Conserved residue lysine¹⁶⁵ is essential for the ability of *O*⁶-alkylguanine–DNA alkyltransferase to react with *O*⁶-benzylguanine

Meng XU-WELLIVER, Sreenivas KANUGULA, Natalia A. LOKTIONOVA, Tina M. CRONE and Anthony E. PEGG¹

Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, P.O. Box 850, 500 University Drive, Hershey, PA 17033-0850, U.S.A.

The role of lysine¹⁶⁵ in the activity of the DNA repair protein, *O*⁶-alkylguanine–DNA alkyltransferase (AGT), and the ability of AGT to react with the pseudosubstrate inhibitor, *O*⁶-benzylguanine (BG), was investigated by changing this lysine to all other 19 possibilities. All of these mutants (except for K165T, which could not be tested as it was too poorly active for assay in crude cell extracts) gave BG-resistant AGTs with increases in the amount of inhibitor needed to produce a 50 % loss of activity in a 30 min incubation (ED₅₀) from 100-fold (K165A) to 2400-fold (K165F). Lys¹⁶⁵ is a completely conserved residue in AGTs from many species, and all of the mutations at this site also reduced the ability to repair methylated DNA. The least deleterious change was that to arginine, which reduced the rate constant for DNA repair by approx. 2.5-fold. Mutant K165R resembled all of the other mutants in being highly resistant to BG, with an ED₅₀ value for inactivation by BG > 200-fold greater than wild-type. Detailed studies of purified K165A AGT showed that the rate constant for repair and the binding to methylated DNA sub-

strates were reduced by 10–20-fold. Despite this, the K165A mutant AGT was able to protect cells from alkylating agents and this protection was not abolished by BG. These results show that, firstly, lysine at position 165 is needed for optimal activity of AGT towards methylated DNA substrates and is essential for efficient reaction with BG; and second, even if the AGT activity towards methylated DNA substrates is impaired by mutations at codon 165, such mutants can protect tumour cells from therapeutic alkylating agents. These results raise the possibility that the conservation of Lys¹⁶⁵ is due to the need for AGT activity towards substrates containing more bulky adducts than *O*⁶-methylguanine. They also suggest that alterations at Lys¹⁶⁵ may occur during chemotherapy with BG and alkylating agents and could limit the effectiveness of this therapy.

Key words: alkylating agents, chemotherapy, DNA repair, *O*⁶-methylguanine.

INTRODUCTION

The DNA repair protein, *O*⁶-alkylguanine–DNA alkyltransferase (AGT), which is a key factor in protecting cells from certain alkylating agents, including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and the therapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [1–5], has been found in many species. Twenty-eight DNA sequences coding for human AGT genes representing 25 different species have now been reported [6,7]. The protein sequences encoded by these genes have substantial homology but there are only 8 absolutely invariant residues (Gly¹⁰⁹, Asn¹³⁷, Pro¹⁴⁴, Cys¹⁴⁵, His¹⁴⁶, Arg¹⁴⁷, Lys¹⁶⁵ and Glu¹⁷², numbered according to the human AGT sequence) in these sequences (Figure 1). As expected, the cysteine acceptor residue is one of the totally conserved residues along with the surrounding 3 amino acids in the sequence -PCHR-. These residues are thought to contribute to the activation of the cysteine residue that acts as a thiolate anion to attack an alkyl group on the *O*⁶-position of guanine [6,8,9]. Mutation of the cysteine acceptor site leads to a total loss of AGT activity [10–12], and mutants of the adjacent residues greatly reduce the stability of the protein and its ability to repair methylated DNA [10,13,14].

The invariant residues Asn¹³⁷ and Glu¹⁷² are critical for maintaining the AGT structure [14,15]. Residues Arg¹²⁸ and Tyr¹¹⁴, which are highly conserved but not absolutely invariant, have been shown to be involved in DNA binding and catalytic

activity [7,16]. Alterations at all of the sites mentioned above lead to a loss of AGT activity. It was therefore somewhat unexpected that two mutants representing highly unrelated substitutions at Lys¹⁶⁵, a totally conserved residue (Figure 1), were found during the screening of a library of plasmids encoding AGT molecules, in which a 24 amino acid region was subjected to random mutagenesis [17]. As described in the preceding paper, two mutations at Lys¹⁶⁵ were found (two colonies with K165S and one with K165A) when 107 surviving colonies, which contained 70 different mutants, were analysed during this screening. Plasmids were selected from this library based on the ability of the mutated AGT to protect against killing by MNNG in the presence of the AGT inactivator *O*⁶-benzylguanine (BG). The

1	MDKDC	EMKRT	TLDSPL	GKLE	LSGCE	QGLHE	IKLLG	KGTSA	40
41	ADAVE	VPAPA	AVLGG	PEPLM	QCTAW	LNAYF	HQPEA	IEEFP	80
81	VPALH	HPVFQ	QESFT	RQVLW	KLLKV	VKFGE	VISYQ	QLAAL	120
121	AGNPK	AA RA V	GGAMR	GNPVP	ILIP	PCHR	VVC	SSGAV	160
161	GLAV	K EWLLA	HEGHR	LKGKPG	LGGSS	GLAGA	WLKG	GATSG	200
201	SPP	AGR							

Figure 1 Sequence of human AGT

Residues that are invariant in all of the known AGT sequences are shown in **bold**. Residues Tyr¹¹⁴ and Arg¹²⁸, which are discussed in the text with regard to DNA binding and reaction with BG, are underlined. Residue Lys¹⁶⁵ is shown with double underlining.

Abbreviations used: AGT, human *O*⁶-alkylguanine–DNA alkyltransferase; BG, *O*⁶-benzylguanine; DTT, dithiothreitol; LB, Luria–Bertani; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; WT, wild-type; ED₅₀, the amount of inhibitor needed to produce a 50 % loss of activity in a 30 min incubation.

¹ To whom correspondence should be addressed (e-mail aep1@psu.edu).

two mutant AGTs at Lys¹⁶⁵ (K165A and K165S) found were indeed active in repairing methylated DNA *in vitro* and highly resistant to inactivation by BG. Although there was some reduction in the activity of *Escherichia coli* extracts expressing these mutant AGTs, instead of wild-type (WT) AGT towards methylated DNA, these mutants show that Lys¹⁶⁵ cannot be essential for AGT activity.

The crystal structures of the alkyltransferases from *E. coli* [6,18], *Pyrococcus kodakaraensis* [19], and human [20] have been solved. These structures are consistent with suggestions that AGT acts via a base-flipping mechanism in which the O⁶-alkylguanine is flipped out of the DNA helix to permit the reaction to occur [21–23]. Such base flipping is now well established for DNA repair glycosylases [24–27]. These models place Lys¹⁶⁵ in close proximity to the flipped-out nucleoside.

In the work presented in this present paper, a complete spectrum of mutants at Lys¹⁶⁵ was generated. All of these mutants were active but were resistant to inactivation by BG. Detailed studies were carried out with mutant K165A. Despite a significant reduction in the rate constant for DNA repair, this mutant was able to protect bacterial cells from MNNG and mammalian cells from 1,3-bis(2-chloroethyl)-1-nitrosourea. This protection was not abolished by BG. These results indicate that a positively charged side chain at residue 165 is needed for optimal repair of DNA, but that there is an even more stringent requirement for lysine at this position in order for the protein to react with the bulky substrate, BG. The 165 codon may be a hot spot for the generation of BG-resistant mutants of AGT that would overcome the advantages of this therapeutic agent.

EXPERIMENTAL

Materials

All oligodeoxyribonucleotides were purchased from Life Technologies. *E. coli* strain TRG8 was derived from strain GWR109 [28], kindly provided by Dr L. Samson, Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA, U.S.A., XL1-Blue bacterial strain and competent cells were purchased from Stratagene (La Jolla, CA, U.S.A.). pQE30 plasmid and the DNA isolation kits were obtained from Qiagen (Chatsworth, CA, U.S.A.). Restriction enzymes and *Pfu* polymerase were purchased from New England Biolabs (Beverly, MA, U.S.A.) and Stratagene. T4-ligase with the 'ligation express kit' and the Talon Metal Affinity Resin were purchased from Clontech Laboratories. Ampicillin, kanamycin, isopropyl β -D-thiogalactoside, haemocyanin, MNNG and most other biochemical reagents were purchased from Sigma. BG, synthesized as described in [29], was generously provided by Dr R. C. Moschel, ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD, U.S.A.

Plasmids pUC-AGTm [30], pIN-AGT [31], pIN-C145A [11], pQE-AGT [32], pCMV-G160R [33], pIN-K165A [34] and pUC-inAGT2 [35] were made as previously described.

Construction of pCMV-K165A mutant plasmid

Plasmid pCMV-K165A was made by ligation of the 85-bp DNA fragment containing a K165A mutation (at 495), obtained by digestion of pIN-K165A with restriction enzymes *Dra*III, which cuts at 436, and *Age*I, which cuts at 521, into the large fragment of pCMV-G160R (mutation at 480) digested with the same enzymes.

Construction of mutants K165F, K165H, K165I, K165L, K165M, K165P, K165Q, K165R and K165V in pUC vector

These mutants were created by PCR using 5'-CCCGTTTTC-CAGCAAGAGTCGTTAC-3' as sense primer matching pUC-AGTm plasmid from nucleotides 259 to 284 and mutagenic antisense primers 5'-GGCTTCCCCAACCGGTGGCCTTCA-TGGGCCAGAAGCCATTCXXXCACGGCTAGCCCTCC-3' (*Age*I site shown in italics, mismatches underlined, where XXX = GAA for phenylalanine, XXX = GTG for histidine, XXX = GAT for isoleucine, XXX = GAG for leucine, XXX = CAT for methionine, XXX = GGG for proline, XXX = CTG for glutamine, XXX = CCG for arginine, and XXX = CAC for valine) matching from nucleotides 536 to 478. The PCR reactions were carried out using *Pfu* polymerase and pUC-AGTm as template. The PCR products were purified with the Gel Purification Kit (Qiagen), digested with *Mlu*I (cut at 283) and *Age*I (cut at 521) enzymes, and ligated into the large fragment of pUC-inAGT2 plasmid digested with the same enzymes.

Subcloning of K165R mutant from the pUC vector to the pQE vector

The K165R mutation-containing 85-bp DNA fragment from pUC-K165R plasmid was isolated by digestion with *Dra*III and *Age*I restriction enzymes and ligated into the large fragment of pQE-AGT digested with the same enzymes to form pQE-K165R.

Construction and selection of K156X library containing a random codon at position Lys¹⁶⁵

The K156X construct was created by PCR using as sense primer 5'-CCGTGCCACAGAGTGG-3' (*Dra*III site shown in italics) and mutagenic antisense primers 5'-GGCTTCCCCAACCGGTGGCCTTCATGGGCCAGAAGCCATTCNNACGGCT-AGCCCTCC-3' (*Age*I site shown in italics, mismatches underlined). The PCR product was synthesized using pUC-AGTm as a template under the following conditions: initial denaturation for 2 min at 92 °C; 30 cycles of denaturation (1 min at 92 °C); annealing (1 min at 54 °C); extension (1 min at 72 °C); followed by a final extension at 72 °C for 5 min. The PCR product was gel purified using the Gel Extraction Kit (Qiagen), digested with *Dra*III and *Age*I enzymes, and ligated into pUC-inAGT2 digested with the same enzymes. The ligated products were transformed into XL1-Blue cells; an aliquot of the transformation mixture was plated on Luria-Bertani (LB) plates supplemented with 50 μ g/ml ampicillin to determine the number of plasmid-containing bacteria, and the remainder was amplified by growing overnight. The plasmid DNA was isolated from the overnight culture, subjected to sequencing analysis to confirm that the randomized sequence was present and introduced into the TRG8 strain by electroporation. An aliquot of the second transformant mixture was plated to determine the efficiency of transformation (2×10^8), and the rest was amplified overnight in LB medium containing ampicillin and kanamycin (50 μ g/ml each). Aliquots of this library were subjected to the screening process or stored at -70 °C for further use.

Active and BG-resistant alkyltransferase mutants were selected by treating exponentially growing random-library-transformed-TRG-8 bacteria ($A_{600} = 0.5$) with 50 μ M BG for 1.5 h, followed by 40 μ g/ml MNNG for 0.5 h at 25 °C as previously described [30]. Plasmid DNA was isolated from individual surviving colonies and subjected to sequencing analysis. Crude extracts for determination of AGT activity were prepared from each novel mutant grown overnight in 10 ml of LB medium by sonicating the

bacteria pellet resuspended in 50 mM Tris/HCl (pH 7.5), 0.1 mM EDTA and 5 mM dithiothreitol (DTT) for 2 min at 0 °C, using an ultrasonic cell disruptor (model W-225-R) on pulse setting 50% duty cycle. Cell debris was pelleted by centrifugation at 4 °C for 15 min at 15000 *g* and the supernatant was used to determine the alkyltransferase activity level and its sensitivity to BG.

Purification of mutant proteins

The WT and K165R mutant AGT were expressed using the pQE vector, which adds a 12 amino acid sequence (MRGS[H]₆GS-) to the N-terminus of the AGT protein and purified by immobilized metal-affinity chromatography as previously described [32]. The recombinant K165A and C145A mutant proteins were produced using the pIN plasmid and purified by FPLC [31].

Determination of effects of BG on AGT activity

Inactivation of the AGT activity by BG in crude bacterial extracts or purified protein preparations was measured by incubating aliquots of the protein with different concentrations of BG at 37 °C for 30 min in 0.5 ml of 50 mM Tris/HCl (pH 7.6), 5 mM DTT, 0.1 mM EDTA containing 50 µg haemocyanin and 10 µg of calf-thymus DNA. The residual AGT activity was determined by incubating the samples with [³H]methylated DNA substrate for 30 min at 37 °C and measuring [³H]methylated protein formed after collection on nitrocellulose filters [30]. The results were expressed as the percentage of the AGT activity remaining. Plots of remaining AGT activity versus inhibitor concentration were used to calculate an ED₅₀ value representing the amount of inhibitor needed to produce a 50% loss of activity.

Reaction of AGT with BG

Measurements of the formation of [8-³H]guanine from [8-³H]BG were carried out using various amounts of the purified AGT proteins in an assay buffer consisting of 0.7 µM *O*⁶-benzyl-[³H]guanine, 25 µg of calf-thymus DNA, 50 mM Tris/HCl (pH 7.6), 0.1 mM EDTA and 5 mM DTT in an assay volume of 0.25 ml. After incubation at 37 °C for 20 min, the reaction was stopped by the addition of 0.5 ml of the same buffer containing 0.2 mM guanine and 0.2 mM BG, and the extent of formation of [8-³H]guanine was determined by HPLC [16].

Kinetics of repair by AGT

The rate constant for the AGT reaction was determined by measuring the appearance of the [³H]methylated AGT at various times using concentrations of AGT protein determined in preliminary experiments to readily measurable rates under the assay conditions. The reaction mixture (1 ml) for each time point contained AGT (4.5 × 10⁻¹⁰ M for non-His-tagged WT, 1.35 × 10⁻⁹ M for His-tagged WT, 4.7 × 10⁻⁹ M for K165A and 5.6 × 10⁻⁹ M for K165R), 3.6 × 10⁻¹⁰ M *O*⁶-[³H]methylguanine in [³H]methylated calf thymus DNA substrate, and 50 µg of cold calf-thymus DNA in a buffer containing 50 mM Tris/HCl (pH 7.6), 5 mM DTT and 0.5 mM EDTA. The rate constant was determined as described in [7].

Electrophoretic mobility-shift assay

Electrophoretic mobility-shift assays were carried out as previously described [7], using oligodeoxynucleotides 5'-AACAGCCATATXGCCC 3' (16-mer), where X represents *O*⁶-methyl-

guanine or guanine. Increasing concentrations of AGT protein were incubated with 10 nM ³²P-labelled DNA probe in 20 µl of reaction mixture containing 20 mM Tris/HCl (pH 7.6), 5 mM DTT, 0.1 mM EDTA and 3% glycerol. The reaction mixtures were incubated for 10 min on ice and loaded on to pre-run, pre-equilibrated (4 °C) 6% (75:1 acrylamide/bis) polyacrylamide gels and electrophoresed in 10 mM Tris/acetate, 0.25 mM EDTA (pH 7.6) buffer for 100 min at 125 V. The amount of radioactivity in the unbound and bound DNA probe was quantified by exposing the dried gel to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The dissociation constant (*K*_d) was defined as the protein concentration required for half-maximal binding when excess protein was used in the reaction [36].

Cell culture, transfection and selection procedure

CHO cells were grown in α-minimal essential medium containing 36 mM NaHCO₃, 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin, as described in [33]. The CHO clones expressing AGT or its mutants were grown in the presence of 0.5 mg/ml geneticin (Gibco BRL) to prevent the loss of the plasmids.

The plasmids described above were used for the transfection of CHO cells to obtain clones expressing the mutant forms of AGT essentially as previously described for selection of CHO cells expressing AGT mutant G156A [33]. Briefly, CHO cells were transfected using Lipofectin (Gibco BRL) for stable transfection of adherent cells using 5 µg of DNA and 10 µl of Lipofectin. Cells which had taken up the plasmid were selected with geneticin at a final concentration of 1 mg/ml. Clones from individual cell colonies were isolated, examined for the level of AGT protein expression and used for further experiments.

Cytotoxicity assay

The colony-forming ability of cells was determined using a colony-forming assay as described in [33]. The CHO cells were plated using 10⁶ cells/25 cm² flask and grown for 24 h. Cells were incubated for 2 h with different concentrations of freshly prepared solutions of BCNU. The medium was replaced and the cells were grown for an additional 16–18 h. The cells were then re-plated at densities of 100–1000 cells/25 cm² flask and grown for 7–8 days until discrete colonies could be stained and counted. The colonies were washed with 0.9% NaCl solution, stained with 0.5% Crystal Violet and counted. The plating efficiency for CHO cells not treated with drugs was about 50%. To assess the effect of AGT inactivation on sensitivity to killing by BCNU, cells were treated with BG or 8-oxo-BG 2 h before exposure to 80 µM BCNU. After the BCNU treatment, all replacement medium contained BG or 8-oxo-BG at the same concentrations as the initial treatment to ensure that an AGT inhibitor was present in the cell during the entire period in which DNA adducts formed by BCNU were present.

Inactivation of cellular AGTs by BG or 8-oxo-BG

Inactivation of AGT by BG or 8-oxo-BG was determined in cells that reached 80% confluence after 4 h exposure to the AGT inhibitor, as described in [37]. Cells were harvested, washed with PBS and the cell pellets stored at -80 °C until analysis. Cell extracts were prepared as previously described [33], and residual AGT activity was measured by incubation for 30 min at 37 °C with the [³H]methylated DNA substrate and by measuring the [³H]methylated protein formed, which was collected on nitrocellulose filters. The results were expressed as the percentage of

the AGT activity present in the control cells to which no drugs was added.

Western blot analysis

The expression of WT and K165A proteins in transfected CHO cells was determined using immunoblots. After SDS/PAGE in 12.5% acrylamide gels, the protein immobilized on the nitrocellulose membrane was determined with a Vistra ECF Western Blotting Kit (Amersham) using antibody MAP-1 [33]. The intensity of chemiluminescence was measured using a FluorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

MNNG survival assay in the presence of BG

TRG8 bacteria containing plasmids expressing WT and K165A AGT were grown in 5 ml of LB media containing 50 µg/ml ampicillin and 50 µg/ml kanamycin with agitation at 250 rev./min in a water bath at 37 °C until the absorbance at 600 nm (A_{600}) reached 0.5. The cultures were treated with 0 or 50 µM BG for 1.5 h at 25 °C followed by a 0.5-h MNNG treatment as described in [30]. Reactions were stopped by diluting small aliquots of the bacteria cultures in M9 media on ice. The bacteria were further diluted and spread on LB plates containing 50 µg/ml ampicillin and 50 µg/ml kanamycin with or without 50 µM BG. The plates were incubated at 37 °C for 16 h, and the colony numbers were determined. The percentage survival was determined by using the number of colonies per ml of culture exposed to MNNG divided by the number of colonies per ml of culture when MNNG was absent.

RESULTS

Mutants at Lys¹⁶⁵

A plasmid library containing K165X mutations in the AGT sequence was constructed in the pUC vector and screened for mutants that had activity to protect cells from MNNG in the presence or absence of 50 µM BG, as described in the accompanying paper [17]. Site-directed mutagenesis was used to produce the mutants not found in this screen in the same vector, so that all 19 mutant AGT proteins could be assessed for their effects on AGT activity. Expression from the pUC-AGT vector (which contains a weak promoter and provides only a modest level of recombinant protein expression [30]) in TRG8 cells that have no endogenous AGT activity, provides a good system to test the ability of mutant AGT proteins to protect cells from the toxic effects of methylating agents such as MNNG [17,30]. None of the Lys¹⁶⁵ substitutions prevented the AGT from protecting against MNNG toxicity.

The amount of AGT activity expressed in the *E. coli* cells by the mutant AGTs was then assayed using cell extracts and testing the transfer of [³H]methyl groups from a [³H]methylated DNA substrate to protein (Table 1). The presence of the additional *E. coli* proteins had no effect on this reaction in control experiments when protein from TRG8 cells was added to pure recombinant WT human AGT at amounts up to or exceeding that present in the assays from the mutant cells. Activity could readily be measured in 18 of the 19 mutants, the exception being mutant K165T (Table 1). However, all mutants were less active than WT, based on the amount of total *E. coli* protein needed to obtain the same level of repair of methylated DNA (Table 1). In most cases, 40–100-fold higher amounts of crude bacterial extract had to be used. The only exception was the conservative change to arginine (K165R), where only approx. 5–6-fold more protein was required. The least active extracts were obtained with bacteria expressing mutants K165T, K165Q

Table 1 BG-resistant AGT mutants at lysine-165

Mutants K165A, K165C, K165D, K165G, K165S and K165T were isolated from the K165X plasmid library screened with BG plus MNNG. Mutant K165Y was obtained from this library screened with MNNG alone and mutants K165E, K165N and K165W were isolated from this library without selection. All other mutants were produced by site-directed mutagenesis. ND, not determined.

Amino acid	µg of protein*	ED ₅₀ (µM)
Tyr	119	240
Phe	101	113
Asn	200† (72%)	68
Ser	98	62
Gln	98‡	52
Trp	110	50
His	108	49
Met	164	49
Glu	98‡	30
Leu	37	29
Arg	8.6	21
Val	137	21
Ile	128	20
Asp	200† (72%)	18
Pro	119	12
Gly	200† (53%)	11
Cys	125	10
Ala	200† (64%)	10
Lys (WT)	1.5	0.1
Thr	200‡ (< 10%)	ND

* The amount of crude protein extract needed to get 80% removal of O⁶-methylguanine in the standard assay.

† The maximum protein that can be used in the assay is 0.2 mg. This gave less than 80% removal of O⁶-methylguanine and the amount removed is shown in parentheses.

‡ The AGT activity level was too low for accurate assays with crude extracts prepared under the standard conditions. Therefore the bacterial cultures were grown in the presence of 0.15 mM isopropyl β-D-thiogalactoside to increase AGT expression. These extracts were then used for the assay. The expression of AGT obtained in the presence of isopropyl β-D-thiogalactoside with these conditions is increased about 4-fold.

and K165E. Even with the addition of isopropyl β-D-thiogalactoside, mutant K165T did not produce sufficient activity in crude extracts for accurate measurement. It is not clear why K165T had such a low activity, especially since this mutant was selected multiple times in the screening of the K165X library with BG plus MNNG, and is therefore capable of protecting *E. coli* from killing by MNNG. It is possible that this mutant protein is very unstable in broken cell preparations, such that its activity could not be detectable in an *in vitro* assay, although this activity was sufficient to protect the bacterial hosts from killing by MNNG. Previous work shows that several AGT mutants produced at conserved residues, such as N137A and H146A mutations cause great instability in AGT, preventing assay in cell extracts but these mutants can still provide some protection from MNNG *in vivo* [14].

