Ubiquitination-Dependent Proteolysis of O^6-Methylguanine-DNA Methyltransferase in Human and Murine Tumor Cells following Inactivation with O^6-Benzylguanine or 1,3-Bis(2-chloroethyl)-1-nitrosourea[†]

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ABSTRACT: In this study, we investigated the role of ubiquitination in the disposition of the inactivated O^6-methylguanine-DNA methyltransferase (MGMT) protein in human (HT-29 and CEM) and murine (ts85) tumor cells. Using a combination of immunoprecipitation and immunoblotting techniques with antibodies against ubiquitin and MGMT, and anti-ubiquitin immunoaffinity chromatography, the MGMT protein was found to coexist with small amounts of its ubiquitinated species in both human and mouse tumor cells, suggesting the presence of endogenous inactivated MGMT. Further, treatment of HT-29 and CEM cells with MGMT-inactivating compounds, O^6-benzylguanine (O^6-BG, 20 μM) or 1,3-bis-(chloroethyl)-1-nitrosourea (BCNU, 100 μM), resulted in increased levels of ubiquitinated MGMT within 1.5−3 h of drug exposure. Kinetic studies in HT-29 cells treated with O^6-BG indicated a slow and gradual conversion of the inactivated MGMT to its polyubiquitinated forms over a course of 3−18 h, with a concomitant disappearance of the parent MGMT protein. We also characterized the previously reported O^6-BG-induced degradation of MGMT in HT-29 cell extracts [Pegg et al. (1991) *Carcinogenesis 12*, 1679−1683] and showed the extracts to be active in conjugation of the MGMT protein with ubiquitin. The proteolysis of O^6-BG-inactivated MGMT in HT-29 cell extracts was energy-dependent and was markedly stimulated by ATP and Mg^{2+} ions. Using the ts85 temperature-sensitive mutant cell line, which expresses a thermolabile ubiquitin-activating enzyme, we observed a differential stability of the inactivated MGMT protein at permissive and nonpermissive temperatures. These results provide conclusive evidence that the MGMT protein, following its inactivation, is degraded via the ubiquitin proteolytic pathway.

Guanine modified by alkylation at its O^6-position is the most significant DNA lesion accounting for the mutagenic, carcinogenic, and cytotoxic properties of many *N*-nitroso compounds and chloroethylating anticancer agents (Lindahl et al., 1988; Pegg, 1990; Pegg & Byers, 1992; Mitra & Kaina, 1993; Tong et al., 1982; Dumenco et al., 1993). Cellular repair of O^6-methylguanine and O^6-alkylguanines in DNA is mediated by O^6-methylguanine-DNA methyltransferase (EC 2.1.1.63, MGMT[1]) in all organisms, including humans. MGMT has the unique mechanism of action that involves transfer of methyl and other alkyl groups from the O^6-position of guanine in DNA to a cysteine residue within its active site. Because the alkyl group is irreversibly bound to the protein, MGMT is functionally inactivated after one reaction cycle (Lindahl et al., 1988; Pegg & Byers, 1992). This mechanism of action of MGMT provides the basis for its inactivation in cells by a variety of alkylating agents and free O^6-alkylated guanines (Dolan et al., 1990; Pegg, 1990; Moschel et al., 1992). The recovery of MGMT activity after its inactivation with these agents is slow and results entirely from de novo protein synthesis (Pieper et al., 1991; Marathi et al., 1993, 1994; Fritz & Kaina, 1992). The most potent O^6-alkylguanine analogue for the inactivation of MGMT is O^6-benzylguanine (O^6-BG), which, on a molar basis, is approximately 2000-fold more active than O^6-methylguanine in the depletion of MGMT in HT-29 cells (Moschel et al., 1992).

Although it is well-established that alkylating agents and the O^6-alkylguanine analogues effectively inactivate cellular MGMT, very little is known about the fate of the inactivated MGMT and whether it has other intracellular biological effects. Pegg et al. (1991) have demonstrated that MGMT in HT-29 cells treated with O^6-BG is sensitized to proteolytic degradation. Another study (Ayi et al., 1994) has reported that alkylation of MGMT makes it susceptible to V8 protease. Despite these reports, the specific mechanisms involved in the cellular processing of and subsequent degradation of inactivated MGMT are still unclear.

In eukaryotes, the nonlysosomal, ATP-requiring ubiquitin proteolytic pathway is a major route for intracellular protein

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[1] Abbreviations: MGMT, O^6-methylguanine-DNA methyltransferase; O^6-BG, O^6-benzylguanine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; Ub, ubiquitin; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TDEG buffer, 40 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol, 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 5% glycerol. Please note that MGMT has been abbreviated as AGT (O^6-alkylguanine-DNA alkyltransferase) in some figures.

