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FAST TRACK

Characterization of DNA Demethylation in Normal and Cancerous Cell Lines and the Regulatory Role of Cell Cycle Proteins in Human DNA Demethylase Activity

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Abstract DNA methylation/demethylation constitutes a major consequence in all biological processes involving transcription, differentiation, development, DNA repair, recombination, and chromosome organization. Our earlier studies established that demethylation of CpG rich sequence by human DNA demethylase activity (5-methylcytosine-DNA glycosylase (5MeC-DNA glycosylase)) resembles ''base excision DNA repair activity'' and creates single-strand breaks on DNA that is associated with proliferating cell nuclear antigen (PCNA). Here in this report, we have identified differential DNA demethylation targets (hemi-methylated vs. fully-methylated) in normal cell lines and cancerous cell lines, and a shortened G_0/G_1 resting time in cancerous cell lines than the normal cell lines. We have identified that in normal HFL1 fibroblast cell line, DNA demethylase activity targets hemi-methylated CpG specific sites on DNA. This normal cell line DNA demethylase activity associates with PCNA immune complex that is inhibited by CDKI proteins $p21^{waf1}$/Gadd45α and Gadd45β. While in cancerous LnCap and BT20 cell lines DNA demethylase activity targets fully-methylated CpG specific sites on DNA. This cancer cell line DNA demethylase activity is not associated with PCNA immune complex and is not inhibited by CDKI proteins $p21^{waf1}$/Gadd45α and Gadd45β. We have also identified that the fully-methylated CpG specific DNA demethylase activity from cancerous cell lines to associate with p300/CBP protein. These significant observations of variable targets of DNA demethylation and alternate partner proteins for DNA demethylase activity in cancerous cell lines are discussed in terms of double-strand DNA breaks versus single-strand DNA breaks and their role in the exit of G_1/G_2 cell cycle stages. Also, the inability of cell cycle regulatory proteins like PCNA, $p21^{waf1}$, and Gadd45 to control DNA demethylase activity in cancerous cell lines is discussed in terms of accelerated G_1/G_2 cell cycle stage exit to facilitate unregulated cellular proliferation, loss of control of chromosomal organization, and the development of oncogenesis in cancerous cell lines. J. Cell. Biochem. 91: 572–583, 2004.

Key words: DNA demethylase; PCNA; p300/CBP protein; Gadd45; DNA strand breaks; G_1/G_2 cell cycle exit; chromosomal organization

Methylation of DNA cytosine is found almost ubiquitously in nature that is purely post-replicative and catalyzed by DNA methyltransferase. The presence of 5-methylcytosine (5-MeC) in DNA is an epigenetic marker and key recognition signal for the regulation of DNA–protein interactions. DNA methylation/demethylation constitutes a major consequence in all biological processes involving transcription, differentiation, development, DNA repair, recombination, and chromosome organization. Our earlier studies established that demethylation of CG rich sequence by human DNA demethylase activity (5MeC-DNA glycosylase) resembles "base excision DNA repair activity" and creates single-strand breaks on DNA that is associated with proliferating cell nuclear antigen (PCNA). So far two types of DNA demethylase (5MeC-DNA glycosylase) activity have been demonstrated. The human DNA

Abbreviations used: PCNA, proliferating cell nuclear antigen; Gadd45, growth arrest and DNA damage induced protein-45; CDKI, cyclin dependant kinase inhibitory proteins; DNMT, DNA-(cytosine-5)-methyl transferase.

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demethylase (5MeC-DNA glycosylase) activity from HeLa cellular extracts demonstrated by us, specifically acted at fully-methylated CpG sites [Vairapandi and Duker, 1993, 1996; Vairapandi et al., 2000a]. Second chick embryo glycosylase has a ten-fold preference for hemi-methylated DNA sites over fully-methylated loci [Jost et al., 1995].

5-MeC-DNA glycosylase activity towards hemi-methylated DNA would result in a single-strand DNA break and totally unmethylated DNA CpG site, with loss of the methylation pattern. By contrast, glycosylic removal of 5-MeC from one strand of fully-methylated DNA would result in single-strand DNA break and formation of hemi-methylated DNA preserving the methylation pattern. While glycosylic removal of 5-MeC from both strands will lead to double-strand DNA break and formation of unmethylated DNA and erasure of methylation pattern.

The substrate specificity of this glycosylase for hemi-methylated or fully-methylated DNA may reflect either the events of cellular development or the stage of the cell cycle. The action of DNA demethylation is progressive in the sense that the fully-methylated CpG islands on the DNA become hemi-methylated and then to unmethylated status. So a comparative analysis for DNA demethylase activity's target as fully-methylated or hemi-methylated CpG sites on DNA in normal and malignant cells will provide valuable insight into the understanding of the regulatory mechanisms operative in the cell cycle to maintain and preserve the methylation pattern on the genomic DNA.

MATERIALS AND METHODS

Cell Lines and Tissue Culture Conditions

We used HFL1—human fibroblast cell line as the non-transformed, normal cell line for our studies. LnCap—human prostate cancer cell line and BT20—human breast cancer line were used as malignant cell lines in the study. HFL1 and LnCap cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics and 10% CO_2. BT20 cell line was cultured in RPMI supplemented with 10% FBS and 1% antibiotics and 5% CO_2. For synchronization of the cultures at late G_1/early S phase of the cell cycle, 3×10^5 cells were seeded on 100 mm cell culture dishes with Aphidicolin (Calbiochem, Cat no. 178273, San Diego, CA) at a concentration of 1 μg/ml tissue culture medium. This aphidicolin treatment was given for 24 h to synchronize the cultures at late G_1/S phase. Then the medium with aphidicolin was removed and washed three times with phosphate buffered saline (PBS) and fresh medium was replenished to start the time course experiment as 0 h. To synchronize the cultures at G_0/G_1 stage of the cell cycle, serum starvation was done for 24 h by keeping the cultures on serum free medium. The experiments bagan at 0 h by removing the serum free medium and replenishing the cell cultures with fresh medium with serum. Taxol (Paclitaxel (T 7402), Sigma, Aldrich Corp. Inc., St. Louis, MO) treatment was done at a concentration of 100 nM final concentration in the medium.