Comparative studies of the inactivation of the K165X mutants by BG were carried out by measuring ED₅₀ values in a standard assay using extracts from the TRG8 cells expressing the recombinant AGT proteins. Although these assays did contain additional *E. coli* proteins, these proteins did not alter the ED₅₀ values, as described in the accompanying paper [17]. All mutations at Lys¹⁶⁵ greatly increased the resistance of AGT to BG, with increases in ED₅₀ values of 100–2400-fold (Table 1). These included the K165R mutant (ED₅₀ of 21 µM), compared with WT (ED₅₀ of 0.1 µM). The greatest increase occurred when Lys¹⁶⁵ was substituted with tyrosine or phenylalanine (Table 1). The smallest increases, which still amounted to 100–120-fold,

Table 2 Effect of K165A mutation on the DNA binding and activity of AGT

Abbreviations: ND, not determined; ds, double-stranded; ss, single-stranded.

Protein	Rate constant for repair of methylated DNA (M ⁻¹ ·min ⁻¹)	Apparent <i>K</i> _d (μM) for binding to 16-mer oligodeoxynucleotides			
		ds m ⁶ G-16mer	ss m ⁶ G-16mer	ds-16mer	ss-16mer
Wild-type	74 × 10 ⁶	ND	ND	0.61	1.1
K165A	5.6 × 10 ⁶	3.3	9.3	4.2	11.3
C145A	0.0	0.13	0.7	0.69	0.9

occurred with substitutions by alanine, cysteine, glycine and proline.

Properties of purified K165A and K165R mutant AGT proteins

In order to study the involvement of the conserved residue Lys¹⁶⁵ in more detail, the K165A and K165R AGT mutant proteins were purified to homogeneity after subcloning the cDNAs into the pQE vector. This vector has a much stronger promoter than that present in the pUC vector, allowing the isolation of recombinant AGT protein that is readily purified by immobilized-metal-affinity chromatography. These purified mutant proteins gave identical ED₅₀ values of 20 μM (for K165R) and 10 μM (for K165A) for inactivation by BG to those presented in Table 1 for the crude bacterial extracts from cells expressing these proteins. The effect of K165A and K165R mutations to greatly reduce the ability to react with BG was confirmed by measuring the production of 8-[³H]guanine from 8-[³H]BG by the purified recombinant proteins. The rate of reaction was reduced from 800 c.p.m./min/μg protein of BG converted into guanine by WT AGT to 29 c.p.m./min/μg protein for K165A and 22 c.p.m./min/μg protein for K165R.

The second-order rate constant for repair of methylated DNA by the K165A mutant was decreased by approx. 13-fold (Table 2), whereas that for K165R was reduced only slightly by 2.5-fold (results not shown). The ability of the K165A mutant to bind to a DNA substrate was also examined using 16-mer oligodeoxynucleotides containing either a normal guanine or an O⁶-methylguanine residue [7]. Previous studies have shown that it is very difficult to measure the binding of WT AGT to oligodeoxynucleotides containing O⁶-methylguanine, because the AGT reacts so rapidly with the substrate that reaction cannot be prevented during the binding assay. The C145A AGT mutant in which the cysteine acceptor site is mutated to alanine was therefore used as a surrogate since it has similar DNA-binding properties to WT AGT [7,12,34]. The binding affinity of WT AGT was measured only with the unmethylated oligodeoxynucleotides. For the binding experiments with methylated oligodeoxynucleotides, the *K*_d of K165A was 21-fold higher than C145A for double-stranded (ds)DNA, and 13-fold higher than C145A for single-stranded (ss)DNA. When the binding affinity to unmethylated oligodeoxynucleotides were measured, the *K*_d of K165A was 7-fold higher than WT AGT for dsDNA and 10-fold higher for ssDNA. These results strongly suggest a role of Lys¹⁶⁵ in DNA interaction (Table 2).

Ability of K165A mutant AGT to protect *E. coli* from MNNG

The TRG8 *E. coli* strain that lacks endogenous alkyltransferase activity was very sensitive to killing by MNNG, with a 10 %

Figure 2 Ability of K165A mutant AGT to protect *E. coli* from MNNG in the presence and absence of BG

Survival was measured as described in the Experimental section using strain TRG8 with no AGT (□), expressing WT AGT (○) or mutant K165A AGT (△), and in the presence (filled symbols) and absence (open symbols) of 50 μM BG. The cells not expressing active AGT contained the plasmid pUC-inAGT2 [35], which has an insertion replacing much of the AGT coding region. The results are means ± S.D. for at least 4 measurements at each concentration. The values for cells expressing WT and cells expressing K165A and K165A plus BG are significantly different from those expressing no AGT or WT plus BG at every concentration tested (*P* < 0.01).

survival at about 7 μg/ml MNNG (Figure 2). Expression of either WT AGT or K165A mutant AGT in this strain from the pUC18 vector protected the cells from MNNG, increasing the 10 % survival values to 24 and 20 μg/ml respectively. The protection by WT AGT was almost completely abolished by the addition of 50 μM BG (10 % survival at 8 μM MNNG) but the addition of BG at this concentration had a much smaller effect on the ability of the K165A mutant to provide protection, reducing the 10 % survival level from 20 μM to 14 μM of MNNG (Figure 2).

Ability of K165A mutant AGT to protect mammalian cells from BCNU

In order to test the ability of AGT mutants with impaired repair function to protect mammalian cells from the alkylating agents, the cDNA for the K165A mutant was subcloned into a mammalian expression vector under the control of the cytomegalovirus promoter and used for transfection of CHO cells that lack endogenous AGT activity. Stable clones expressing approximately similar amounts of protein as WT were used. As determined by Western blots, the amount of the K165A mutant protein was approx. 75 % of that of WT (Figure 3a). The AGT activity of CHO cells expressing K165A and WT AGT was determined by using the crude cell extracts. The AGT activity in cells expressing K165A mutant was about 365 fmol of methyl groups accepted/mg of total protein in comparison with the cells expressing WT AGT which had activities of approx. 11 000 fmol/mg of total protein. Part of this difference may be due to the reduced rate constant of the K165A mutant and the fact that slightly less K165A mutant AGT protein than WT protein was present (Figure 3a). However, these differences cannot account for all of the reduction in activity and it appears that some of the K165A mutant AGT protein in the cell may be inactive when cell extracts are prepared.

The ED₅₀ value for inactivation by BG of the AGT protein in the CHO cells was determined by incubating the cells with different concentrations of BG for 4 h and measuring the remaining AGT activity in the extracts prepared from them (Figure 3b). The AGT activity in cells expressing K165A was more resistant to BG (ED₅₀ of 5.0 μM) than that in cells

Figure 3 Effects of expression of K165A AGT in CHO cells

(a) Levels of control and K165A mutant protein in the clones of transfected CHO cells used. (b) Loss of AGT activity in response to BG treatment in cells transfected with plasmids expressing control (●) and K165A (○) AGT. (c) Ability of BCNU to kill control CHO cells (■), CHO cells expressing WT AGT (●), and CHO cells expressing K165A mutant AGT (○). (d) Effect of BG treatment on the killing of CHO cells expressing WT AGT (●) and CHO cells expressing K165A mutant AGT (○) by 80 μM BCNU.

expressing WT AGT (ED_{50} of 0.6 μM). However, it is noteworthy that this difference (8-fold) was much less than the 100-fold difference seen when assays were carried out on AGT *in vitro*.

Both WT and K165A mutant AGTs had a major effect in protection of CHO cells from killing by the chloroethylating agent BCNU (Figure 3c). Although K165A mutant showed considerable deficiency in the repair of DNA measured *in vitro* with cell extracts, CHO cells expressing this mutant were just as resistant to the BCNU treatment as those expressing WT AGT (Figure 3c).

As reported previously [33], the ability of WT AGT to protect CHO cells from BCNU was abolished by treatment with BG and > 99% of the cells were killed by the combination of 100 μM BG and 80 μM BCNU (Figure 3d). In contrast, the protection provided by the K165A mutant was virtually intact when 100 μM BG was used before the BCNU treatment (Figure 3d). Significantly higher concentrations of BG were needed (> 400 μM) to obtain killing of > 90% of the cells. Thus despite the reduced activity, the K165A mutant AGT protein was able to protect cells from killing by a therapeutic chloroethylating agent even when BG was present. The WT AGT, on the contrary, was ineffective at protecting cells from killing by BCNU when BG was added.

DISCUSSION

Although most of the K165X mutant AGT proteins were only assayed for their ability to protect the host *E. coli* cells from MNNG *in vivo*, and to repair methylated DNA or react with BG *in vitro* with crude extracts, the results clearly indicate that all substitutions at this position do allow activity in DNA repair but render the protein resistant to inactivation by BG. The two

representative mutants that were purified to homogeneity were K165R, representing the most conservative change, and K165A as an example of one of the least active mutants. These mutants showed major reductions in the ability to react with BG that were identical, whether assayed in crude extracts or with purified protein. K165A was sufficiently active to allow survival of *E. coli* or CHO cells treated with MNNG or BCNU respectively. Since this was one of the least active mutants, it is likely that all of the others would also provide protection. It is noteworthy that the level of resistance to BCNU imparted by expressing the K165A mutant protein in CHO cells was very similar to that produced by WT AGT even though the activity was much less when measured in cell extracts. Since AGT acts only once, it would be expected that the survival is proportional to the number of active molecules present in the cell. The most likely explanation of this inconsistency is that K165A protein is fully active within the cell, perhaps due to stabilization by interaction with other nuclear components, but that the protein is readily inactivated by extraction and/or incubation during the *in vitro* assays. Another possibility is that the level of AGT expression in the transfected cells is so high that it provides an excess repair capacity, but this seems less likely since the protection by WT AGT is reduced in a linear manner by increasing levels of BG (Figure 3d).

Residue Lys¹⁶⁵ may have an important function in the alkyl transfer reaction with either alkylated-DNA or free base BG, since all 19 substitutions resulted in reduced activity towards methylated DNA substrates and a great decrease in sensitivity to BG. It seems probable that the side chain of residue 165 plays an important role in interaction with the DNA substrate. The results with the purified mutant K165A are in agreement with this hypothesis. The rate constant for repair of methylated DNA by the purified mutant K165A was 13-fold less than for WT

AGT. This is in good agreement with the fact that the K_d for K165A mutant was 21-fold higher for methylated dsDNA, and 13-fold higher for methylated ssDNA. These results indicate that the decreased rate of repair for methylated DNA might be due to the impaired interaction between DNA and protein.

The slightly greater amount of cell protein needed for repair of a fixed amount of methylated DNA, when extracts were prepared from cells expressing K165R compared with WT AGT (Table 1), is consistent with the 2.5-fold reduction in the rate constant for the K165R mutant. These results suggest that the lysine side chain has a structural role in maintaining the conformation of the AGT protein, which can also be fulfilled by arginine at this position. This is in agreement with the recently described crystal structure of human AGT [20], where Lys¹⁶⁵ appears to interact with another highly conserved residue, Tyr¹⁵⁸. In contrast, a much larger, > 140-fold, increase in protein extract was needed for the K165A mutant to get equivalent repair to WT AGT, even though the reduction in rate constant was only 13-fold. This is consistent with the possibility raised above that the K165A protein is unstable when extracted from cells and the Ala¹⁶⁵ side chain would not be able to interact with Tyr¹⁵⁸.

The results shown in Table 1 indicate that, although the lysine side chain at position 165 is important, it is not essential for the interaction between AGT and the DNA substrate. All of the Lys¹⁶⁵ AGT mutants had sufficient activity to provide protection of bacteria to MNNG killing, and (except for K165T) they also produced measurable activities *in vitro*. Mutant K165A was one of the least active mutants, but it conferred a large degree of resistance to MNNG in *E. coli* (Figure 2). It is likely that the interactions between AGT and the DNA substrates, as well as the resulting stabilization of the AGT–DNA complex, are mediated by several amino acids, including Lys¹⁶⁵. When only Lys¹⁶⁵ is mutated, such interactions are reduced but not completely abolished. Residue Arg¹²⁸ is known to be involved in DNA binding and Tyr¹¹⁴ also has been shown to aid in the stabilization of the AGT–DNA complex [7]. These interactions may be sufficient to permit repair of methylated DNA by the K165X mutants.

It is widely accepted that the primary physiological role of AGT in cells is to deal with endogenous methylation damage and several sources of endogenous methyl groups have been proposed [38–40]. The complete conservation of lysine at position 165 raises the possibility that other more bulky adducts at the O⁶-position [41,42] are also physiological AGT substrates. If only methyl adducts are the normal AGT substrates, alterations at this position would not be deleterious.

BG acts to inactivate AGT by virtue of its ability to serve as a pseudosubstrate for the protein [31,43]. BG is accepted into the active-site pocket of AGT and the benzyl group is transferred to residue Cys¹⁴⁵ forming an S-benzylcysteine residue at the active site, which abolishes AGT activity. All mutants of AGT at Lys¹⁶⁵, regardless of the size and/or hydrophobicity of the side chain of the substitution, had a dramatically reduced ability to react with BG, and thus were highly resistant to this inhibitor. It appears, therefore, that the interaction with the BG substrate is strongly dependent on the presence of a lysine at position 165, and that no other amino acids, including arginine (which is able to substitute effectively in repair of methylated DNA), can be used. It is noteworthy that a similar phenomenon exists with AGT mutants at conserved residue Tyr¹¹⁴ [7]. Most mutations at this site led to a large reduction in activity towards methylated DNA but the conservative change Y114F produced only a 3-fold reduction. However, all alterations in Tyr¹¹⁴, including the mutant Y114F, had large reductions (approx. 100-fold) in their ability to react with BG [7]. These results suggest that both Tyr¹¹⁴

and Lys¹⁶⁵ interact with the substrate base and also with other parts of the DNA substrate. Tyr¹¹⁴, like Lys¹⁶⁵, is located close to the flipped-out base in the proposed models of AGT mechanism [20–23]. The recently described crystal structure of human AGT [20] is consistent with this suggestion.

In the case of alkylated DNA substrates, the other components of the DNA-binding domain may allow adequate interaction with the substrate for reaction to take place in the K165X mutants, albeit at a slightly reduced rate. In contrast, these interactions would not be present with the free base BG and a much greater reduction in the rate of alkyl transfer may result. The nature and size of the side chain in the Lys¹⁶⁵ substitutions do appear to have some effect on the protein's ability to react with BG. The two mutants with the highest ED₅₀ values for inactivation by BG (K165Y and K165F) both contain a bulky, aromatic side chain, and the mutants with the lowest ED₅₀ values showed changes to the small amino acids (K165A, K165G, K165C and K165P). The side chain of Lys¹⁶⁵ appears to maintain the ring position of Tyr¹⁵⁸, which is likely to interact with the phenyl ring of BG [20]. Mutation of K165 to bulky amino acids would push the side chain of Tyr¹⁵⁸ into the BG binding pocket. This would have a greater effect than the mutation to Ala or Gly where the resistance to BG may be due to the lack of the positioning of Tyr¹⁵⁸ lessening the ability of the phenyl rings from this residue and BG to interact.

BG is currently undergoing clinical trials to enhance the cancer chemotherapeutic activity against BCNU [44,45]. The development of resistance to this strategy by the appearance of mutant forms of AGT with reduced affinity for BG may be a serious problem, since multiple sites for such mutations have been observed [17,30]. Our results add Lys¹⁶⁵ to this list. We show that any alteration at Lys¹⁶⁵ would be likely to cause resistance to therapy with BG plus an alkylating agent. It might appear that the reduced activity of most K165X mutants would act against the development of such resistance, but the results in which K165A mutant AGT was expressed in CHO cells argue strongly against this. Despite the reduced activity towards methylated DNA and the possibility that not all of the protein is correctly folded in the cell [since the ratio of activity to protein was reduced by a factor of 30, whereas the rate constant is reduced by only 13-fold (Table 2)], the K165A mutant AGT was as effective as WT AGT in providing protection to BCNU when expressed in CHO cells (Figure 3). Furthermore, this protection was highly refractory to BG. The BG-resistance observed in the *in vitro* assays with this mutant protein is therefore present within the cell and is not an artifact of these assays. The K165A mutant was one of the most poorly active on the basis of the large amount of protein needed to measure activity *in vitro* with bacterial extracts. It is therefore very likely that all of the other BG-resistant mutants identified here would be effective in counteracting the actions of chemotherapeutic agents such as BCNU and temozolomide, even in the presence of BG.

This research was supported by grants CA-18138, CA-57725 and CA-71976 from the National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.

REFERENCES

- 1 Lindahl, T., Sedgwick, B., Sekiguchi, M. and Nakabeppu, Y. (1988) *Annu. Rev. Biochem.* **57**, 133–157
- 2 Samson, L. (1992) *Mol. Microbiol.* **6**, 825–831
- 3 Pegg, A. E. and Byers, T. L. (1992) *FASEB J.* **6**, 2302–2310
- 4 Pegg, A. E., Dolan, M. E. and Moschel, R. C. (1995) *Progr. Nucleic Acid Res. Mol. Biol.* **51**, 167–223
- 5 Mitra, S. and Kaina, B. (1993) *Progr. Nucleic Acid Res. Mol. Biol.* **44**, 109–142

- 6 Moody, P. C. E. and Moore, M. E. (1995) in *Novel Approaches in Anticancer Drug Design Molecular Modelling-New Treatment Strategies*, vol. 49 (Zeller, W. J., D'Incalci, M. and Newell, D. R., eds.), pp. 16–24, Karger, Basel
- 7 Goodtzova, K., Kanugula, S., Edara, S. and Pegg, A. E. (1998) *Biochemistry* **37**, 12489–12495
- 8 Demple, B. (1990) in *Protein Methylation* (Paik, W. K. and Kim, S., eds.), pp. 285–304, CRC Press, Boca Raton, Florida
- 9 Spratt, T. E. and Campbell, C. R. (1994) *Biochemistry* **33**, 11364–11371
- 10 Harris, L. C., Potter, P. M. and Margison, G. P. (1992) *Biochem. Biophys. Res. Comm.* **187**, 425–431
- 11 Crone, T. M. and Pegg, A. E. (1993) *Cancer Res.* **53**, 4750–4753
- 12 Hazra, T. K., Roy, R., Biswas, T., Grabowski, D. T., Pegg, A. E. and Mitra, S. (1997) *Biochemistry* **36**, 5769–5776
- 13 Ihara, K., Kawate, H., Chueh, L. L., Hayakawa, H. and Sekiguchi, M. (1994) *Mol. Gen. Genet.* **243**, 379–389
- 14 Crone, T. M., Goodtzova, K. and Pegg, A. E. (1996) *Mutation Res.* **363**, 15–25
- 15 Pieper, R. O., Morgan, S. E. and Kelley, M. R. (1994) *Carcinogenesis* **15**, 1895–1902
- 16 Kanugula, S., Goodtzova, K., Edara, S. and Pegg, A. E. (1995) *Biochemistry* **34**, 7113–7119
- 17 Xu-Welliver, M. and Pegg, A. E. (2000) *Biochem. J.* **347**, 519–526
- 18 Moore, M. H., Gulbus, J. M., Dodson, E. J., Demple, B. and Moody, P. C. E. (1994) *EMBO J.* **13**, 1495–1501
- 19 Hashimoto, H., Inoue, T., Nishioka, M., Fujiwara, S., Takagi, M., Imanaka, T. and Kai, Y. (1999) *J. Mol. Biol.* **292**, 707–716
- 20 Wibley, J. E. A., Pegg, A. E. and Moody, P. C. E. (2000) *Nucleic Acids Res.* **28**, 393–401
- 21 Demple, B. (1995) *Curr. Biol.* **5**, 719–721
- 22 Roberts, R. J. and Cheng, X. (1998) *Annu. Rev. Biochem.* **67**, 181–198
- 23 Vora, R., Pegg, A. E. and Ealick, S. E. (1998) *Proteins* **32**, 3–6
- 24 Parikh, S. S., Mol, C. D. and Tainer, J. A. (1997) *Structure* **5**, 1543–1550
- 25 Parikh, S. S., Mol, C. D., Slupphaug, G., Bharati, S., Krokan, H. E. and Tainer, J. A. (1998) *EMBO J.* **17**, 5214–5226
- 26 Yamagata, Y., Kato, M., Odawara, K., Tokuno, Y., Nakashima, Y., Matsushima, N. and Yasamura, K. (1996) *Cell* **86**, 311–319
- 27 Labahn, J., Schärer, O. D., Long, A., Ezaz-Nikpay, K., Verdine, G. L. and Ellenberger, T. E. (1996) *Cell* **86**, 321–329
- 28 Rebeck, G. W. and Samson, L. (1991) *J. Bacteriol.* **173**, 2068–2076
- 29 Dolan, M. E., Moschel, R. C. and Pegg, A. E. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5368–5372
- 30 Xu-Welliver, M., Kanugula, S. and Pegg, A. E. (1998) *Cancer Res.* **59**, 1936–1945
- 31 Pegg, A. E., Boosalis, M., Samson, L., Moschel, R. C., Byers, T. L., Swenn, K. and Dolan, M. E. (1993) *Biochemistry* **32**, 11998–12006
- 32 Edara, S., Kanugula, S., Goodtzova, K. and Pegg, A. E. (1996) *Cancer Res.* **56**, 5571–5575
- 33 Loktionova, N. A. and Pegg, A. E. (1996) *Cancer Res.* **56**, 1578–1583
- 34 Kanugula, S., Goodtzova, K. and Pegg, A. E. (1998) *Biochem. J.* **329**, 545–550
- 35 Xu-Welliver, M., Leitao, J., Kanugula, S. and Pegg, A. E. (1999) *Cancer Res.* **59**, 1514–1519
- 36 Trimmer, E. E., Zamble, D. B., Lippard, S. J. and Essigmann, J. M. (1998) *Biochemistry* **37**, 352–362
- 37 Loktionova, N. A., Xu-Welliver, M., Crone, T., Kanugula, S. and Pegg, A. E. (1999) *Biochem. Pharmacol.* **58**, 237–244
- 38 Lindahl, T. (1993) *Nature (London)* **362**, 709–715
- 39 Lindahl, T. (1996) *Philos. Trans. R. Soc. London Ser. B* **351**, 1529–1538
- 40 Taverna, P. and Sedgwick, B. (1996) *J. Bacteriol.* **178**, 5105–5111
- 41 Goodtzova, K., Kanugula, S., Edara, S., Pauly, G. T., Moschel, R. C. and Pegg, A. E. (1997) *J. Biol. Chem.* **272**, 8332–8339
- 42 Wang, L., Spratt, T. E., Liu, X.-L., Hecht, S. S., Pegg, A. E. and Peterson, L. A. (1997) *Chem. Res. Toxicol.* **10**, 562–567
- 43 Ciocco, G. M., Pegg, A. E., Moschel, R. C., Chae, M.-Y., McLaughlin, P. J., Zagon, I. S. and Pegg, A. E. (1995) *Cancer Res.* **55**, 4085–4091
- 44 Dolan, M. E. and Pegg, A. E. (1997) *Clin. Cancer Res.* **3**, 837–847
- 45 Dolan, M. E. (1997) *Adv. Drug Delivery Rev.* **26**, 105–118

Received 9 November 1999/18 January 2000; accepted 8 February 2000