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S-phase Specific Downregulation of Human O^6-Methylguanine DNA Methyltransferase (MGMT) and its Serendipitous Interactions with PCNA and $p21^{cip1}$ Proteins in Glioma Cells

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Abstract

Whether the antimutagenic DNA repair protein MGMT works solo in human cells and if it has other cellular functions is not known. Here, we show that human MGMT associates with PCNA and in turn, with the cell cycle inhibitor, $p21^{cip1}$ in glioblastoma and other cancer cell lines. MGMT protein was shown to harbor a nearly perfect PCNA-Interacting Protein (PIP box) motif. Isogenic p53-null H1299 cells were engineered to express the p21 protein by two different procedures. Reciprocal immunoprecipitation/western blotting, Far-western blotting, and confocal microscopy confirmed the specific association of MGMT with PCNA and the ability of p21 to strongly disrupt the MGMT-PCNA complexes in tumor cells. Alkylation DNA damage resulted in a greater colocalization of MGMT and PCNA proteins, particularly in HCT116 cells deficient in p21 expression. p21 expression in isogenic cell lines directly correlated with markedly higher levels of MGMT mRNA, protein, activity and greater resistance to alkylating agents. In other experiments, four glioblastoma cell lines synchronized at the G1/S phase using either double thymidine or thymidine-mimosine blocks and subsequent cycling consistently showed a loss of MGMT protein at mid- to late S-phase, irrespective of the cell line, suggesting such a downregulation is fundamental to cell cycle control. MGMT protein was also specifically degraded in extracts from S-phase cells and evidence strongly suggested the involvement of PCNA-dependent $CRL4^{Cdt2}$ ubiquitin-ligase in the reaction. Overall, these data provide the first evidence for non-repair functions of MGMT in cell cycle and highlight the involvement of PCNA in MGMT downregulation, with p21 attenuating the process.

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Introduction

O^6-Methylguanine DNA methyltransferase (MGMT) is a DNA repair protein that stoichiometrically removes the mutagenic alkyl adducts introduced at the O6-position of guanine and O4-of thymine by various exogenous and endogenous agents [1]. This unique and conserved direct reversal repair prevents the GC to AT transitions and maintains the stability of human genome [2]. Unlike the other DNA repair pathways, human MGMT works as a single protein to remove the guanine O^6-bound alkyl groups to cysteine145 at its active site [3]. Because the alkyl groups are covalently linked to Cys145, MGMT is self-inactivated after each reaction and the inactive protein is degraded through the ubiquitin/proteasome pathway [4,5]. The alkylation DNA damage introduced by the clinically used anticancer alkylating agents is also targeted and scavenged by MGMT. These drugs

Abbreviations: MGMT, O^6-methylguanine DNA methyltransferase; PCNA, proliferating nuclear antigen; His Tag, Hexahistidine tag; DAPI, 4,6-Diamidino-2-phenylindole; rMGMT, recombinant MGMT protein; tet, tetracycline; IB, immunoblot; WB, western blot; LV, lenti-viral transfection; AS, asynchronous cells; CS0, G1/S synchronized cells before release for cycling; CS2, CS4 and CS8, synchronized cells 2, 4 and 8 hours after release into cycling; TMZ, temozolomide; BCNU, 1,3-bis (2-chloroethyl)-1-nitrosourea; Conc, concentration; $CRL4^{Cdt2}$, PCNA-dependent ubiquitin ligase; Ub, ubiquitin; IP, immunoprecipitation; PIP-box, PCNA interacting protein motif.

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bearing a single (temozolomide) or two (chloroethylnitrosoureas) electrophilic groups generate mutagenic lesions and cytotoxic G-C interstrand crosslinks respectively to exert the antitumor effects. Paradoxically, however, the human cancers express higher levels of MGMT, and the resulting repair of alkyl lesions confers a high level of tumor drug resistance and failure of chemotherapy [6]. Diverse groups of MGMT inhibitors have emerged, and O^6-benzylguanine, a pseudo-substrate inhibitor has been extensively studied [7,8]. Therefore, understanding the molecular aspects of MGMT function is highly important from several angles ranging from the mechanism of DNA repair, carcinogenesis, and cancer therapy.

The stoichiometric reaction mechanism of MGMT raises many puzzling questions such as why human cells synthesize a protein for a single repair event at a significant cost, why does it undergo several posttranslational modifications [9–11], whether MGMT serves other functions in the cell, and whether MGMT accomplishes the de-alkylation all by itself *in vivo* or does it associate with accessory / replication proteins? Although answers to these are unknown, evidence from our laboratory and others suggests that MGMT specifically interacts with many cellular proteins and may have other functions [12,13]. For example, using MGMT-Sepharose affinity chromatography and tandem mass spectrometry, we showed the specific interaction of MGMT with a diverse group of proteins involved in DNA replication (ORC1, MCM helicases, PCNA, DNA pol δ) and cell cycle progression (CDKs, $p21^{cip1,}$ and ubiquitin pathway components) [12]. A similar study by another group confirmed the specific binding of MGMT with various regulatory proteins in human glioma cells [13]. Recently, we showed a specific association and fine interplay between human MGMT and estrogen receptor-α proteins and their co-degradation after tumor cell treatments with either O^6-benzylguanine or fulvestrant, their respective inhibitors [14]. A previous study implied that the alkylated (inactivated) human MGMT is a negative regulator of ER-mediated transcription following DNA alkylation damage [15].