Cellular extracts used in the DNA demethylase activity assay were prepared by harvesting the cells by trypsinization and the cells were washed three times in PBS. The cell pellets were taken in DNA demethylase activity assay buffer and sonicated three times for 30 s each with a gap of 1 min in between the pulses. All the time the sonication was done on an ice bath. After sonication, the cellular debris was removed by centrifugation at 10,000*g* for 15 min. The supernatant was saved and used in the later experiments as the enzyme source. Protein estimation was done by micro-Bradford assay method.

For cell cycle analysis, the cells were harvested by trypsinization, washed three times in PBS, and fixed in 70% ethanol on ice for 30 min. The cells were washed three times with PBS. The cells were treated with DNAse free RNAse A at a concentration of 5 μg/ml PBS at 37°C for 1 h. Propidium iodide was added at a concentration of 35 μg/ml of PBS to stain the DNA for FACS analysis. FACS analysis was done in a BD scan cell sorter at the common facility of the Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia. Cell quest program was used to analyze the cells in various phases of the cell cycle. At least 10,000 cells were collected for each FACS analysis.

Preparation of Labeled Synthetic Double-Stranded DNA and the Specific Nicking Assay for the DNA Demethylase (5-MeC-DNA Glycosylase) Activity Assay

Fifty micrograms each of 37mer; (5′ GGG AGA GAG GGA AGC ^{5Me}CGG AGG AAG GAA C5MeCG GGA AAG G 3′) and 40 mer; (5′ TCC CCT TTC CCG GTT CCT TCC TC^{5Me}C GGC TTC CCT CTC TCC C 3′), synthetic oligomer

single-stranded DNA were hybridized in 6× SSC for 30 min at 88 and at 70°C for 1 h. Once again this cycle is repeated for 30 min at 88°C and another hour at 70°C. Then the hybridized double-stranded DNA was allowed to cool at room temperature for 30 min. The DNA was desalted by centricon-10 (amicon) filtration once. Further the double-stranded DNA, with a 3′ recessive end on only one strand, was purified from single-stranded DNA by separation on 20% polyacrylamide gel and eluted out of the gel [Vairapandi and Duker, 1996]. This synthetic double-stranded DNA was used in the DNA demethylase activity assay. This synthetic DNA substrate has a fully-methylated CpG site on the 25th base pair and a hemi-methylated CpG on the 12th base pair from the 3′ recessed end. DNA cleavage at the fully-methylated site will give a 25 base pair product and DNA cleavage at hemi-methylated site will give 12 base pair product.

The 3′ end of the hybridized, double-stranded DNA (5 μg) was filled in by Klenow fragment of DNA polymerase I with cold dGTP and α^{32}P or ^{35}S labeled dATP as described [Vairapandi et al., 2000a]. This reaction produced a 40 bp double-stranded DNA labeled on only one strand at the 3′ end with α^{32}P or ^{35}S labeled dATP. This labeled 40 bp DNA was again purified on 20% polyacrylamide gel and eluted out of the gel (specific activity was 1.1×10^6 ^{32}P cpm/μg DNA), and was used in the specific nicking assays for the purified 5-MeC-DNA glycosylase activity assays. Fifty thousand ^{32}P cpm were used in each reaction in a volume of 100 μl of glycosylase assay buffer (50 mM Tris-Hcl (pH 7.8), 5 mM $MgCl_2$, 10 μg/ml BSA). The incubation time was for 18 h at 37°C. After the incubation time the DNA was phenol:chloroform extracted and ethanol precipitated. The DNA was taken in 5 μl of sterilized water. Twenty microliters of formamide was added to the DNA. The DNA samples were heated at 55°C for 5 min, before it was loaded onto the denaturing 20% urea–acrylamide gel [Sambrook et al., 1989]. After the electrophoresis the gel was removed, dried, and exposed to X-ray autoradiography

Immunoprecipitation of PCNA or p300/CBP Complex and Demonstration of PCNA or p300/CBP Bound DNA Demethylase Activity

Two hundred mirogram of cellular extracts were immuno-precipitated with anti-PCNA (PC10, Santacruz Biotechnology, Santa Cruz, CA, SC-56) or p300 antibody, (N-15, Santacruz, SC-584) in PBS, with 3% non-fat milk. The immunocomplex was pulled down with protein A agarose beads (Calbiochem) and washed thrice (PBS with 0.1% Tween-20). Two final washes were given with 5-MeC-DNA glycosylase assay buffer. Later, the beads with the bound immune complex were used directly as the enzyme source, in the site specific nicking assay reaction with ^{35}S 1,00,000 cpm synthetic DNA substrate as described above. Mouse anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) was used for non-specific immuno complex preparation and used as negative control in the site specific nicking assay. After overnight (O/N) incubation at 37°C, the PCNA/p300 immune complex was separated from the reaction mix. The PCNA/p300/CBP protein associated with the beads was separated by extraction with 100 mM glycine (pH 2.5). The collected extract's acidic pH was neutralized with the addition of one third volume of 1 M Tris (pH 9). This extract was mixed with reaction mix. The pooled together mix was subjected to phenol:chloroform extraction and ethanol precipitation of DNA. This precipitated DNA was taken up in formamide buffer and analyzed on 20% urea–acrylamide denaturing gel. After the gel resolution, the gel was amplified with NENhance and dried for X-ray autoradiography. Another parallel set of immunocomplexes were also prepared to check for the presence of PCNA or p300/CBP protein by Western probing.

RESULTS

DNA Demethylase Activity in Normal Cell Line Targeted Hemi-Methylated CpG Sites, While Malignant Cells' DNA Demethylase Activity Targeted Fully-Methylated and Hemi-Methylated CpG Sites on DNA; Only the Hemi-Methylated CpG Specific Activity Is Associated With PCNA Immune Complex

We used HFL1 cell line as the non-transformed, normal cell line for our studies. LnCap cell line and BT20 line were used as malignant cell lines in the study. The synthetic 40 base pair DNA substrate with one fully-methylated CpG site on both DNA strands and one hemi-methylated CpG site on the 3′ end labeled strand was used to test the target of demethylation in normal/malignant cellular extracts in an in vitro assay. Five micrograms protein extract prepared from various cell lines was used with

^{35}S 100,000 cpm synthetic DNA in each assay as described. The results are given in Figure 1. Interestingly, HFL1 normal cellular extract exclusively used hemi-methylated CpG site as the target. At the same time malignant cell lines (LnCap and BT20) preferred the fully-methylated CpG site as the target (Fig. 1A). Thus, the target for DNA demethylation is apparently different in malignant cell lines than the normal cell lines.

