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Directed Evolution of O^6-Alkylguanine-DNA Alkyltransferase for Efficient Labeling of Fusion Proteins with Small Molecules In Vivo

Alexandre Juillerat,[1,2] Thomas Gronemeyer,[1,2] Antje Keppler,[1,2] Susanne Gendreizig,[1,2] Horst Pick,[2] Horst Vogel,[2] and Kai Johnsson[1,2,*]
[1]Institute of Molecular and Biological Chemistry
[2]Institute of Biomolecular Sciences
Swiss Federal Institute of Technology
CH-1015 Lausanne
Switzerland

Summary

We report here the generation of mutants of the human O^6-alkylguanine-DNA alkyltransferase (hAGT) for the efficient in vivo labeling of fusion proteins with synthetic reporter molecules. Libraries of hAGT were displayed on phage, and mutants capable of efficiently reacting with the inhibitor O^6-benzylguanine were selected based on their ability to irreversibly transfer the benzyl group to a reactive cysteine residue. Using synthetic O^6-benzylguanine derivatives, the selected mutant proteins allow for a highly efficient covalent labeling of hAGT fusion proteins in vivo and in vitro with small molecules and therefore should become important tools for studying protein function in living cells. In addition to various applications in proteomics, the selected mutants also yield insight into the interaction of the DNA repair protein hAGT with its inhibitor O^6-benzylguanine.

Introduction

The specific in vivo labeling of proteins with small reporter molecules has great potential for studying protein function. So far, in vivo protein labeling is achieved by either introducing the label as an unnatural amino acid using nonsense codon suppression technology [1], or the protein of interest is expressed as a fusion protein with a polypeptide or protein that mediates the labeling [2–9]. Examples of polypeptides or proteins that mediate the in vivo labeling of fusion proteins are proteins that noncovalently bind a reporter molecule [2–5] or polypeptides containing cysteines that form stable complexes with biarsenical compounds [6–8]. We have recently introduced a method for the specific labeling of fusion proteins in vivo [9] which is based on the reaction of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT, 207 residues) with O^6-benzylguanine (BG) derivatives of type **1** (Figure 1). The normal function of hAGT is to repair O^6-alkylated guanine in DNA by transferring the alkyl group in an S_N2 reaction to a reactive cysteine residue (Cys145) [10]. The repair mechanism is unusual, as the protein is irreversibly inactivated [10]. Consequently, the reaction of hAGT fusion proteins with BG derivatives of type **1** leads to the irreversible and covalent labeling of the fusion proteins as the label is transferred with the benzyl group to the reactive cysteine, yielding a stable thioether. Labeling in bacteria and yeast is specific, as the respective endogenous AGTs do not accept BG as substrates, whereas for the labeling in mammalian cells, AGT-deficient cell lines can be used [10, 11].

Important for the versatility of the approach is the observation that the reaction of hAGT fusion proteins with **1** (i.e., **2**–**5**) does not depend on the nature of the label, allowing the covalent attachment of a wide variety of different reporter molecules [9]. However, the efficiency of the in vivo labeling is determined by the speed of the reaction, and the rate constant for the reaction of hAGT with BG derivatives such as **3a** is only 400 $s^{-1}M^{-1}$, four orders of magnitude below the reaction of hAGT with its natural substrate, O^6-methylguanine-DNA [9, 10]. Here, we report the generation of hAGT mutants with significantly increased activities against substrates of type **1** and the use of these mutants for the efficient labeling of hAGT fusion proteins in vivo and in vitro. The characterization of the selected mutants also adds to our understanding of the interaction of hAGT with its inhibitor BG.

Results and Discussion

To increase the activity of hAGT against BG derivatives of type **1**, we envisioned using directed evolution based on phage display [12]. Active hAGT displayed on phage can be covalently labeled with digoxigenin or biotin using substrates such as **2** or **3** and subsequently enriched out of libraries of phage displaying no or less active hAGT using immobilized anti-digoxigenin antibody or avidin. We have previously shown that hAGT can be displayed functionally on phage λ [13]; however, in our hands this system was not suitable for directed evolution of hAGT (A.K., K.J., unpublished data). We therefore decided to display hAGT libraries on filamentous phage M13 as a fusion protein with g3p using phagemid pAK100 [14]. Although proteins displayed on filamentous phage have to fold under the oxidizing conditions of the periplasm, functional display of proteins with highly reactive cysteine residues on filamentous phage has already been demonstrated [15, 16]. To investigate if hAGT is active as a g3p fusion protein on the surface of filamentous phage, model selections were performed; mixtures of phage displaying hAGT and phage displaying no hAGT were incubated with **2** and phage labeled with digoxigenin subsequently captured using magnetic beads covered with anti-digoxigenin antibody. In these experiments, phage displaying hAGT were enriched by a factor of about 400 per round, indicating that hAGT can indeed be functionally displayed on filamentous phage.

For the creation of a library of hAGT mutants, we made use of structural and biochemical information available on hAGT (Figure 2) [10, 17–20], identifying four amino acids that could affect the reactivity of hAGT toward

*Correspondence: kai.johnsson@epfl.ch

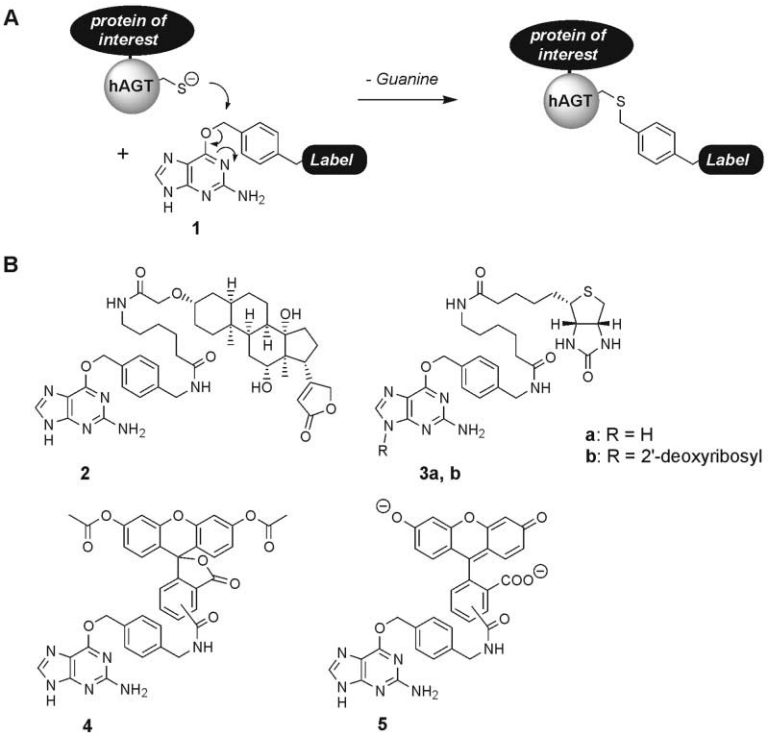

Figure 1. Labeling of hAGT Fusion Proteins

(A) Labeling of hAGT fusion proteins with **1**. The protein of interest can be fused either to the N or C terminus of hAGT.

(B) BG derivatives used throughout this work for labeling with (i) digoxigenin (**2**), (ii) biotin (**3a**, **b**) and (iii) fluorescein (**4** and **5**).

BG (Pro140, Asn157, Ser159, Gly160). The side chain of Pro140 is believed to undergo an important hydrophobic interaction with the benzyl ring of BG [17–19], and the mutation Gly160Trp has been shown to increase the reactivity of hAGT toward BG, presumably through hydrophobic interactions [17, 20]. The side chains of Asn157 [17] and Ser159 [17, 18] have both been proposed to form a hydrogen bond with the N7 of BG, and the Cβ of Ser159 has been proposed to make contact with the benzyl ring of BG [19].

The codons for residues 140, 157, 159, and 160 were randomized using synthetic oligonucleotides, yielding a library of 2.6×10^5 independent hAGT mutants after electroporation of the corresponding pAK100 vector into *E. coli* XL1-Blue. Sequencing of five randomly picked clones confirmed the randomization at residues 140, 157, 159, and 160. To ensure enrichment of highly active hAGT mutants in the selections, the phage library was incubated with low concentrations of **2** for short periods of time; in the first round, phage were incubated with **2** (1 μM) for 10 min and quenched with BG (100 μM), and labeled phage was captured with magnetic beads covered with anti-digoxigenin antibody, whereas in the second and third round, the incubation time was reduced to 5 min and 40 s, respectively. The rate for the reaction of wild-type with BG derivatives of the type **1** is sufficiently slow so that the employed selection conditions should indeed lead to an enrichment of mutant proteins with activities higher than wild-type. After the third round, nine clones were analyzed by DNA sequencing (Table 1). Seven of the nine selected clones

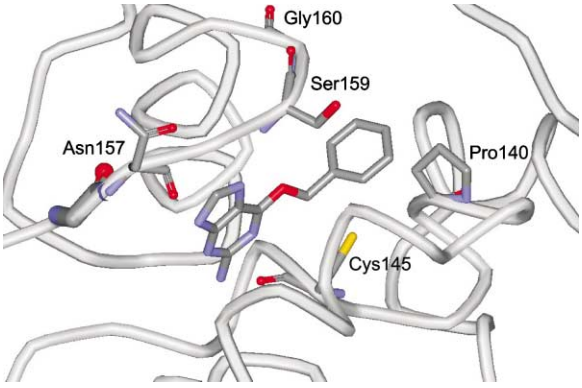

Figure 2. BG Docked into the Active Site of hAGT [16, 17]

Highlighted are the reactive Cys145 and the randomized residues Pro140, Asn157, Ser159, and Gly160. The following distance constraints (<3 Å) were used for docking of BG into the active site of hAGT: OH group of Tyr114 to N3 of BG; carbonyl of Cys145 to N2 of BG; carbonyl of Val148 to N2 of BG; NH of Ser159 to O6 of BG. Docking was done using the software MOLOC.

Table 1. Sequences of Selected hAGT Mutants at Randomized Residues and Their Activities (k_{obs}) as GST-hAGT Fusion Proteins against **3a**

	Res. 140	Res. 157	Res. 159	Res. 160	k_{obs} ($s^{-1}M^{-1}$)
Wild-type	Pro	Asn	Ser	Gly	400
PGSG	Pro	Gly	Ser	Gly	1200
PGEG	Pro	Gly	Glu	Gly	8000
PGEA	Pro	Gly	Glu	Ala	6000
PGNW	Pro	Gly	Asn	Trp	3200
PGQW	Pro	Gly	Gln	Trp	500
PPQC	Pro	Pro	Gln	Cys	800
MWSV	Met	Trp	Ser	Val	2400
PRSG	Pro	Arg	Ser	Gly	n.d.
FREG	Phe	Arg	Glu	Gly	n.d.
^{W160}hAGT	Pro	Asn	Ser	Trp	1000

were expressed and purified as glutathion S-transferase (GST) fusion proteins [9]. For the determination of the activity of the selected mutant proteins, the second-order rate constants (k_{obs}) of the reaction of the GST-hAGT fusion proteins with **3a** were measured (Table 1). **3a** was used as a substrate in these measurements, as its reaction with hAGT can be readily followed by Western blotting, using a streptavidin-peroxidase conjugate to detect and quantify biotinylation [9]. The second-order rate constants were determined at concentrations of **3a** (<5 μM) where the measured rate depended linearly on the concentration of **3a**.

All of the characterized mutants possessed enhanced activities against **3a**, with ^{PGEG}hAGT being a factor of 20 more active than wild-type hAGT, demonstrating the success of the selection scheme. The sequences of the active clones yield interesting insights into the interaction of hAGT with BG; at position 140, seven out of nine clones possess a proline. The two other amino acids identified at this position, phenylalanine and methionine, would also allow for the proposed hydrophobic interaction with the benzyl ring of BG [17–19]. At position 157, glycine was found in five out of nine clones, whereas at position 159, only polar side chains capable of hydrogen bonding were found, indicating that in the interaction of wild-type hAGT with BG, Ser159 and not Asp157 undergoes hydrogen bonding with the N7 of BG. The two most active clones, ^{PGEG}hAGT and ^{PGEA}hAGT, possess a glutamic acid at position 159, and a favorable interaction between this residue and BG might result from hydrogen bonding between deprotonated Glu159 and a tautomer of BG that has a hydrogen atom bound to the N7 of BG. To test this hypothesis, we investigated the activity of wild-type hAGT and ^{PGEA}hAGT against the nucleoside **3b** in which the N7 can only act as a hydrogen bond acceptor. Compared to their respective activities against **3a**, the activity of wild-type hAGT against **3b** (340 $s^{-1}M^{-1}$) was not significantly affected, whereas the activity of ^{PGEG}hAGT against **3b** (1200 $s^{-1}M^{-1}$) decreased by a factor of 5. Thus, Glu159 might indeed act as a hydrogen bond acceptor in the interaction of ^{PGEG}hAGT with BG. Gly160 was either conserved or mutated to hydrophobic residues, underlining previous observations that polar amino acids at this position reduce the reaction rate of hAGT with BG [20].

It is instructive to compare the rate of the labeling of the selected hAGT mutants with the rates of complex formation between biarsenical ligands and tetracysteine motifs [8], a reaction that has been successfully used for in vivo protein labeling. Second-order rate constants for the reaction of biarsenical ligands complexed by simple dithiols with tetracysteine motifs of the type Cys-Cys-Xxx-Xxx-Cys-Cys (where Xxx is any amino acid except cysteine) have been reported to be around 10^4 $s^{-1}M^{-1}$ [8] and are thus comparable to those measured for ^{PGEG}hAGT and ^{PGEA}hAGT.

We then investigated if the selected mutants allow for a more efficient labeling of hAGT fusion proteins in vivo. Toward this end, we compared the fluorescence labeling of nuclear targeted ^{PGEA}hAGT and ^{W160}hAGT with **4** in AGT-deficient CHO cells [11]. **4** contains the membrane-permeable diacetate of fluorescein as label, which inside the cell is hydrolyzed into the corresponding fluorescein

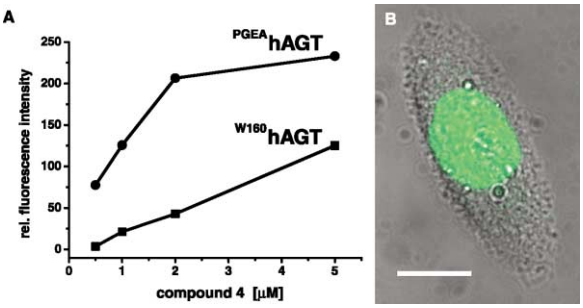

Figure 3. Labeling of Nuclear Targeted hAGT in AGT-Deficient CHO Cells

(A) Relative fluorescence intensity in the nucleus of AGT-deficient CHO cells expressing nuclear targeted ^{PGEA}hAGT or ^{W160}hAGT and labeled with varying concentrations of **4**. Shown data are the average for more than ten cells.

(B) Confocal micrograph of nuclear targeted ^{PGEA}hAGT in AGT-deficient CHO cells showing an overlay of transmission and fluorescence channel (ex. 488 nm). The image was recorded 30 min after pulse labeling with **4** (500 nM) for 5 min and three washes with PBS buffer. Bar = 10 μm.

derivative **5** [2, 9]. ^{W160}hAGT possesses an about 2-fold higher activity than wild-type against BG derivatives (Table 1) and has been previously used in the in vivo fluorescence labeling of hAGT fusion proteins [9]. To ensure localization of the two proteins in the nucleus of the cell, they were fused to three consecutive simian virus 40 large antigen nuclear localization domains [21], yielding ^{W160}hAGT-NLS_3 and ^{PGEA}hAGT-NLS_3, respectively. AGT-deficient CHO cells transiently expressing ^{W160}hAGT-NLS_3 or ^{PGEA}hAGT-NLS_3 were incubated for 5 min with varying concentrations of **4** and washed three times to remove excess fluorophore, and the intensity of the fluorescence signal in the nucleus of the cell was measured using laser scanning confocal microscopy (Figure 3A).

Cells transiently expressing ^{PGEA}hAGT-NLS_3 displayed a fluorescence signal in the nucleus that was significantly stronger than that of cells transiently expressing ^{W160}hAGT-NLS_3 (Figure 3A). In particular, fluorescence labeling of ^{PGEA}hAGT was still clearly detectable after incubations with **4** at concentrations as low as 500 nM (Figure 3B), whereas no significant labeling of ^{W160}hAGT-NLS_3 could be detected under these conditions. The data thus confirm the in vitro measurements and clearly demonstrate the superiority of the selected mutant proteins for the in vivo labeling of hAGT fusion proteins.

The in vivo labeling of fusion proteins for functional studies has the potential to become an attractive alternative to more traditional approaches in which autofluorescent proteins or epitope tags are fused to the protein of interest. In particular, in vivo labeling opens up the possibility to provide proteins with properties that cannot be genetically encoded, and a single fusion protein can be labeled with a variety of different molecules for completely different purposes [2–9]. Furthermore, simple pulse-chase labeling experiments can discriminate between old and new copies of a fusion protein in a living cell, thereby allowing study of its life cycle, mobility, and stability as well as that of the macromolecular assemblies it participates in [7]. Currently, there are two tags that seem to be particularly well suited for the in vivo

labeling of fusion proteins with small molecules: the tetracysteine motif which forms reversible complexes with biarsenical compounds [6–8] and the here discussed hAGT which irreversibly reacts with BG derivatives [9]. In general, the scope of applications of such tags is affected by a possible interference of the tag with the natural function of the protein to be studied, by the possibility to use them in different cell types and organelles as well as by the selectivity, efficiency, and speed of the labeling reaction. Up to now, the main restriction for the use of hAGT fusion proteins in mammalian cells was the necessity to work in AGT-deficient cell lines [9]. However, the here demonstrated possibility to engineer the specificity and reactivity of hAGT through directed evolution not only improves the efficiency and speed of the labeling of hAGT fusion proteins but also points toward one potential solution of this problem.

Significance

The in vivo labeling of proteins with synthetic molecules capable of probing and controlling protein function has the potential to become an important method in proteomics. We previously introduced a method for the in vivo labeling of fusion proteins of the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) that is based on the irreversible reaction of hAGT with O^6-benzylguanine derivatives. Using directed evolution, we report here the generation and characterization of hAGT mutants with significantly increased activity against substituted BG derivatives and their use in the in vivo labeling of hAGT fusion proteins. The increased in vitro activity of hAGT against BG derivatives directly translates into a much more efficient in vivo protein labeling, making the selected mutants valuable tools for studying protein function in the living cell. In particular, genetically encoded tags such as hAGT that allow for the selective labeling of fusion proteins with a variety of different probes in vivo and in vitro should be attractive choices for genome-wide applications. In addition to these practical applications, the results also provide insight into the interaction of hAGT with its inhibitor BG.

Experimental Procedures

General

Chemicals were purchased from Sigma or Roche. Flash column chromatography was performed on silica gel 60 (40–63 μm, Fluka). Mass spectra were recorded by electrospray ionization on a Finnigan MAT APCI/ESI SSQ 710C spectrometer. UV spectra were measured on a Perkin Elmer Lambda10 UV/VIS spectrometer. Compounds **3a**, **3b**, **4**, and **5** were synthesized as described previously [9, 13].

O^6-[4-(Digoxigenin-3-*O*-Methyl Carbonyl-ε-Aminocaproic Acid)-Amidomethyl)]-Benzylguanine 2

10.3 mg (0.038 mmol) of O^6-(4-aminomethyl-benzyl)guanine was dissolved in 1 ml dry DMF (40°C, sonicated for 30 min) under argon atmosphere. After cooling to room temperature, 2.5 μl triethylamine (0.018 mmol) and 5 mg (0.0076 mmol) digoxigenin-3-*O*-methyl carbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester in 0.5 ml DMF were added. After stirring at room temperature for 3 hr, the reaction was quenched with 100 μl water, and the product was purified by flash column chromatography using a stepwise gradient of methanol in dichloromethane (0%, 5%, 20% methanol). Solvent was evaporated in vacuo, and the product dissolved in 100 μl DMSO. The concentration of **2** (66 mM) was determined by measuring UV absorbance at $\lambda = 280$ nm using the extinction coefficient of O^6-(4-aminomethyl-benzyl)guanine ($\epsilon_{280} = 7.1\ mM^{-1}cm^{-1}$). Yield: 5.4 mg (0.0066 mmol, 87%). $R_f = 0.35$ (20% methanol in dichloromethane). MS(ESI) *m/z* (%) = 814.37 (100 $[M+H]^+$), 1628.32 (12 $[2M+H]^+$).

Library Preparation and Phage Selections

For the library generation, saturation mutagenesis of residues 140, 157, 159, and 160 was performed by overlap extension PCR using the following four synthetic oligonucleotides in which the codons of the four residues 140, 157, 159, and 160 were randomized as NNK: primer 1: CTA CTC GCG GCC CAG CCG GCC ATG GCG GAC TAC AAA GAC ATG GAC AAG GAT TGT GAA ATG; primer 2: GGA ATT CGG CCC CCG AGG CCG *C* GTT TCG GCC AGC AGG CGG; primer 3: C AGA GTG GTC TGC AGC AGC GGA GCC GTG GGC NNK TAC NNK NNK GGA CTG GCC GTG AAG GA; and primer 4: G CTC CGC TGC TGC AGA CCAC TCT GTG GCA CGG GAT GAG GAT MNN GAC AGG ATT GCC TCT. The theoretical number of possible mutants on the DNA level is 10^6. Primers 1 and 2 contain SfiI restriction sites (underlined), primers 3 and 4 contain the randomized bases. The PCR product was ligated into phage display vector pAK100 and electroporated into *E. coli* XL1-Blue, yielding a library of 4.4×10^5 independent clones. In about 60% of the clones, i.e., 2.6×10^5, the tetracycline resistance cassette in pAK100 was replaced by the hAGT insert. Colonies were scraped off the plates and grown in 2YT medium (25 mg/ml chloramphenicol, 1% glucose, 1 mM $MgCl_2$) at 37°C until the optical density OD_{600} reached 0.6. VCS M13 helper phage were added to a final concentration of 4×10^9 particles/ml, and the culture was incubated for 30 min at 37°C without shaking and 3 hr at 37°C at 180 rpm. Cells were harvested by centrifugation (2500 × g, 5 min), resuspended in SB-MOPS (50 mM 3-morpholinopropanesulfonic acid, 25 mg/ml chloramphenicol, 70 mg/ml kanamycin, 1 mM $MgCl_2$), and subsequently incubated overnight at 24°C, 200 rpm. Cells were pelleted, and the supernatant containing the phage was adjusted to 1 mM DTT and stored at 4°C prior to selections. Phage titers were typically around 10^{12}–10^{13} cfu/ml at this point (colony forming unit). For the selections, **2** was added to phage (10^{12} cfu/ml) to a final concentration of 1 μM and gently rotated for 10 min. In the second and third rounds of the selections, the incubation time was reduced to 5 min and 40 s, respectively. The reaction was quenched by addition of free BG to a final concentration of 100 μM. Phage were precipitated using polyethylene glycol 8000 (4% w/v) and NaCl (3% w/v), centrifuged, and resuspended in 500 μl PBS supplemented with 1 mM DTT. To this solution, 500 μl PBSMM (PBS supplemented with 4% skimmed milk powder) was added and gently rotated for 60 min at 24°C. 75 μl of anti-digoxigenin antibody-coated magnetic beads (Roche; washed three times with PBS and blocked for 60 min with PBSMM) were added to the phage preparation and rotated at 4°C for 25 min. After immobilization of labeled phage, the beads were washed three time with PBSMM, once with PBST (PBS supplemented with 0.05% Tween-20), three times with 50 mM Tris (pH 7.4), 150 mM NaCl, 0.005% TX100, and 2 mM $CaCl_2$, and twice with PBS. Phage were eluted by incubating the beads with 100 μl 0.1 M glycine (pH 2.5) for 5 min, and the solution was neutralized with 50 μl 1 M Tris-Cl (pH 8.0). *E. coli* JM101 were infected with eluted phage, plated on 2YT plates (supplemented with 1% glucose, 25 mg/ml chloramphenicol), and incubated overnight at 37°C. In general, between 10^5 and 10^6 phage were eluted after each round. The next day, colonies were scraped off the plates, aliquoted, and stored at −80°C prior to the next round of selection. After the third round of selections, the percentage of phage conferring tetracycline resistance to *E. coli* JM101 dropped from 40% to about 0.03%.

Characterization of Selected hAGT Mutants as GST Fusion Proteins

The gene of the selected mutants was amplified by PCR and cloned into the pGEX-2T expression vector (Pharmacia) as described for wild-type [9]. Expression was done at 24°C, and the protein was purified as a GST-hAGT fusion protein. For the measurements of the reaction rates between the hAGT mutant proteins and **3a**, protein

(0.2–0.4 μM) was incubated with **3a** (1–5 μM) in reaction buffer (50 mM HEPES [pH 7.2], 1 mM DTT) at 24°C, and aliquots were taken at defined times. The aliquots were quenched with O^6-benzylguanine (1 mM final concentration) and analyzed by Western blotting using a Neutravidin peroxidase conjugate (Pierce) and a chemiluminescent peroxidase substrate (Renaissance Reagent Plus, NEN). The intensities of the bands on the Western blot were analyzed using an image station (Kodak 440), and the data fitted to a pseudo first-order reaction model. Second-order rate constants were obtained by dividing the pseudo first-order rate constants by the concentration of **3a**.

Fluorescence Labeling of hAGT in AGT-Deficient CHO Cells

Fusion proteins ^{W160}hAGT-NLS$_3$ and ^{PGEA}hAGT-NLS$_3$ were constructed by ligation of the two corresponding hAGT genes into the mammalian expression vector pECFP-Nuc (Clontech) using the restriction sites NheI and BglII. Chinese hamster ovary (CHO) cells deficient in O^6-alkylguanine-DNA alkyltransferase (AGT) were transfected with the resulting plasmids as described [9, 22]. After transient expression of the fusion proteins for 24 hr, cells were pulse labeled with **4** at varying concentrations for 5 min in a microscope chamber. Excess substrate was removed by three washes with PBS, and the cells were incubated for 30 min with PBS. Laser scanning confocal micrographs were recorded using a 488 nm argon/krypton laser line on a Zeiss LSM 510 microscope (Carl Zeiss AG) with a 63× water (1.2 numerical aperture) objective. Scanning speed and laser intensity were adjusted to avoid photobleaching of the fluorescent probes and damage or morphological changes of the cells. For the determination of the relative intensity of the fluorescence signal in the nucleus of the cell as a function of the concentration of **4**, more than 10 cells were analyzed at each concentration of **4**, and the mean value was calculated.

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