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degradation. This pathway mediates a selective degradation of abnormal proteins and proteins with short half-lives (Finley & Chau, 1991; Hershko & Ciechanover, 1992; Ciechanover & Schwartz, 1994; Glotzer et al., 1991). These include some DNA repair proteins (Bailly et al., 1994) and topoisomerase proteins that are covalently linked to DNA following cellular drug exposure.[2] In this process, ubiquitin, a highly conserved 8.5 kDa protein, is covalently bound to the proteins in multiple numbers, and the resulting targeted proteins are degraded by a high-mass proteasome (Rechsteiner, 1991; Ciechanover & Schwartz, 1994). In the present study, we examined whether in human tumor cells the ubiquitin pathway is involved in the disposition of MGMT that had been inactivated by either O^6-BG or the chloroethylating antitumor drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).

MATERIALS AND METHODS

Chemicals, Cell Lines, and Antibodies. O^6-BG was a kind gift from Dr. Robert Moschel (Frederick Cancer Research and Development Center, Frederick, MD). It was dissolved in dimethyl sulfoxide (DMSO) as a 50 mM stock solution. BCNU (Bristol-Myers, Wallingford, CT) was dissolved in ethanol and diluted with culture medium prior to addition to cultures. The HT-29 human colon carcinoma and CEM human lymphoblastic leukemia cell lines obtained from American Type Culture Collection were maintained by routine passage in Dulbecco's minimum essential medium (DMEM) and RPMI-1640 medium, respectively, that had been supplemented with 10% fetal bovine serum and antibiotics. The mouse mammary carcinoma cell line, ts85, is a cell cycle mutant (Mita et al., 1980) that is temperature-sensitive with respect to ubiquitin-protein conjugation due to a mutation in the ubiquitin-activating enzyme E1 (Finley et al., 1984; Deveraux et al., 1990). The ts85 cell line was obtained from Dr. Martin Rechsteiner of the University of Utah and maintained at the permissive temperature of 30 °C in DMEM with 10% serum. Monoclonal antibodies to human MGMT (MT 4.A1; Brent et al., 1990) were kindly provided by Dr. Darrel Bigner of Duke University; these antibodies also cross-react with the mouse MGMT protein (Ostrowski et al., 1991). Anti-ubiquitin monoclonal antibodies were from Chemicon International (Temecula, CA). All other chemicals were purchased from Sigma Co. (St. Louis, MO).

Drug Treatment, Cell Extract Preparation, and Immunoprecipitation. HT-29 and CEM cells in their exponential phase growth were treated with 100 μM BCNU for 1 h and postincubated in fresh media for 30 min. O^6-BG was added to 20 μM, and the cells were incubated for 90 min. In experiments to analyze the kinetics of MGMT ubiquitination, HT-29 cells were treated with O^6-BG (20 μM) continuously for 3, 6, and 18 h. After they were washed twice with cold PBS, the cell pellets were suspended in 40 mM Tris-HC1 (pH 8.0) containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 5 mM *N*-ethylmaleimide (NEM). NEM was used to inhibit the many thiol group-dependent enzymes in the ubiquitin proteolytic pathway, including the ubiquitin hydrolase and the proteasome, and enhance the detection of ubiquitinated proteins (Goebl et al., 1994); it was inactivated in cell extracts by addition of cysteine to 10 mM. Cell-free extracts were prepared by sonication followed by centrifugation at 10000*g* for 15 min at 4 °C. To eliminate nonspecific binding of proteins, 400 μg protein aliquots were precleared with protein A Sepharose (Harlow & Lane, 1988) and allowed to react with 2 μg of monoclonal antibodies against MGMT at 4 °C for 6 h. This immunoprecipitation step enabled us to acquire the total MGMT protein (native and ubiquitinated) and to further analyze the ubiquitination pattern in these samples. Antigen−antibody complexes were collected after addition of protein A Sepharose and continued mixing for 2 h. Immunoprecipitates were washed three times with Tris-buffered saline and suspended in 100 μL of nonreducing SDS gel loading buffer. After they were boiled for 5 min, the samples from each treatment were divided into two equal portions and subjected to SDS−PAGE. The IgGs used for immunoprecipitation migrated in their undissociated form (approximately 160 kDa) under these conditions and allowed the detection of ubiquitin conjugates with a range of higher molecular masses.

MGMT Degradation in ts85 Cells. ts85 cells were treated with 20 μM O^6-BG for 90 min at their normal growth temperature (30 °C) or 1 h after transfer to the restricted temperature (42 °C). Preincubation at the restricted temperature is required to inactivate the E1 ubiquitin-activating enzyme in ts85 cells (Finley et al., 1984; Deveraux et al., 1990). The cells were either processed immediately for extract preparation or allowed to remain at their corresponding temperatures for 3, 6, and 9 h. Cell extracts were prepared in the lysis buffer containing NEM (described above) and protein content quantified (Bradford, 1976), and samples were subjected to Western blot analysis using antibodies to MGMT.

Immunoaffinity Chromatography of Ubiquitinated Proteins. Anti-ubiquitin monoclonal antibodies were linked to cyanogen bromide-activated Sepharose according to the manufacturer's (Pharmacia) instructions. Preliminary experiments showed the matrix to bind purified ubiquitin when examined by Western blot analysis. HT-29 cells were treated with 20 μM O^6-BG or 100 μM BCNU for 1.5 h and cell extracts prepared as described in the previous section. They were precleared with protein A Sepharose and diluted with Tris-buffered saline before application on the immunoaffinity column. Bound proteins were desorbed by elution with 0.2 M glycine buffer (pH 2.8). The eluted fractions were dialyzed, freeze-dried, and dissolved in SDS gel loading buffer for electrophoresis and Western blotting using MGMT antibodies.

SDS−PAGE and Western Blotting. Proteins were electrophoresed on 10 or 12% SDS−polyacrylamide gels under nonreducing conditions. Following electrophoretic transfer of proteins onto poly(vinyl difluoride) membranes, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 8.0) and then reacted with MGMT or ubiquitin antibodies (2 μg/mL). Immunocomplexes were visualized by incubation with [^{125}I]protein A and autoradiography.

Assay for MGMT Activity. Cell-free extracts were prepared in a buffer containing 40 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, 1 mM EDTA, and 5% glycerol (TDEG buffer). MGMT activity was measured by the transfer of a [^{3}H]-labeled methyl group from the O^6-position of guanine in DNA to the MGMT protein according to Myrnes et al.

[2] P. D'Arpa and L. F. Liu, personal communication.

(1984). Cell extracts were supplemented with the DNA substrate (1 μg, 10 000 cpm) incubated at 37 °C for 30 min, followed by the hydrolysis of DNA in trichloroacetic acid at 80 °C and collection of the protein precipitates for scintillation counting.

MGMT Conjugation with Ubiquitin in HT-29 Extracts. To examine if the ubiquitin−MGMT conjugates are formed *in vitro*, we modified the established procedure involving [^{125}I]ubiquitin as a substrate (Laub & Jennissen, 1991) by using unlabeled ubiquitin. HT-29 cell extracts prepared in TDEG buffer (100 μg of protein) were incubated in the presence of 5 mM $MgCl_2$ and 3 μg of purified ubiquitin with and without addition of 20 μM O^6-BG. After 5 min at 37 °C, 5 mM NEM was added to stop the reaction. Such a short-term incubation is critical because ubiquitin conjugation reactions occur rapidly and the resulting conjugates are highly labile (Laub & Jennissen, 1991). The samples were precleared with protein A Sepharose, immunoprecipitated with AGT antibodies, and immunoblotted using MGMT antibodies.

Effect of ATP on MGMT Degradation in HT-29 Cell Extracts. Cell-free extracts in TDEG buffer were prepared from exponentially growing HT-29 cells and used to study the degradation of inactivated MGMT as described previously (Pegg et al., 1991). Protein aliquots (100 μg) were treated with and without O^6-BG in the presence of 5 mM $MgCl_2$. ATP and other nucleotides were supplemented at 2 mM. The reaction mixtures were incubated at 37 °C for 10−30 min. SDS gel loading buffer was added to terminate the reactions, and the samples were electrophoresed. Western blotting was performed using MGMT antibodies, as described earlier.

RESULTS

Ubiquitination of MGMT Protein and Its Kinetics in Human Tumor Cells. Our initial Western blot studies showed that some faint higher molecular bands in addition to the expected 22−25 kDa protein were detected by antibodies specific to MGMT protein. Some MGMT Western blots shown in earlier publications also indicate the presence of these additional bands (Pegg et al., 1991; Zhukovskaya et al., 1992). We found that inclusion of NEM at 5 mM in cell extraction buffers resulted in a highly enhanced detection of these slow-migrating protein bands on immunoblots, and therefore, NEM was routinely included during extract preparation. The results of immunoprecipitation/immunoblot analyses to detect the presence of ubiquitin−MGMT conjugates in HT-29 cells are shown in panels a and b of Figure 1. The 22−25 kDa polypeptide representing the unmodified form of MGMT was insensitive to ubiquitin staining (lane 1 in panels a and b of Figure 1). However, even without drug treatment, three high-molecular mass species of MGMT corresponding to 100, 50, and 38 kDa were recognized by MGMT antibodies; these large forms of MGMT were also recognized by ubiquitin antibodies (compare lane 1 in panels a and b of Figure 1). A higher molecular mass ubiquitinated MGMT (50 kDa) was also present in control CEM cells (lane 1 in panels a and b of Figure 2). These results point to a significant level of MGMT ubiquitination occurring naturally in tumor cells. On the basis of the molecular mass of unmodified MGMT protein, we estimate that the MGMT−ubiquitin complexes

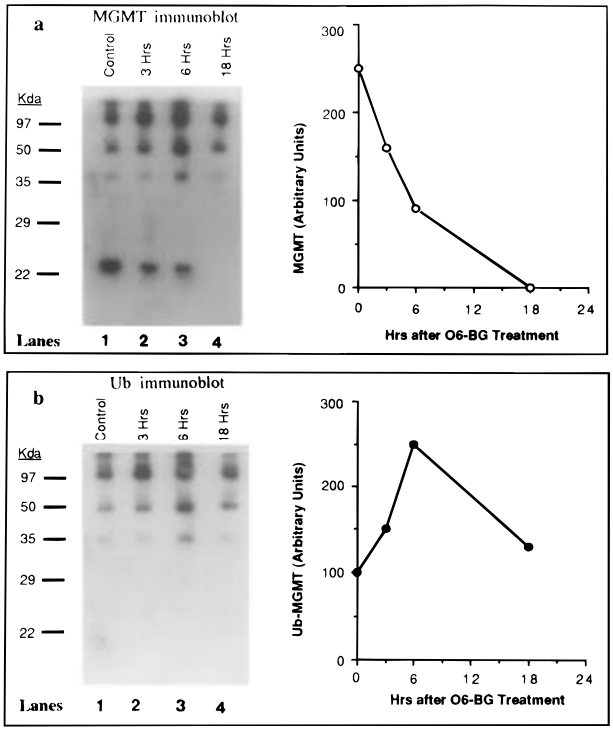

FIGURE 1: Kinetics of the MGMT protein ubiquitination and its proteolysis in HT-29 cells treated with O^6-BG. Cells were incubated continuously with O^6-BG (20 μM) for 3, 6, and 18 h. The MGMT protein was immunoprecipitated from cell extracts (400 μg of protein at each time point) as described in Materials and Methods. The solubilized immunoprecipitates were divided into two equal portions and electrophoresed separately to obtain the Western blots, a and b. Blot a was reacted with MGMT antibodies and blot b with ubiquitin antibodies. The band corresponding to the unconjugated MGMT protein (22.5 kDa) in a and all the bands in b were quantitated by densitometry to obtain the data shown on the right side of the Western blots; in b, the peak areas of different bands were combined to represent the net amount of ubiquitinated MGMT.

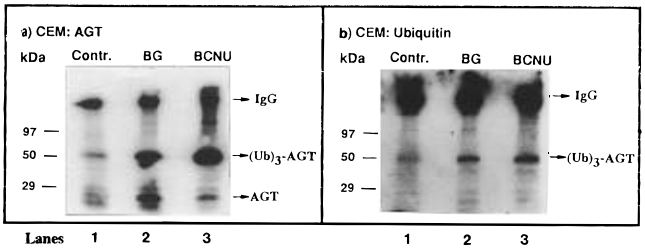

FIGURE 2: Western blots showing ubiquitination of the MGMT protein in CEM cells. Cells were treated with O^6-BG or BCNU for 1.5 h and extracts immunoprecipitated using MGMT antibodies as described in Materials and Methods. The immunoprecipitates were dissolved in SDS sample buffer and equal amounts processed for MGMT (a) and Ub (b) Western blots as described in the legend to Figure 1. AGT = MGMT, Contr. = control, and BG = O^6-BG. Positions of the undissociated IgG, unmodified MGMT, and triubiquitinated [$(Ub)_3$] MGMT are shown.

contain two, three, and nine ubiquitin moities linked to the parent protein. Using a sensitive assay for AGT (Myrnes et al., 1984), we observed a 100% and approximately 50% reduction of MGMT activity in human tumor cell lines (HT-29 and CEM) treated with 20 μM O^6-BG and 100 μM BCNU, respectively (Table 1). This inactivation of MGMT was associated with a significant increase in the amount of high-molecular mass protein bands that were immunoreactive with both MGMT and ubiquitin antibodies in HT-29 and CEM cells (Figure 1a, &1b and Figures 2a,b). The Western blots of the MGMT immunoprecipitates performed with both

Table 1: Inhibition of MGMT Activity in Tumor Cell Lines by O^6-BG and BCNU[a]

	MGMT activity (pmol/mg protein)		
cell line	control	O^6-BG (% inh)	BCNU (% inh)
HT-29	2.10 ± 0.07[b]	0 (100)	1.0 ± 0.10 (52.4)
CEM	2.80 ± 0.05	0 (100)	1.5 ± 0.10 (46.4)
ts85	0.10 ± 0.02	0 (100)	nd[c]

[a] inh is inhibition. Tumor cells were treated with 20 μM O^6-BG or 100 μM BCNU for 90 min and extracts prepared in TDEG buffer. MGMT activity was quantitated at different protein concentrations in two independent experiments as described in Materials and Methods. [b] Mean ± standard error of the mean. [c] Not determined.

MGMT and ubiquitin antibodies showed a similar banding pattern; this and the similar staining intensities of the higher molecular mass bands on the two immunoblots (compare panels a with b in Figures 1 and 2) thus establish the protein bands to be ubiquitin−MGMT protein complexes. A key feature of the drug-induced ubiquitination was an increase in the level of the preexisting ubiquitin−MGMT conjugates as evident from the enhanced band intensities on Western blots. DNase treatment of the extracts prior to immunoprecipitation did not change the pattern of protein bands, indicating that the higher molecular mass MGMT bands were not covalently complexed with DNA (Gonzaga et al., 1992). The ubiquitination pattern of total cellular proteins from the control and O^6-BG- and BCNU-treated cells remained largely unchanged, indicating the specificity of the Ub modification of the MGMT protein (not shown).

Figure 1a also shows the changes over time in the levels of polyubiquitinated MGMT and the parent MGMT in O^6-BG-treated HT-29 cells. This base line level of ubiquitinated MGMT did not change over a time period of 20 h (not shown). However, O^6-BG treatment induced a discernible increase of the ubiquitinated forms at 3 h and an even higher increase at 6 h of incubation, as evident from the increased intensity of the corresponding bands (Figure 1a, lanes 2 and 3, respectively). The ubiquitination pattern returned to near base line levels at 18 h (Figure 1a, lane 4). Simultaneously, the levels of the parent 22 kDa (unconjugated) MGMT decreased gradually over 3 and 6 h, reaching undetectable levels at 18 h. The kinetics of formation of the ubiquitin antibody-reactive MGMT protein (Figure 1b) was similar to that of the higher molecular mass MGMT species shown in Figure 1a. The disappearance of the MGMT protein seen in Figure 1a and the alterations in the level of the ubiquitinated MGMT observed in Figure 1b were quantitated by densitometry, and these profiles are shown with the corresponding Western blots.

Immunoaffinity Chromatography. To obtain further evidence for the modification of MGMT by ubiquitination, we performed the immunoaffinity chromatography on Sepharose linked with anti-ubiquitin antibodies. Figure 3 shows the pattern of MGMT protein from untreated and O^6-BG- or BCNU-treated HT-29 cells specifically bound to the immunoaffinity column. Drug exposure resulted in the formation of ubiquitinated MGMT proteins of 52, 75, and 98 kDa. Binding of a 98 kDa MGMT species to the immunoaffinity column in control cell extracts is also evident (Figure 3, lane 1). These results are consistent with the Western blot results shown for HT-29 cells in panels a and b of Figure 1. Although BCNU inhibited MGMT activity to a lesser extent than O^6-BG in human tumor cells (Table 1), the level of

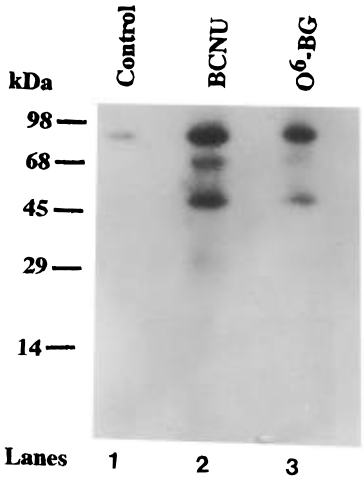

FIGURE 3: Evidence for ubiquitination of MGMT by anti-ubiquitin affinity chromatography. HT-29 cells were treated with O^6-BG or BCNU for 1.5 h and cell extracts prepared and processed on the immunoaffinity column as described in Materials and Methods. The column eluates were subjected to Western blotting using MGMT antibodies.

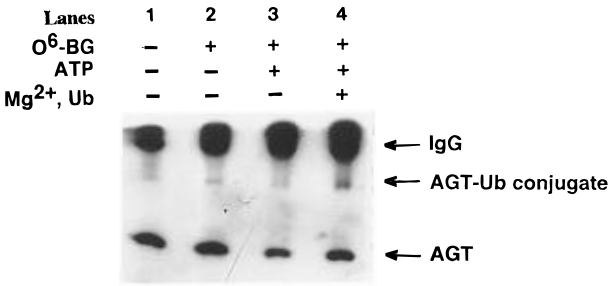

FIGURE 4: Conjugation of ubiquitin with MGMT in HT-29 cell extracts. Protein aliquots (100 μg) were incubated with and without O^6-BG (20 μM), ATP (2 mM), $MgCl_2$ (5 mM), and ubiquitin (3 μg) as indicated for 5 min (see Materials and Methods for details). Samples were immunoprecipitated and immunoblotted with AGT antibodies. AGT = MGMT. Positions of the undissociated IgG, 100 kDa AGT−Ub conjugate, and unmodified AGT are indicated.

MGMT ubiquitination induced by BCNU was higher than that observed with O^6-BG in both HT-29 cells (Figure 3, lanes 2 amd 3) and CEM cells (Figure 2a,b, lanes 2 and 3).

Ubiquitination of the MGMT Protein in HT-29 Cell Extracts. The generation of ubiquitin−MGMT conjugates *in vitro* is shown in Figure 4. A protein band corresponding to 100 kDa increased in intensity when exogenous ubiquitin was added to the reaction mixtures (Figure 4, lane 4). The presence of a ubiquitinated MGMT species of similar molecular mass in intact HT-29 cells (Figures 1a,b and 3) indicates that the pattern of ubiquitin conjugation with the MGMT protein is similar *in vivo* and *in vitro*. A ladder of MGMT protein bands as expected for a substrate with different levels of ubiquitination was, however, not evident in our *in vitro* study, presumably because of their formation in very small amounts during the 5 min reaction.

ATP-Stimulated Degradation of MGMT in HT-29 Cell Extracts. Consistent with the previous observations of Pegg et al. (1991), we found that exposure of HT-29 extracts to O^6-BG resulted in a rapid disappearance of MGMT protein (Figure 5, lane 5). The reaction kinetics of the MGMT breakdown showed a lag period of 15 min (Figure 5, lane 2), after which there was a rapid increase in its degradation (Figure 5, lane 5), indicating that the process may involve several intermediate enzymatic reactions before the final

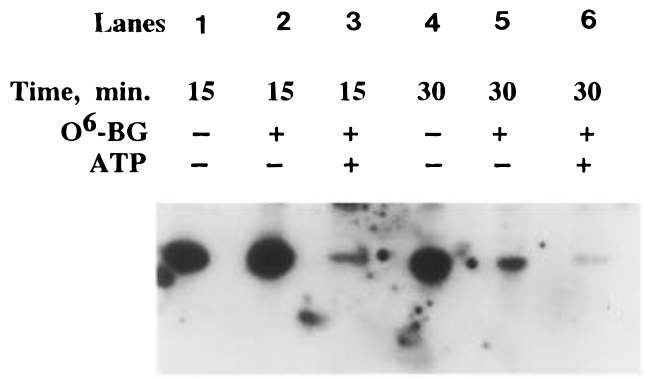

FIGURE 5: Western blots showing the stimulation of MGMT degradation by ATP in HT-29 cell extracts. Protein aliquots (100 μg) containing 5 mM $MgCl_2$ were treated with and without 20 μM O^6-BG for the periods shown. The reaction mixtures in lanes 3 and 6 contained 2 mM ATP. All samples were processed for immunoblotting using MGMT antibodies.

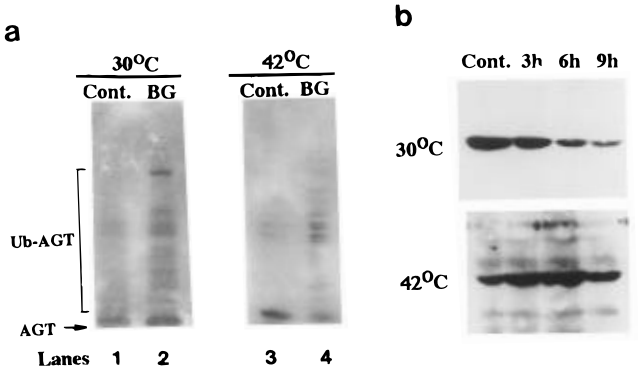

FIGURE 6: Ubiquitination and loss of the AGT protein in ts85 cells treated with O^6-BG at permissive (30 °C) and nonpermissive (42 °C) temperatures. (a) Coexistence of the MGMT protein with its ubiquitinated forms and the effect of O^6-BG treatment for 90 min thereon. (b) Pattern of the 22 kDa MGMT polypeptide in ts85 cells 3, 6, and 9 h after O^6-BG treatment. Cells were treated with 20 μM O^6-BG at permissive or nonpermissive temperatures and incubated for the periods shown. Cell extracts were prepared, and 100 μg of protein was electrophoresed in each lane and processed for Western blotting using MGMT antibodies. AGT = MGMT.

proteolytic step. Our initial studies showed that Mg^{2+} ions stimulate this proteolytic process, and thus, $MgCl_2$ at 5 mM was included in all the reactions shown in Figure 5. Addition of ATP to the reaction mixtures containing O^6-BG markedly increased the rate of MGMT degradation (Figure 5, lanes 3 and 6), most evident after a 15 min incubation. We interpret the breakdown of MGMT in the absence of added ATP (Figure 5, lane 5) as due, in part, to endogenous ATP present in the cell extracts. This is supported by the observation that addition of O^6-BG to dialyzed cell extracts did not result in MGMT degradation (data not shown). GTP and UTP also stimulated the disappearance of the inactive MGMT protein at rates similar to those of ATP (not shown), indicating a broad nucleotide specifcity for this proteolytic process.

Ubiquitination of the MGMT Protein and Its Degradation in Mouse ts85 Cells. We took advantage of the temperature-sensitive defect prevalent in ts85 cells for the ligation of ubiquitin to proteins (Finley et al., 1984; Deveraux et al., 1990) and examined the influence of ubiquitination on the breakdown of the MGMT protein after O^6-BG treatment. Consistent with previous observations of lower MGMT levels in murine cell lines (Foote & Mitra, 1984; Mitra & Kaina, 1993), ts85 cells showed 21−28-fold less MGMT activity than human tumor cells (Table 1). Figure 6a shows the Western blot pattern of MGMT and the effect of O^6-BG thereon in ts85 cells at permissive (30 °C) and nonpermissive (42 °C) temperatures. The native MGMT protein in control ts85 cells coexisted with a range of ubiquitinated forms as represented by bands of higher molecular masses (Figure 6a, lane 1). This is consistent with similar observations we made with HT-29 and CEM human tumor cells (Figures 1−3). O^6-BG treatment of ts85 cells maintained at 30 °C resulted in a slight increase of MGMT ubiquitination as seen by an enhanced intensity of the preexisting bands and the appearance of a slow-migrating band (Figure 6a, lane 2). Compared to cells at 30 °C, ts85 cells kept at 42 °C showed a significantly reduced level of MGMT−Ub complexes in both control and O^6-BG-treated cells (Figure 6a, lanes 3 and 4). O^6-BG has been shown to reduce the content of immunoreactive MGMT protein in HT-29 cells (Pegg et al., 1991). In line with this report, O^6-BG treatment of ts85 cells at 30 °C decreased the intensity of the 22 kDa MGMT band in a time-dependent manner (Figure 6b, upper panel). In contrast, the native MGMT polypeptide remained largely undegraded in ts85 cells heated to 42 °C and treated with O^6-BG during the same time course (Figure 6b, lower panel).

DISCUSSION

Protein ubiquitination is a common posttranslational modification that plays an essential role in the regulation of many cellular functions, such as cell cycle control, ribosome biogenesis, chromatin structure, DNA repair, and stress response. Proteolysis mediated by ubiquitinatation is the best understood cellular function of the ubiquitin system. In this pathway, a series of ubiquitin moieties become covalently attached to the free amino groups of proteins destined for catabolism in a sequence of ATP-dependent reactions catalyzed by ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes. The multi-ubiquitin chain then serves as a reusable recognition signal for selective proteolysis of these target proteins by a large (26S) ATP-dependent protease complex. The mitotic cyclins, p53 tumor suppressor, c-myc oncoprotein, thalassemic hemoglobins, and proteins synthesized with unusual amino acids like canavanine are some examples of the short-lived and/or abnormal cellular proteins whose degradation occurs by ubiquitination [reviewed in Finley and Chau (1991), Hershko and Ciechanover (1992), and Ciechanover and Schwartz (1994)].

MGMT inhibition by O^6-BG has been shown to enhance the cytotoxicity of (2-chloroethyl)nitrosoureas both *in vitro* and *in vivo* (Dolan et al., 1990, 1991, 1993a,b; Friedman et al., 1992; Mitchell et al., 1992; Gerson et al., 1993, 1994). Currently, clinical trials are being initiated to exploit this strategy in cancer therapy. The present study was designed to understand better the mechanisms underlying the cellular processing of MGMT following its inactivation in tumor cells. We provide several lines of evidence, both in cells and in a cell-free system, that MGMT inactivated by either O^6-BG or BCNU undergoes ubiquitination and is degraded by an ATP-dependent pathway. Our initial studies showed that NEM was essential for an enhanced detection of the ubiquitinated forms of the MGMT protein. The failure of earlier workers to detect this modification might be due to the fact that the cell extracts were prepared in buffers without

NEM or other sulfhydryl blockers in which the steady-state levels of the MGMT−Ub conjugates are low, because of their rapid degradation.

Of the two MGMT-inactivating compounds studied, O^6-BG, by being a specific pseudosubstrate, inactivates MGMT (Moschel et al., 1992), irrespective of the nuclear or cytoplasmic location of the enzyme, while BCNU primarily provides a DNA-bound substrate [O^6-(chloroethyl)guanine, O^6-ethanoguanine, or O^6-(hydroxyethyl)guanine] to inactivate MGMT. Although BCNU inactivation of MGMT was only partial (Table 1), it was more potent than the complete inactivator, O^6-BG, in inducing MGMT ubiquitination (Figures 1−3). Several mechanisms may account for this differential ubiquitination. First, it may be related to the ability of BCNU to inactivate the nuclear pool of MGMT more rapidly than O^6-BG. This is supported by the reported rapid loss of nuclear MGMT relative to that in the cytoplasm after cell exposure to ACNU (Ishibashi et al., 1994). Second, the affinity of the enzymes involved in ubiquitin conjugation for the chloro and/or hydroxyethyl *S*-MGMT produced by BCNU and the benzyl *S*-MGMT generated by O^6-BG may differ. Additionally, differences in the half-lives of the inactivated MGMT produced by these two compounds might also contribute to this phenomenon.

The ladder pattern of bands that we observed on Western blots is clearly indicative of polyubiquitination of MGMT, a hallmark of proteins targeted for catabolism via this proteolytic pathway. The presence of MGMT ubiquitination in human tumor cells, HT-29 colon carcinoma and CEM leukemic cells, as well as the mouse mammary carcinoma (ts85) suggests that the process may be conserved in mammalian cells and is independent of tumor types. We observed that untreated human tumor cells possess polyubiquitinated MGMT, with approximate molecular masses of 38, 50, and 100 kDa, reflecting different levels of ubiquitination and that the native MGMT protein once inactivated is further processed into these multiple species. The kinetics of MGMT ubiquitination in HT-29 cells following its inactivation by O^6-BG indicated that this process occurs over a relatively long period, with a peak at 6 h lasting up to 18 h after drug treatment. A progressive disappearance of the parent MGMT protein together with its increased ubiquitination indicates that the ubiquitin proteolytic pathway is likely to be the only route for MGMT degradation. The results of Pegg et al. (1991) that, in HT-29 cells, the inactivated MGMT protein is eliminated gradually with a 50% loss at 6 h and a 70% loss at 16 h are consistent with our data. Ayi et al. (1994) also demonstrated that the alkylated MGMT was stable for at least 3 h in CEM cells and that the inactive MGMT was retained in the leukocytes of patients undergoing chemotherapy with CCNU.