We had earlier demonstrated human PCNA immune complex associated DNA demethylase activity [Vairapandi et al., 2000a]. PCNA association with DNA demethylase activity in malignant cell lines in comparison with normal cell line was tested in an in vitro assay. PCNA immune complex was prepared from 200 μg protein extract prepared from various cell lines. This immune complex was used as the enzyme source in demethylase reaction with ^{35}S 100,000 cpm synthetic DNA substrate as described in the "Materials and Methods." Interestingly, only the hemi-methylated CpG specific DNA demethylase activity was associated with PCNA in HFL1, BT20, and LnCap cells as demonstrated in Figure 1B. Fully-methylated CpG specific DNA demethylase activity seen in the malignant cell lines did not associate with PCNA immune complex. Although, PCNA immune complexes prepared from normal cell lines had more hemi-methylated site specific activity than the cancerous cell lines. Thus, malignant cell lines have a fully-methylated CpG specific DNA demethylase activity that is not associated with PCNA. Loss of PCNA association with the demethylase activity and differential target for DNA demethylation may have consequences for cellular transformation.

p300/CBP Protein Interacted With PCNA and p300 Protein Immune Complex Is Associated With the Fully-Methylated CpG Specific DNA Demethylase Activity

In normal cell cycle progression, G_0/G_1 exit is brought out by the inactivation of pRB and it's association with p300-CBP and PCNA protein [Shikama et al., 1997; Hasan et al., 2001; Ngan et al., 2001]. Recently PCNA was shown to bind the p300/CBP protein and the p300 domain responsible for that association is localized at

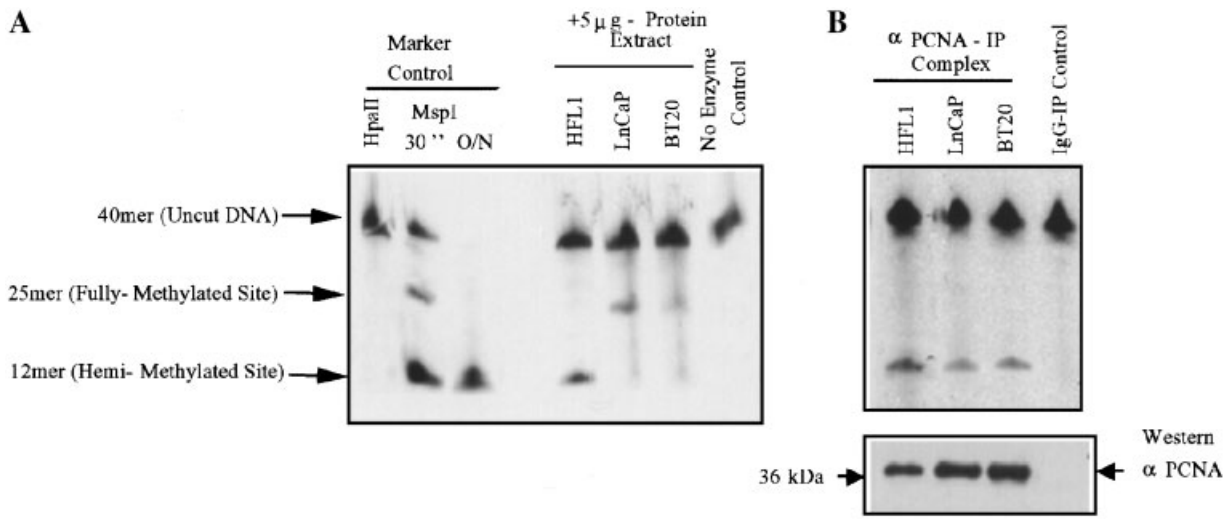

Fig. 1. Analysis of DNA demethylase activity's target site in various cell lines and the association of proliferating cell nuclear antigen PCNA with hemi-methylated CpG specific DNA demethylase activity: DNA demethylase activity was tested in various cell lines by the in-vitro 5-MeC-DNA-glycosylase activity assay with the synthetic ^{32}P radio labeled 40mer DNA substrate. Five micrograms protein extract from various cell lines was used as the enzyme source and the specific DNA nicking assay for DNA demethylase was performed as described in ''Materials and Methods.'' HpaII did not cut this DNA substrate. MspI control digestion was done to identify the 25 base and 12 base products as the markers. MspI partial digestion for 30 min resulted in both 25 base and 12 base products. 25 base DNA product represents the nick created on the fully-methylated CpG site on DNA and 12 base DNA product represents the nick created on the hemi-methylated CpG site on DNA. **A**: shows the DNA demethylase activity created nicks on the ^{32}P radio labeled 40mer DNA substrate by the added protein extract from various cell lines. HFL1 normal cell line extract created nicks on the 12 base hemi-methylated CpG DNA site and the cancerous BT20 and LnCap cell line extract predominantly created nicks on the 25 base fully-methylated CpG DNA site. **B**: shows the PCNA IP complex associated DNA demethylase activity from these cell lines. Only the 12 base hemi-methylated DNA site nicks were created by the PCNA IP complexes. The PCNA IP complex from normal HFL1 cell line created more nicks on hemi-methylated CpG site than from the cancerous cell lines.

the carboxy terminus. Recent studies on human flap endonuclease-1 activity has implicated that the p300/CBP protein is not only a component of the chromatin remodeling machinery but might also play a critical role in regulating DNA metabolic events [Hasan et al., 2002]. Also, association of CBP/p300 acetylase and thymine DNA glycosylase and DNA demethylation has been found and linked to DNA repair and transcription [Cervoni and Szyf, 2001; Tini et al., 2002].