Previously, we reported briefly on MGMT binding with PCNA [16] and the presence of MGMT in $p21^{cip1}$-PCNA complexes [17]. Human PCNA is a homotrimeric protein that encircles the duplex DNA forming a ring-shaped clamp and functions as a processivity factor by tethering replicative DNA polymerases [18]. PCNA also provides a molecular platform that facilitates the myriad protein-protein and protein–DNA interactions that occur at the replication fork. Numerous PCNA-associated proteins such as the FEN1 nuclease, DNA cytosine methyltransferase, and topoisomerase II compete for binding to a common surface on PCNA [19]. Many of these partners contain a highly conserved PCNA-binding motif, QXXhXXaa (where 'h' is a hydrophobic, 'aa' are aromatic and X is any amino acid), referred to as a PCNA interacting protein (PIP) box [18,19]. Besides being an essential part of DNA replication machinery, PCNA also plays important roles in cell cycle regulation (primarily in S-phase), translesion synthesis, long-patch base excision repair and recombination [20].

All DNA repair pathways including nucleotide excision repair (XP-A, XP-G), mismatch repair (MSH2, MSH6), and PARP-1 are associated with PCNA in some way or another [20]. While PCNA exists in free and chromatin-bound states, the abundance of cellular PCNA is strictly controlled by the cyclin-dependent kinase (CDK) inhibitor $p21^{CDKN1A,}$ which is an avid binder and sequestrator of the former [21]. $p21^{cip1}$, which is activated by the p53 tumor suppressor, plays essential roles in the DNA damage response by inducing cell cycle arrest, direct inhibition of DNA replication and apoptotic regulation. p21 interferes with PCNA-dependent DNA polymerase activity, thereby inhibiting DNA replication, however, DNA repair processes dependent on PCNA appear to be largely unaffected [22,23]. Additionally, PCNA interfaces with the cell cycle by forming PCNA-p21/CDK-cyclin quaternary complexes with both positive and negative signaling roles in cell cycle progression. Furthermore, PCNA is an integral component of the regulated and timely destruction of proteins during the S-phase and facilitates the formation of pre-replication complexes for the next cell cycle [24,25]. In this process, PCNA assists with the recognition of target proteins by $CRL4^{Cdt2}$ ub-ligase [26,27]. No information is available on the involvement of either PCNA or other cell cycle proteins in the regulation of the one-step direct reversal reaction performed by MGMT. Here we describe a detailed analysis of MGMT interaction with PCNA and $p21^{cip1}$ in isogenic cancer cell lines and demonstrate a PCNA-dependent downregulation of MGMT protein in S-phase.

Materials and Methods

Cell Culture and Reagents

All cell lines were obtained within 6 months of time and were authenticated by the original sources and investigators. The human glioblastoma cell lines, SF188 (Univ. of California Brain Tumor Bank, San Francisco, CA), GBM10 (Dr. Jann Sarkaria, Mayo Clinic, Rochester, MN), and T98G (ATCC). Other cell lines, HT29 colon cancer, H1299 lung cancer All cell lines were from ATCC. The human medulloblastoma cell line UW228 was provided by Dr. Francis Ali-Osman (Duke University, Durham, NC). All tumor cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and antibiotics at 37°C in 95% humidified air, and 5% CO_2. Thymidine, mimosine and all other chemicals were obtained from Sigma-Aldrich. Monoclonal antibodies to MGMT were purchased from EMD Millipore. Other antibodies were purchased as follows: actin, PCNA, p21 (Cell Signaling), Ubiquitin (Santa Cruz Biotechnology).

Isogenic Cell Lines

Three different isogenic cell line pairs, with one member in each group differing in p21 expression level, were used in this study. The first model was a p21-inducible cell line generated using the tetracycline (Tet)-regulated vector system (H1299-p21) that repressed p21 expression in the presence of Tet [28]. The plasmid constructs for this conditional gene expression were from Clonetech Laboratories (Mountain View, CA). Full-length cDNA for human p21cip1 was cloned into the HindIII-SpeI sites of the pTET-SPLICE plasmid. The p53-null H1299 cells were first transfected with the 'regulatory plasmid', pUDH15-1 and the clones selected in the presence of 450 μg/ml of G418. These resulting clones were next transfected with the pTET-p21 'response plasmid' and selected against zeocin. The resistant clones were screened for p21 expression and maintained in a medium containing Tet (1 μg/ml). The second isogenic cell line was developed by stable transfection of H1299 cells with p21 lentiviral particles (H1299-LV/p21) obtained from the Vigene Biosciences (Rockville, MD). Blasticidin was used for selecting the transfected cells and clones were created. The third cell line model was the HCT116 colon cancer cells proficient or deficient in p21 expression. HCT116 $p21^{++}$ and HCT116 $p21^{--}$cells [29] were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD).