the replacement of Cys-145 with tyrosine or tryptophan has a similar effect on AGT degradation to replacement with phenylalanine. The mutation that caused a large increase in the susceptibility of AGT to proteasomal degradation was C145R. In this case, it is probable that the positively charged side chain of the arginine residue also produces a change in conformation by repulsion from the positively charged side chains of residues His-146 and Arg-147.

The only mutants at position 145 that were similar to WT AGT in their proteasomal degradation were changes to serine in which the hydrogen-bonding network described above can be maintained and the alteration to glutamic acid. Although the latter is at first sight surprising, the interaction of this negatively charged residue with His-146 or possibly Arg-147 might prevent the helical displacement.

Mutants C145A, C145G, C145I and C145M were slightly less resistant than WT to proteasomal degradation in the reticulocyte lysate system but were not sensitized to the extent shown by the class of mutants typified by C145F. The slightly enhanced rate of degradation of the C145A mutant AGT compared with WT was not reflected in the studies in CHO cells but it should be noted that this system was only studied for a relatively short 6 h period in which cycloheximide could be used to inhibit protein synthesis without causing toxic effects. The modest increase in susceptibility to degradation seen in mutants like C145A may be explained by the fact that the hydrogen-bonded network is disrupted by the changes but the side chains of the mutant amino acids can be inserted without the maximal level of steric displacement produced by more bulky residues.

Conversely, the introduction of a steric displacement without disruption of the hydrogen-bonding network also partially sensitized the AGT to proteasomal degradation. This is demonstrated by the results with mutant M134F. As described above, Met-134 is located in the helix H6 that also forms part of the active site pocket. The increase in the size of the side chain when Met-134 is replaced by phenylalanine would mimic the displacement caused by the addition of an alkyl group to the Cys-145 acceptor site described above. As shown in Figures 2 and 3, this mutant is more readily ubiquitinated and degraded than WT AGT but is less labile than mutant C145F unless also allowed to react with BG. [Mutant M134F is capable of reacting with BG under the conditions used in our experiments (33)] The results suggest that both steric displacement factors and the interruption of the hydrogen-bond network that involves Cys-145 is necessary to maximize the ubiquitination and degradation of the alkyl-AGT.

The results shown in Figure 6D indicate that only mono-ubiquitinated AGT was formed from C145F when ubiquitin analogs that are unable to form extended chains due to methylation or mutation of the Lys-48 residue in ubiquitin needed for chain elongation were used for the conjugation reaction. This implies that there is normally one preferred site for ubiquitination. However, the results in Figure 6A–C show that when this site is mutated to arginine, another lysine can be used instead as no single lysine to arginine mutant was not converted to ubiquitinated products. It is noteworthy that even the triple K101R/K104R/K107R alteration did not prevent ubiquitination although it has been reported that upon alkylation, AGT becomes susceptible to immunoprecipitation with a monoclonal antibody for which the epitope has been identified as residues 101–107, KLLKVKV (48). This rules out the possibility that this is the ubiquitination site but it remains possible that it is involved in recognition by E2/E3 conjugating enzymes.

Comparison of the C145F and C145S mutant forms of AGT with respect to protein degradation and the ability to act as substrates for the formation of multi-ubiquitinated chains should provide a valuable model system in which the pathway of AGT degradation can be characterized more fully. As discussed above, the former mutant represents the alkylated form of the protein. The C145S mutant should be an excellent model for the WT protein that is free from the disadvantage that repair of endogenous alkylation damage is continually converting the WT AGT to the alkylated and readily degraded form. Screening for proteins that are able to recognize the C145F protein but not the C145S protein would be a powerful way to identify the proteins playing a critical role in the degradation of alkyl-AGT.

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