We wanted to test the association of PCNA and p300/CBP in relation to cell cycle stages. Such an association of p300/CBP with PCNA was comparably tested in a normal cell line (HFL1) and malignant LnCap cell line in synchronized cells. The cells were synchronized at late G_1/early S phase of the cell cycle by aphidicolin treatment for 24 h. Later, the medium along with the drug was removed and fresh medium with 10% FBS was replenished and the time was set as 0 h after drug release. Then cells were harvested at 0, 4, 8, 12, and 24 h and cellular extract was prepared by RIPA lysis and extraction. Aphidicolin resulted in the enrichment of more than 80% of cells in 'late G_1/S' boundary at 0 h, 'S' phase at 4 h, G_2/M phase at 8 h and mitotic exit at 12 h, and G_0/G_1 at 24 h that was ascertained by FACS cell sorter analysis (data not provided). Consequently cellular extract preparation from these time points, represents various stages of cell cycle.

p300 immune complexes were prepared from 200 μg of cellular extract from each of these stages and anti-p300 was used for immune-precipitation along with proteinA/G beads. These p300 IP complexes were resolved on 6.5% top and 10% bottom SDS–PAGE gels and Western analysis was done after transfer of the resolved proteins onto hi-bond nitrocellulose membrane. Top part of the membrane was analyzed for p300/CBP protein and bottom part of the membrane was analyzed for PCNA protein by anti PCNA antibody. Visualization of p300/PCNA bound antibody was done by HRP coupled secondary antibodies and ECL reagents. The result for co-immuno precipitation (co-ip) of PCNA protein with anti p300 antibody is given in Figure 2. Normal HFL1 cell line and LnCap cell line demonstrated association of PCNA protein in p300 immuno complexes. In HFL1 normal cell line, PCNA is associated with p300/CBP protein most abundantly at late G_1/S phase (0 and 4 h) of the cell cycle than at later times. In malignant LnCap cell line, PCNA association with p300/CBP protein was prominent at late G_0/G_1 (24 h) and comparable equal association of p300/CBP with PCNA in all other phases (Fig. 2A, Top). The same experiment was repeated with reciprocal PCNA immuno-complex preparation from 200 μg of cellular extract from each cell cycle stages of HFL1 or LnCap cells. These reciprocal results are given in (Fig. 2A, Bottom). This reciprocal experimental approach resulted in the demonstration of p300/CBP association with PCNA immune complex at all the stages of cell cycle. Thus, the association of p300/CBP with PCNA becomes clear from these experiments. Also, it becomes apparent that p300/CBP association with PCNA is preferred at late G_1/S phase (0 and 4 h) in the normal cells, whereas malignant cells have disturbed association of p300/CBP with PCNA which happens preferentially at G_0/G_1 (24 h) stage of the cell cycle.

Since PCNA also associated with p300/CBP protein, we wanted to address the question of the ability of DNA demethylase activity to associate with p300 protein. In an in vitro assay, p300 protein's association with DNA demethylase activity in malignant cell lines in comparison with normal cell line was tested. p300 immune complex was prepared from 200 μg protein extract from various cell lines. This immune complex was used as the enzyme source in demethylase reaction and the results are given in Figure 2B. Interestingly, the fully-methylated CpG specific DNA demethylase activity from the malignant cell lines was seen associated with p300 immune complex and the hemimethylated CpG specific DNA demethylase activity from the normal HFL1 cell line was not. Thus, malignant cell lines have a fully-methylated CpG specific DNA demethylase activity that is associated with p300/CBP and not PCNA. Loss of PCNA association and the ability of p300/CBP protein to gain control of fully-methylated CpG site specific DNA demethylase activity may have consequences for cellular transformation.

CDKI Proteins $p21^{waf1}$/Gadd45α/Gadd45β (Myd118) Have Inhibitory Effect on PCNA Associated Hemi-Methylated CpG Specific DNA Demethylase Activity but not on the p300/CBP Associated Fully-Methylated CpG Specific DNA-Demethylase