The ubiquitin-activating enzyme, E1, catalyzes the first reaction in the ubiquitin pathway involving the formation of a high-energy ubiquitin−adenylate intermediate and its transfer to a thiol site within the E1 molecule (Hershko & Ciechanover, 1992). We used the ts85 cells with an established defect of the E1 enzyme to study the interrelationship between ubiquitination and proteolysis of the MGMT protein. In temperature shift experiments with ts85 cells, we observed a differential stability of the inactivated MGMT protein, providing strong evidence that the ubiquitin pathway is involved in cellular disposition of MGMT. The ts85 cells and other murine cells with thermolabile E1 enzymes have been previously utilized to demonstrate the involvement of ubiquitination in the proteolysis of different proteins (Ciechanover et al., 1984), including the tumor suppressor p53 protein (Chowdary et al., 1994) and DNA topoisomerase I[2].

The Mg^{2+} and ATP stimulation of MGMT degradation we observed in this study is consistent with the energy requirement of the ubiquitin proteolytic pathway both *in vitro* (Ciechanover et al., 1991; DeMartino et al., 1991) and *in vivo* (Finley & Chau, 1991; Ciechanover & Schwartz, 1994). The Mg^{2+} requirement indicates that ATP hydrolysis is necessary for the degradation of MGMT. The endogenous ATP and Mg^{2+} present in HT-29 cell extracts appear sufficient for promotion of the proteolysis of the inactivated MGMT; this may explain the earlier observations of Pegg et al. (1991) and similar data presented here (Figure 5, lane 5). This *in vitro* system should be valuable in the delineation of further details of the cellular disposition of MGMT.

The finding of ubiquitinated MGMT in tumor cells not treated with drugs is interesting and indicates the presence of inactive MGMT protein in cells. The polyubiquitinated forms we observed may represent a population of MGMT undergoing normal turnover. However, MGMT, in its inactive form, may also accumulate in cells under normal physiological conditions through the repair of O^6-alkylguanine DNA adducts generated via nutritional intake or environmental exposure to alkylating agents or their precursors. Demethylation of DNA modified nonenzymatically by *S*-adenosylmethionine is one such example (Rydberg & Lindahl, 1982). The observations of Zhukovskaya et al. (1992), who found a lack of correlation between the level of immunoreactive MGMT and MGMT functional activity, as well as differential chromatographic behavior of MGMT on phosphocellulose in a number of human cell lines, are supportive of the presence of inactive MGMT in tumor cells. Because of the inability of the currently available antibodies to differentiate between the active and inactive forms of MGMT, caution is warranted in interpreting the MGMT content on the basis of immunohistochemical or immunoblot analysis alone. The molecular mechanism by which the MGMT protein becomes a target for ubiquitination is not clear. It is possible that the alkylation of MGMT, such as with O^6-BG or BCNU, alters its tertiary conformation, thus facilitating its conjugation with ubiquitin. On the other hand, the destabilizing amino acid residues present at or near the N-terminus of the inactive MGMT protein may also trigger the process of ubiquitination according to the N-end rule (Ciechanover & Schwartz, 1994; Bartel et al., 1990). Further studies are required to address these mechanisms.

Knowledge of MGMT protein metabolism in cancer cells, particularly, following its inactivation may impact significantly the development of strategies to exploit this in cancer chemotherapy. In this context, a better understanding of the mechanism(s) underlying the de novo synthesis of the MGMT protein following its functional inactivation will be very important. Studies to date have shown no changes in MGMT mRNA levels following MGMT protein depletion by O^6-BG (Fritz & Kaina, 1992; Marathi et al., 1993) or streptozotocin−BCNU combination (Pieper et al., 1991), and yet, in many experimental systems, the MGMT activity recovers to basal levels by 24 to 48 h after drug removal (Pegg, 1990; Lee et al., 1991; Marathi et al., 1993, 1994). The formation of inactive MGMT and/or its degradation may

be a factor in signaling the de novo synthesis of MGMT (Pegg et al., 1991). Consequently, a prolonged inhibition of MGMT is a requirement for significant potentiation of nitrosourea cytotoxicity (Pieper et al., 1991; Marathi et al., 1993, 1994). MGMT ubiquitination may thus provide a rationale by which the repletion of MGMT in tumor cells after treatment with MGMT-inactivating agents such as O^6-BG can be inhibited. For example, since ubiquitination of inactive MGMT is likely to be a rate-limiting step in its proteolysis, a transient inhibition of ubiquitination (Hershko et al., 1991) and/or that of the proteasome (Rock et al., 1994) may extend the retention of inert MGMT in cells and allow improved therapeutic efficacy of 2-(chloroethyl)nitrosoureas and other alkylating agents.

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