The association of human PCNA with hemimethylated CpG specific DNA demethylase

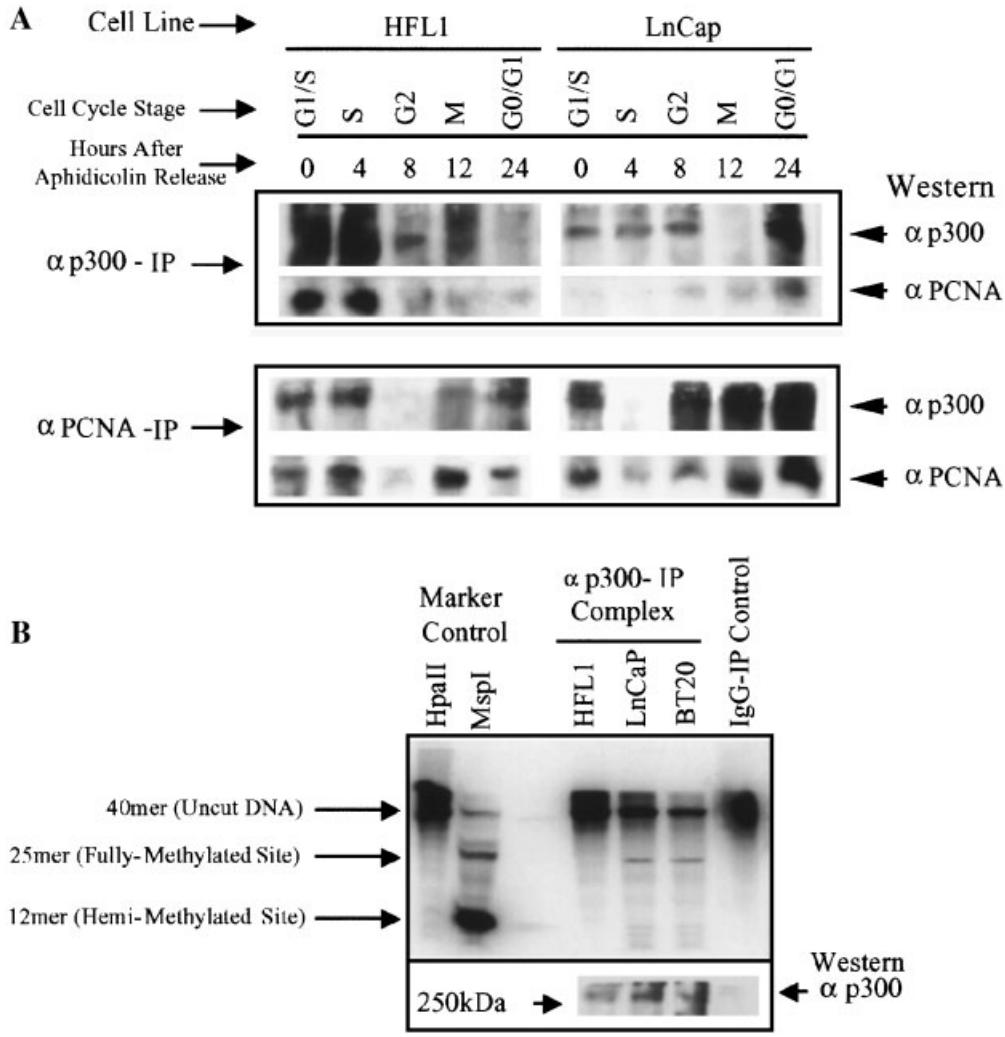

Fig. 2. PCNA IP complex association with p300 protein and the association of p300 protein with fully-methylated CpG specific DNA demethylase activity: Association of PCNA (proliferating cell nuclear antigen) with p300 protein was tested by co-immuno precipitation (co-ip) method as described in ''Materials and Methods.'' Cell extracts were prepared from aphidicolin synchronized cell lines. Cell stage at the harvesting time was ascertained by parallel FACS analysis of a representative population. **A**: shows the result from co-ip and Western results for PCNA association with p300 protein. **Top part** depicts p300 IP Western results and **bottom** part depicts PCNA IP-Western results. The cells were harvested at the times marked and the corresponding cell cycle stage of the harvesting time is also marked on the top. As can be seen PCNA interacted and co-ip with p300 protein at all the time points. This interaction is very prominent at late G_1/S and 'S' phase (0 and 4 h) in normal HFL1 cell line, while cancerous LnCap cell line had abundant interaction of PCNA with p300 protein at G_0/early G_1 stage (24 h). Same results were also obtained with BT20 cancerous cell line. **B**: shows the p300 protein immuno complex associated DNA demethylase activity. p300 protein immune complex was prepared from various cell lines and used as enzyme source in the specific nicking assay for DNA demethylase activity as described in ''Materials and Methods.'' As can be seen from this figure, p300 immune complex from HFL1 normal cell line did not have any DNA demethylase activity and did not produce any nicks on the DNA substrate. While the p300 immune complex prepared from cancerous LnCap and BT20 cell line does have DNA demethylase activity and created nicks on the 25 base fully-methylated CpG specific DNA sites and minor nicks at the 12 base site.

activity and p300/CBP protein's association with fully-methylated CpG specific DNA demethylase activity is demonstrated confirmatively (Figs. 1 and 2). Since PCNA directly interacted with CDKI proteins ($p21^{waf1}$, Gadd45α, and Gadd45β) [Vairapandi et al., 1996, 2000b, 2002] we wanted to test the effect of these proteins on the DNA demethylase activity in various malignant cell lines in comparison with normal cell line in an in vitro assay. The results are given in Figure 3.

CDKI inhibitors $p21^{waf1}$, Gadd45α, and Gadd45β (MyD118) were bacterially expressed and purified as described [Vairapandi et al.,

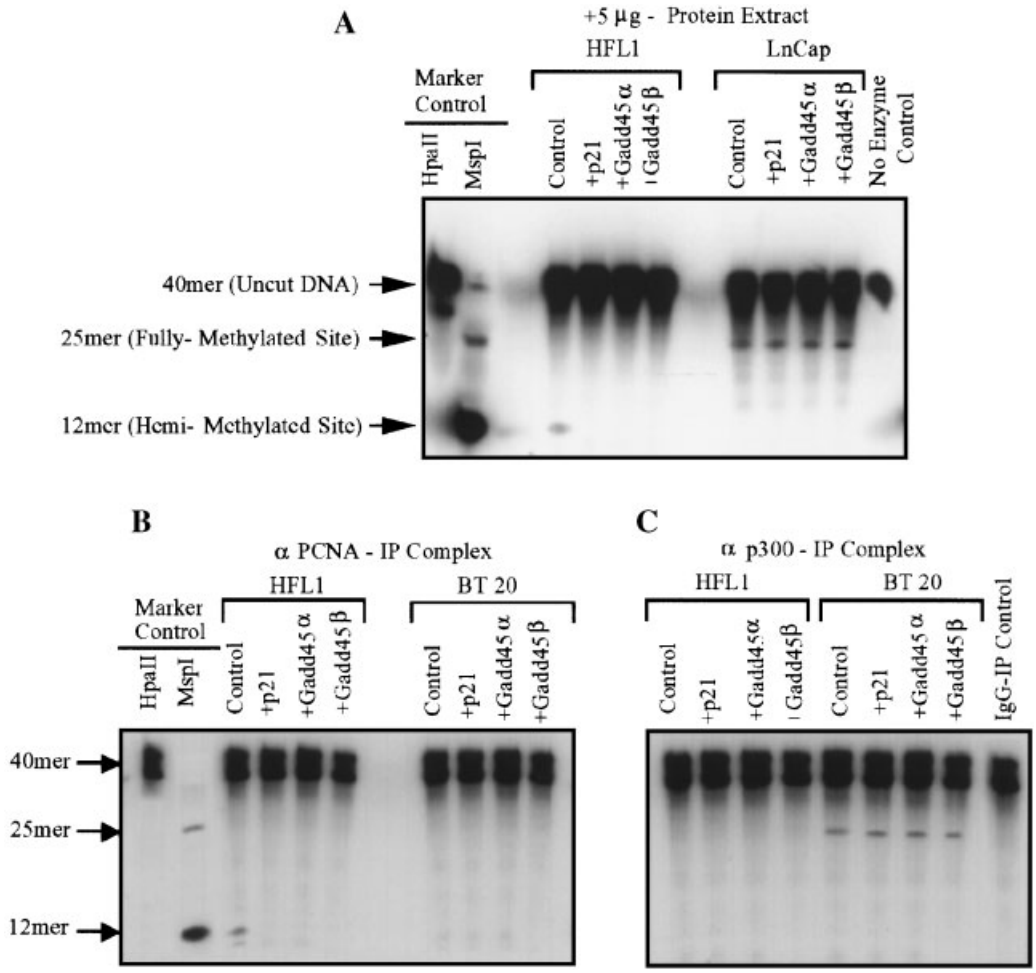

Fig. 3. Effect of CDKI proteins {p21, Gadd45α, and Gadd45β (MyD118)} on hemi- and fully-methylated CpG site specific DNA demethylase activity: PCNA interacting CDKI proteins (p21, Gadd45α, and Gadd45β) effect on fully- and hemi-methylated CpG specific DNA demethylase activity was assessed by in vitro site specific DNA nicking assay as described in ''Materials and Methods.'' 100 ng of the bacterially expressed recombinant inhibitor proteins were incubated with the cellular extracts in the DNA demethylase activity assay. **A**: shows the effect of CDKI proteins on DNA demethylase activity mediated by the cell extract. Normal cell line HFL1 extract created 12 base hemi-methylated CpG site specific nick on the DNA substrate and the addition of CDKI proteins inhibited the creation of this nick and demonstrate the inhibitory effect of CDKI proteins on hemi-methylated CpG specific DNA demethylase activity. Cancerous cell line LnCap extract created prominent 25 base fully-methylated CpG site specific nick on the DNA substrate and the addition of CDKI proteins did not inhibit the creation of this nick. This demonstrated the inability of CDKI proteins on fully-methylated CpG specific DNA demethylase activity. To confirm this observation of CDKI proteins ability to inhibit hemi-methylated CpG specific DNA demethylase but not the fully-methylated CpG specific DNA demethylase activity, PCNA/p300 protein IP complexes were prepared and used as the enzyme source in the DNA demethylase activity assay along with CDKI proteins as described in ''Materials and Methods.'' **B**: shows the PCNA IP complex associated DNA demethylase activity in HFL1 and BT20 cell lines and the effect of CDKI proteins on this activity. PCNA IP complex from normal HFL1 cell line created 12 base hemi-methylated DNA site specific nicks on the DNA substrate which were abolished by the addition of PCNA interacting CDKI proteins. PCNA IP complex from cancerous BT20 cell line had very minimal 12 base product generation which was not inhibited by the addition of CDKI proteins. **C**: shows the p300 protein IP complex associated DNA demethylase activity in HFL1 and BT20 cell lines and the effect of CDKI proteins on this activity. p300 protein IP Complex from normal HFL1 cell line did not create any nicks on the DNA substrate. Whereas, p300 protein IP complex from cancerous BT20 cell line created nicks on the 25 base fully-methylated CpG specific site on the DNA substrate which was not at all inhibited by the addition of CDKI proteins.

2000b]. Five micrograms protein extract prepared from various cell lines were used as the enzyme source in demethylase reaction with ^{35}S 100,000 cpm synthetic DNA substrate along with 100 ng of the various inhibitors. The CDKI proteins were incubated with the enzyme extract for 30 min at room temperature before the addition of the synthetic DNA substrate to begin the DNA demethylase activity assay. The results of this experiment are given in

Figure 3A. As can be seen from this figure, CDKI proteins ($p21^{waf1}$, Gadd45α, and Gadd45β) that can also interact with PCNA completely abolished the hemi-methylated CpG specific DNA demethylase activity in HFL1 normal cellular extract. While, the fully-methylated CpG specific DNA demethlyase activity seen in the malignant LnCap cell line was resistant to these PCNA interacting CDKI proteins ($p21^{waf1}$, Gadd45α, and Gadd45β).

We wanted to confirm this very significant observation that G_1/G_2 restriction point regulatory CDKI proteins' ability to inhibit normal cellular DNA demethylase activity specific for hemi-methylated CpG specific DNA, but not the malignant cellular DNA demethylase activity that is specific for fully-methylated CpG specific DNA. So p300/PCNA immune complexes were prepared from 200 μg protein extract prepared from various cell lines. These immune complexes were used as the enzyme source along with 100 ng of these CDKI proteins as described earlier, in demethylase reaction with ^{35}S 100,000 cpm synthetic DNA substrate. After O/N incubation at 37°C, the p300/PCNA immune complex reacted DNA were processed as described before and were taken up in formamide buffer and analyzed on 20% urea–acrylamide denaturing gel. After the gel resolution, the gel was amplified with NENhance and dried for X-ray autoradiography. The results are given in Figure 3B,C.

Figure 3B demonstrates that PCNA immune complex can associate with hemi-methylated CpG specific DNA demethylase activity seen in HFL1. PCNA immune complex can not associate with the fully-methylated CpG specific DNA demethylase activity seen in LnCap cellular extract. Figure 3B also confirmatively demonstrates that the PCNA associated hemi-methylated CpG specific DNA demethylase activity in HFL1 normal cell line is inhibited by the CDKI proteins that interact with PCNA to regulate G_1/G_2 restriction points. Very little amount of hemi-methylated CpG specific DNA demethylase was seen associated with the PCNA immune complex from the malignant cell line and this was not seem to be inhibited by the CDKI proteins addition. Figure 3C demonstrates that p300/CBP immune complex can associate with fully-methylated CpG specific DNA demethylase activity seen in BT20 cells. p300 immune complex from HFL1 cell lines did not associate with DNA demethylase activity. Figure 3C also confirmatively demonstrates that the p300/CBP protein associated fully-methylated CpG specific DNA demethylase activity in BT20 malignant cell line is not at all inhibited by the added CDKI proteins.

Cancerous Cell Lines Demonstrate Shortened G_0/G_1 Resting Time Than Normal Cell Line

The normal cell lines (HFL1) displayed DNA demethylase activity specific for only hemi-methylated CpG specific DNA and this activity is amenable to the role of G_1/G_2 regulating CDKI proteins. At the same time, the malignant and cancerous cell lines displayed fully-methylated CpG specific DNA demethylase activity and are resistant to the CDKI proteins. So, we wanted to test any possible variations in the cycling time of these various cell lines by cell cycle analysis. Initially cell cultures were synchronized at G_0 phase of the cell cycle by over night serum starvation method. After serum starvation, the medium was replenished with 10% FBS with the addition of taxol at 100 nM final concentration. Taxol is a potent mitotic blocker and will not let the cells to cross the G_2/M and enter into G_0/G_1 stage. This treatment let us to see the serum starved cells move from G_0/G_1 to 'S' phase of the cell cycle without any ambiguity of G_2/M falling back into G_0/G_1. The serum restoration time was set as 0 h and cells were harvested after 8, 12, 18, 24, or 36 h. The cells harvested at various times were nuclear DNA stained with propidium iodide and were subjected to FACS analysis. The results are given in Figure 4. As seen from this Figure 4, more than 70% of the serum starved normal human fibroblast cell line rested and stayed at G_0/G_1 for 12 h, before they moved out of G_1 phase to enter into 'S' phase of the cell cycle by 18 h. Whereas cancerous cell lines demonstrate shortened G_0/G_1 resting time. LnCap cell line exited G_0/G_1 after 12 h. BT20 cell line demonstrate less than 8 h resting time at G_0/G_1, before they moved out of G_1 phase to enter into 'S' phase of the cell cycle. This shortened G_0/G_1 resting time, demonstrated clearly that the malignant cell lines have altered G_0/G_1 check point apparatus. Thus, the cell cycle analysis results point to abnormal perturbance of G_0/G_1 restriction point in the malignant cell lines than the normal cell lines.

These same cell lines were tested further for any variation in the length of time, they spend in either 'S' or G_2/M phase of the cell cycle. To

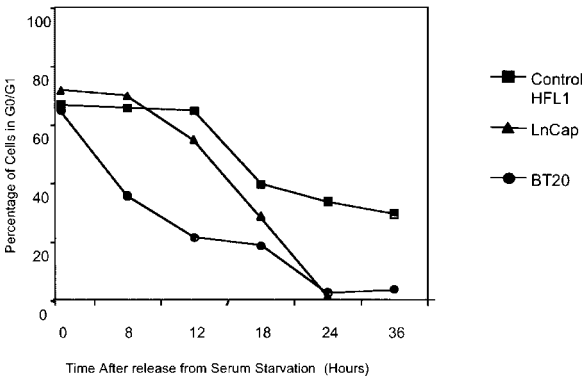

Fig. 4. Cell cycle study to estimate G_0/G_1 resting time in various cell lines by FACS analysis; for cell cycle analysis, cell cultures were synchronized at G_0 phase of the cell cycle by overnight (O/N) serum starvation method. After serum starvation, the medium was replenished with 10% fetal bovine serum (FBS) complemented with 100 nM taxol. Taxol is a potent mitotic blocker and will not let the cells to cross the G_2/M boundary and enter into G_0/G_1 stage. This treatment allows us to see the serum starved cells move from G_0/G_1 to 'S' phase of the cell cycle without any ambiguity of G_2/M falling back into G_0/G_1. The serum restoration time was set as 0 h and cells were harvested after 8, 12, 18, 24, or 36 h. The cells harvested at various times were nuclear DNA stained with propidium iodide and were subjected to FACS analysis. The cell population in G_0/G_1 stage was analyzed and calculated by the cell quest program and the results are given as a line graph. As can be seen from this figure, all the cell lines contained more than 70% cells in G_0 state at 0 h. The normal HFL1 cell line maintained this 70% G_0/G_1 population up to 12 h and only by 18 h they began to exit out of G_1 state into 'S' phase of the cell cycle. The cancerous LnCap cell line began to exit out of G_1 phase into 'S' phase by 12 h and BT20 cell line had the shortest G_0/G_1 resting time and began to exit out of G_1 phase into 'S' phase even by 8 h.

analyze the time spent on 'S' and G_2/M phase of the cell cycle, these cell lines were synchronized at the boundary of G_1/S by aphidicolin treatment. Aphidicolin treatment at 1 μg/ml of tissue culture medium, arrests eukaryotic cells at the border of G_1 exit or entry into 'S' phase. This treatment is completely reversible by simple washing off of the medium and replacement of fresh medium. The fresh medium with replacement time was set as 0 h and the cells were harvested at 0, 4, 8, 12, and 24 h, stained with propidium iodide and subjected to FACS analysis.

Aphidicolin treatment synchronized more than 95% cells at the G_1/S boundary (0 h) in the normal HFL1 cell line whereas malignant cell lines demonstrated about 90% of the cells at G_1/S boundary. All the G_1/S boundary arrested cell lines moved into the 'S' phase at 4 h after release from drug arrest; progressed into 'G_2/M' phase at 8 h; >50% of the G_2/M population exited M phase at 12 h to enter into G_0/G_1 phase; more than 85% of the cells are seen in the G_0/G_1 phase of the cell cycle at 24 h time point (data not given). These results demonstrate that all the normal and malignant cell lines have almost equal time to spend in the S and G_2/M phase of the cell cycle.

DISCUSSION

The mechanism for DNA demethylation remains a controversial field for very long. We were first to identify the human demethylase activity as a 5-MeC-DNA glycosylase [Vairapandi and Duker, 1993] which was contested [Steinberg, 1995]. Later, new mechanisms for demethylation of DNA were proposed. One mechanism involved RNA and a possible ribozyme mediated DNA demethylation [Weiss et al., 1996], and was contested [Swisher et al., 1998]. Another mechanism involving a demethylase activity that transformed methylated cytosine bases to cytosine bases had been identified and cloned [Bhattacharya et al., 1999]. That mechanism was also contested by two groups, which tried but were not able to reproduce their findings [Ng et al., 1999; Wade et al., 1999]. Later the transformation of methylated cytosine bases to cytosine was asserted again in an assay which relied on an incubation time of 48 h [Detich et al., 2002].

We continued in our efforts to purify the 5-MeC-DNA glycosylase activity and demonstrated the glycosylic mechanism of 5-MeC removal from DNA, with well-defined synthetic substrates and demonstrated the activity was free from contaminating nucleases [Vairapandi and Duker, 1996; Vairapandi et al., 2000a]. This mechanism was the only corroborated mechanism with other published reports where removal of 5-MeC as a free base from DNA was by the enzyme 5-MeC-DNA glycosylase. This activity was found in human HeLa cells, chick embryos and differentiating mouse myoblasts [Jost and Jost, 1994, 1995; Fremont et al., 1997]. We were the first to demonstrate human DNA demethylase activity as 5-MeC-DNA glycosylase with associated abasic site nicking activity that resembled "base excision DNA repair activity" in it's action mechanism. Also, we had demonstrated the association of this activity with small RNA moieties and the nuclear protein, PCNA [Vairapandi et al., 2000a].

In this report, we have demonstrated that the DNA demethylase activity in normal cell lines have specificity for hemi-methylated CpG islands. We have also shown the association of hemi-methylated CpG site specific DNA demethylase activity with PCNA which is prevalent in normal cell line (Fig. 1). Whereas the malignant cell lines prefer totally different fully-methylated CpG islands as the substrate for DNA demethylase activity, and the co-ip studies demonstrated the association of this totally different DNA demethylase activity to p300/CBP protein (Fig. 2B). PCNA did not associate with the fully-methylated CpG specific DNA demethylase activity from the malignant cell lines. Also PCNA interacting CDKI proteins ($p21^{waf1}$, Gadd45α, and Gadd45β) have the capability to completely inhibit the PCNA associated DNA demethylase activity (hemi-methylated CpG specific) seen in the normal cell line. Cancerous cell lines have variable fully-methylated CpG specific DNA demethylase activity, that did not bind to PCNA protein, but did bind to p300/CBP protein and are resistant to the inhibitory effect of CDKI proteins (Fig. 3). Thus the DNA demethylase activity seen in the cancerous cell lines are totally different in their specificity and are operative out of the reach of normal cell cycle proteins like PCNA, $p21^{waf1}$, and Gadd45 proteins' which have a regulatory role in the cell cycling.

Our work on the cell cycle study of normal and cancerous cell lines demonstrate comparably equal time in the 'S' and 'G_2/M' phases of the cell cycle. Malignant cell lines do, however, demonstrate shortened G_0/G_1 resting time than do normal cell lines (Fig. 4). Thus, there is a possibility that this coincidence of short G_0/G_1 restriction time and varied CpG demethylation specificity may point to a regulatory lapse in the G_2/G_1 restriction period which resulted in the short G_0/G_1 resting time.

PCNA associated DNA demethylase activity creates single-strand breaks on DNA by 5MeC-DNA glycosylase action mechanism [Vairapandi and Duker, 1993; Vairapandi et al., 1996, 2000a]. PCNA along with DNA demethylase action mediate demethylation of DNA that may be followed by the single-strand DNA breaks that open and unwind DNA, in order to provide optimal transcription and replication conditions. This may mark the early events of the G_0/G_1 stage of the cell cycle that leads to the later G_1 events of the inactivation of pRB and it's association with p300-CBP and PCNA protein. This may signal the chromatin remodeling events to let the cell to exit the G_1 stage and enter the S phase.

Our PCNA and p300 protein co-ip studies from the synchronized cellular extracts pointed to the probability of hurried association of p300/CBP with PCNA at early G_0/G_1 stage in malignant cells rather than at late G_1/S phase as in the normal cells (Fig. 2A,B). This may mean an earlier, p300/CBP accessibility to PCNA and associated DNA demethylase activity that may lead to earlier chromosome remodeling of condensed chromosomes from the end of mitotic exit, to relaxed chromosomal DNA at an earlier G_0/G_1 stage.

Hemi-methylated CpG specific DNA demethylase activity only has been identified in normal cell lines whereas fully-methylated CpG specific DNA demethylase activity has been found to be predominant in cancerous cell lines. p300/CBP protein associated fully-methylated CpG specific DNA demethylase activity has the potential to make double-strand breaks and also open-up hitherto inaccessible regions on DNA to oncogenes and many cell cycle regulatory proteins.

DNA demethylase activity (5-MeC-DNA glycosylase) towards hemi-methylated DNA would result in a single-strand DNA break and totally unmethylated DNA CpG site, with loss of the methylation pattern. This is acceptable erasure of methylation pattern in terminally differentiated cell systems which are destined to apoptose finally. By contrast, glycosylic removal of 5-MeC from one strand of fully-methylated DNA would result in single-strand DNA break and formation of hemi-methylated DNA preserving the methylation pattern. These hemi-methylated CpG islands can be re-methylated by DNMT1 activity after DNA replication and also by recombinatory events.

While glycosylic removal of 5-MeC from both strands will lead to double-strand DNA break and formation of unmethylated DNA and erasure of methylation pattern. These unmethylated CpG islands can not be re-methylated by the DNMT activity which wholly depends on the prior methylation patterns to complete the methylation [Bestor et al., 1988]. These unmethylated CpG islands can only be re-methylated by replicative recombinatory events and sister chromatid exchanges happening at the G_2/M stage of the cell cycle which wholly defend on the double-strand DNA breaks. These

events probably will be accomplished with the help of p300/CBP or unknown protein complexes associated DNA demethylase activity.

Cancerous cell lines have abundant fully-methylated CpG specific DNA demethylase activity, because these cell lines have two to three times more G_2/M population than the normal cycling cells at any time. Although, p300/CBP protein associated with PCNA protein, our results did not demonstrate significant association of hemi-methylated CpG site specific DNA demethylase activity in p300 immune complex that was inhibited by the CDKI proteins. Also, the PCNA immune complex did not have any fully-methylated CpG specific DNA demethylase activity. This may be a result of compartmentalized association of PCNA and p300/CBP in separate complexes with DNA demethylase activity. This other alternative reason might explain that the cancerous cell lines lost their ability to compartmentalize this double-strand break creating activity from the single-strand breaking activity or altogether this activity may be a new transformed activity brought in by oncogenesis.

DNA demethylase activities may play a vital role at G_1/G_2 exit stages of the cell cycle, because of it's capability to produce DNA strand breaks which are intrinsic in the carry over of transcription, replication, and recombination tasks in the regulated stages of cell cycle. Both kinds of target specificity are needed for the mammalian cell to function in a regulated way to fulfill all the tasks in the completion of one cell cycle and to preserve the integrity of the genome. The compartmentalization of these two activities to specific stages of the cell cycle and their regulation to be functional at specific periods within cell cycle is paramount for the cells to cycle in normal growth.

Alterations in DNA methylation are now widely recognized as a contributing factor in human tumorigenesis. Recent research implicates the failure of DNA demethylation and hypermethylation of DNA promotor regions for the transcriptional silencing of many tumor suppressor genes [Szyf et al., 2000; Ehrlich, 2002; Jones and Baylin, 2002; Lynch et al., 2002]. Our results in this report point to the presence of variable DNA demethylase activity in malignant cell lines that are not regulated by normal cell cycle regulatory proteins like PCNA, $p21^{waf1}$, and Gadd45 proteins. This may reflect the presence of altered DNA demethylase activity or altogether different DNA demethylase activity introduced into the cellular system by oncogenesis. This may partially explain the variable reported DNA demethylase activities so far and the difficulty in identifying and cloning the real DNA demethylase protein. Much more focused effort is needed to definitively identify and clone the DNA demethylase activity that will prove as an important tool to define the process of oncogenesis and to develop therapeutical aids to prevent and treat cancer.

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