

The nuclear 16-kD protein methylation increases in the early period of liver regeneration in a hepatectomized rat

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Abbreviations: ADMA, N^GN^G-dimethyl(asymmetric)arginine; AdoMet, S-adenosyl-L-methionine; G-protein, GTP-binding protein; GTP, guanosine triphosphate; hnRNP, heterogeneous nuclear ribonucleoprotein; MMA, N^G-monomethylarginine; PRMT, arginine methyltransferase; SDMA, N^GN^G-dimethyl(symmetric)arginine

Abstract

Methylation events play a critical role in various cellular processes including regulation of gene transcription and proliferation. We observed that methyltransferase activity underwent time-dependent changes in the cytosol of the rat hepatocytes upon partial hepatectomy. However, any change in the methylation of nuclear proteins is not clear during hepatocyte proliferation. The nuclear fraction possesses basal level of methyltransferase to catalyze methylation of several proteins ranging from 7 to 70 kD prior to any hepatectomy. The specific p16 (16 kD) band was transiently and heavily methylated post 1 day hepatectomy, and then became non-detectable, but not in the control liver. Methylation of p16 band was completely inhibited by exogenously added histones, particularly 2AS, 1, 2A and 2B subtypes. The methylated p16 protein remains stable in either acid or alkali-induced demethylation conditions, indicating that

methylation is not likely to occur on isoaspartyl or C-terminal cysteinyl residues. Exogenous addition of non-hydrolyzable GTP caused a dose-dependent suppression of a p16 methylation suggesting that G-proteins might play a role as an endogenous methylation inhibitor *in vivo*. Taken together, we have identified the proliferation event associated-methylation of the nuclear p16 protein in the hepatocytes undergoing liver regeneration.

Keywords: histone; nuclear methyl acceptors; protein methylation; regenerating rat liver

Introduction

Methylation has been shown to play important roles in several biological phenomena occurring in prokaryotes as well as eukaryotes. Methylation is one of the major posttranslational or posttranscriptional modifications of macromolecules including protein, RNA and DNA. Protein methylation is found in the residues, arginine, aspartate, glutamate, lysine and C-terminal cysteine, which has been shown to occur in a variety of cellular processes including protein trafficking, protein-protein interaction, and various signal transduction pathways leading to cellular proliferation (Paik and Kim, 1980; McBride and Silver, 2001). Methylation of proteins is catalyzed by highly specific methyltransferases which transfer a methyl group from S-adenosyl-L-methionine (AdoMet) to the guanidino nitrogen of arginine residues, generating N^G-monomethylarginine (MMA), N^GN^G-dimethyl(asymmetric)arginine (ADMA) and N^GN^G-dimethyl(symmetric)arginine (SDMA) (Paik and Kim, 1980; Gary *et al.*, 1996), or to lysine to produce N^ε-mono-, N^ε-di-, and N^ε-trimethyllysine (Duerre *et al.*, 1977), or to aspartate or glutamate to produce alkali labile ester linkages (Tan *et al.*, 1991), or to C-terminal farnesylated cysteine to produce carboxyl methyl ester (Backlund, 1992).

Proliferation occurs through series of changes in which expression of numerous genes allow progression into the cell cycle. Earlier studies demonstrated that the activity of protein arginine methyltransferase (PRMT) was directly correlated with the degree of proliferation and thus, it was found to be elevated in highly proliferating tissues such as regenerating liver (Kwon *et al.*, 2004), developing fetal brain, rapidly growing hepatomas and continuously dividing HeLa cells (Paik *et al.*, 1975; Paik and Kim, 1980). Indeed,

a 20-kDa protein in the cytosol was found to be a major substrate of methyltransferases activated in proliferating mammalian cells. For example, exponentially growing human cancer cell lines, such as HCT-48, HeLa cells, A549, and Hep G2, all showed increased level of methylation in this 20-kD protein as compared to their normal counterparts, suggesting that increased methylation may be associated with cellular proliferation and transformation (Park *et al.*, 1997; Gu *et al.*, 1999). In addition, we showed recently that methylation of this cytosolic 20-kDa protein was increased in a more physiologic setting, such as proliferating hepatocytes, due to elevated methyltransferase activity, and that histones were likely to play some regulatory roles in this process (Kwon *et al.*, 2004).

Since our previous studies focused on the methylation of cytosolic proteins in association with hepatocyte proliferation, an immediate question arises if the methylation status changes in the nuclear proteins during the course of cell proliferation. Modification of nuclear proteins is important as it can be closely related to gene transcription, which occurs in the nucleus preceding cell proliferation. Our study now finds that nuclear proteins are also subject to methylation by the methyltransferases activated during the course of liver regeneration. In contrast to the cytosolic fraction, which showed persistent methylation of a 20-kD band up to 7 days following hepatectomy, methylation of nuclear substrates do not show any persistent changes in methylation. Rather their level of methylation was more or less consistent before or after hepatectomy. One exception to this was the appearance of a 16-kD protein heavily methylated 1 day following hepatectomy, which then disappeared rapidly within the next 3 days. Our data describing the characteristics and specificity of the methyltransferases regulating methylation of this 16-kD protein proposes the importance of methylation events in the process of liver regeneration.

Materials and Methods

Reagents

S-adenosyl-L-[methyl-³H]methionine (³H]AdoMet; 60 Ci/mmol) was purchased from Amersham Pharmacia Biotech Inc. (Buckinghamshire, England). Calf thymus histone II-AS (bulk histone prepared by extraction in 1 M NaCl, precipitation in water, followed by acid extraction, dialysis and lyophilization), myelin basic protein, cytochrome C, alcohol dehydrogenase, α -lactalbumin, carbonic anhydrase, γ globulin, bovine serum albumin, GTP γ S and S-adenosyl-L-homocysteine were purchased from Sigma Chemical Co. (St. Louis, MO). Calf thymus histones 1, 2A, 2B, 3 and 4 were

purchased from Roche Applied Science (Mannheim, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and LMW electrophoresis calibration kits were purchased from Bio-Rad Laboratories (Hercules, CA).

Partial hepatectomy

Median and left lateral lobes (about 70% of liver) of 6 weeks old Sprague Dawley rats were surgically removed according to the method approved by Institutional Review Board (day 0). Rats were then sacrificed at day 1, 3, 5, 7, 14, 21 and 28 following hepatectomy and the remaining (regenerating) liver (right lateral and caudate lobes) was harvested for analysis (Waynforth and Flecknell, 1992). At each time point, three rats were used for analysis.

Preparation of cytosolic extracts

All procedures were carried out at 4°C unless otherwise stated. Harvested liver was cut into small pieces and homogenized in 4 volumes of 0.32 M sucrose containing 5 mM potassium phosphate (pH 7.6), 5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol (DTT), 1 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 100,000 *g* for 60 min, and the supernatant was used as crude cytosolic extracts which contain endogenous enzymes and substrates of the liver (Gu *et al.*, 1999).

Preparation of nuclear extracts

5 g of rat liver was minced and placed in 10 ml lysis buffer (0.25 M sucrose, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF, 10 mM Tris-HCl, pH 7.4). The minced liver was homogenized with 5-10 strokes of a serrated Teflon pestle. Homogenate was overlaid on to concentrated sucrose solution (2 M sucrose for control liver or 1.7 M sucrose for regenerating livers, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF, 10 mM Tris-HCl, pH 7.4), and centrifuged at 23,000 rpm at 4°C for 30 min on the SW 28 rotor (Beckman). The pellet was resuspended with lysis buffer and used as nuclei fraction (Spector *et al.*, 1998).

In vitro methylation assay

Methylation reaction was carried out by adding 5 μ Ci of [³H]AdoMet into 50 μ g of crude extracts of rat liver (nuclei or cytosol) in a total volume of 50 μ l. Where indicated, 20 μ M of bulk histones (histone II-AS, Sigma) or their individual subtypes, histone 1, 2A, 2B, 3 or 4, were added to the mixture prior to assay. Alternatively, 20 μ M of myelin basic protein, cytochrome C, alcohol dehydrogenase, α -lactalbumin, car-

bonic anhydrase, bovine χ globulin or bovine serum albumin was added to the reaction mixture. S-adenosyl homocysteine, which specifically inhibits AdoMet-dependent methylation, was added at 0.8 mM to the reaction mixture to inhibit methyltransferase activity. Reaction was carried out at 37°C for 30 min, and stopped by adding 5×SDS-PAGE sample buffer (Park *et al.*, 1997). Protein concentration was determined according to Bradford method (Bradford, 1976).

Where indicated, the methylation reaction was terminated by addition of 1 mM S-adenosyl-L-homocysteine (a transmethylation inhibitor). The stability of [³H]AdoMet incorporation into the nuclear methyl acceptors was tested either by treating extracts with 1N HCl, pH 3-4, or 1 N NaOH, pH 10-11, for 60 min at 37°C.

Methylation analysis by SDS-PAGE and autoradiography

The reaction mixture dissolved in SDS-PAGE sample buffer was loaded onto 15% polyacrylamide gels and run under constant current (20 mA) (Laemmli, 1970). The gel was stained with Coomassie Blue, soaked in Amplify solution (NAMP-100, Amersham), dried and exposed to Hyperfilm™ MP (Amersham) diagnostic film at -70°C for 7 days. The density of radioactive bands was measured using Fujix-Bio-Imaging Analyzer BAS2500 (Fuji Photo Film).

Results

Appearance of a novel methylation product in the early period of rat liver regeneration

Upon partial hepatectomy in a rat, the residual hepatic parenchymal stem cells rapidly proliferate and differentiate to reach the normal size of liver within 30 days (Figure 1A, Waynforth and Flecknell, 1992). In our experiment, weight of the remaining liver (right lateral and caudate lobes) increased following partial hepatectomy and reached the normal mass by 28 days. By comparison, weight of right lateral and caudate lobes from age-matched non-hepatectomized mice did not change significantly over time. Similarly, weight of entire liver from non-hepatectomized mice did not change significantly. These data indicate that the remaining liver proliferated and differentiated to restore its original mass.

To examine if regenerating liver cells undergo changes in methylation status, crude cytosolic and nuclear extracts were prepared at various days post hepatectomy and subjected to an *in vitro* methylation assay. Intriguingly, the densities of regenerating nuclei were found to be lower than those of control nuclei, so regenerating nuclei could be centrifuged down

through 1.7 M, but not 2.0 M. As shown previously (Kwon *et al.*, 2004), the major band in the cytosol fraction following an *in vitro* methylation assay migrated at 20 kD (Figure 1). Upon hepatectomy, methylation of this cytosolic 20-kD protein increased more than 5 fold (compare C at day 0 and 1). Addition of bulk histone to the assay completely inhibited methylation of the 20-kD protein (C+H at day 0 and 1), suggesting that histones and this 20-kD protein compete for the same methyltransferase. Interestingly, exogenously added bulk histone also became methylated appearing as a smeary band around 16 kD. In contrast to cytosolic fraction, more bands (~70-kD, ~24-kD, ~18-kD, and ~7-kD) showed basal level of methylation even prior to hepatectomy in the nuclear fraction (Figure 1, see N at day 0). 1 day following hepatectomy, the level of methylation of all three bands remained relatively similar as compared to that before the hepatectomy. However, a new band running around 16 kD was detected as a major methylated protein (compare N at day 0 and 1). Methylation of a band running at 70 kD appeared to be reduced in this particular gel, however, it was not reproducibly detected in the other experiments. Likewise, other methylated product of about 24-kD, 18-kD and 7-kD proteins shown as a heavy band in the gel seemed not to be affected by the process of liver regeneration (Figure 1).

Since histones are abundant nuclear proteins and also run at 16 kD, the 16-kD protein newly appeared

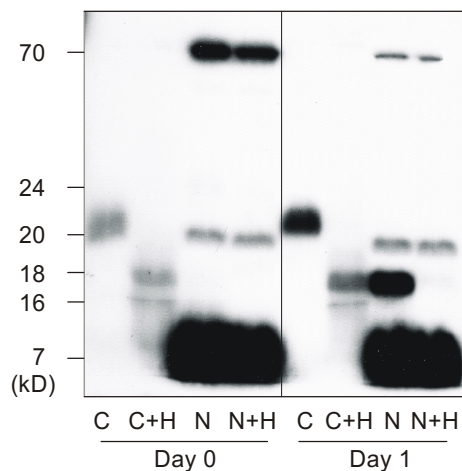


Figure 1. The status of methylation in the regenerating liver following partial hepatectomy. Cytosolic and nuclear extracts were prepared from liver cells prior to or 1 day following partial hepatectomy and subjected to methylation assay as described in the Materials and Method section. Where indicated, 20 μ M bulk histone was added to the reaction mixture. C, N, H denote cytosol, nucleus, and histones, respectively.

to be methylated following hepatectomy could likely be histones. Interestingly, however, addition of exogenous bulk histone did not result in or enhance methylation of added histones, but rather blocked methylation of a 16-kD protein (Figure 1). These data suggest that a 16-kD band may not likely be histones. Since histones can compete with a 16-kD protein for methylation, methyltransferases responsible for meth-

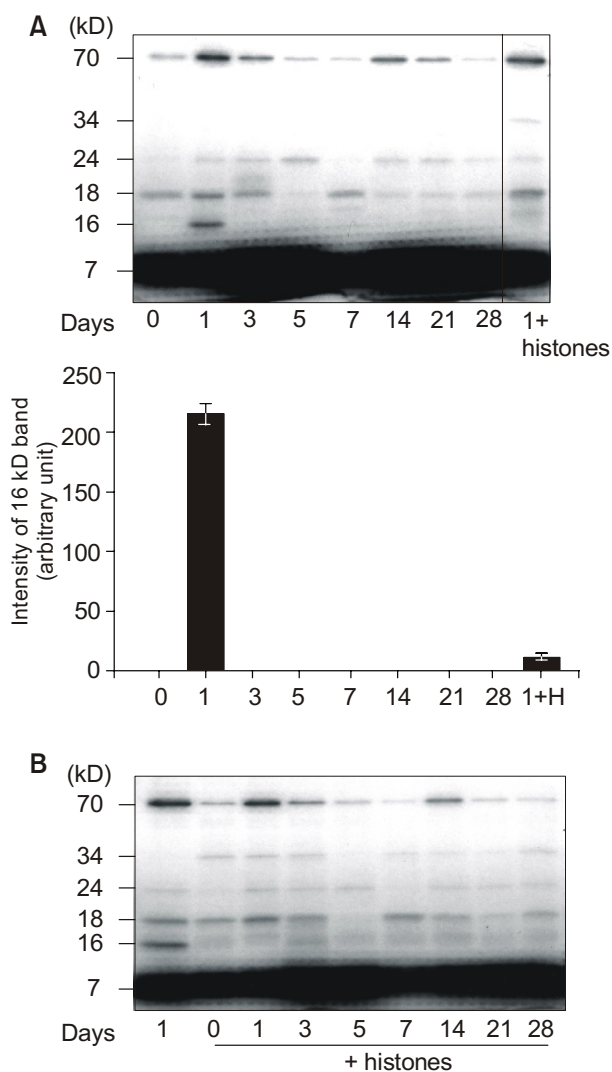


Figure 2. Changes in the methylation status of nuclear proteins in the regenerating liver cells. Nuclear extracts were prepared from rat liver at 0, 1, 3, 5, 7, 14, 21, 28 days following partial hepatectomy and subjected to methylation assay. Where indicated, 20 μ M histones were added to the reaction mixture. (A) Autoradiography of nuclear extracts showing methylation. The level of methylation on a 16-kD protein was measured with densitometer from three individual experiments and plotted below. Error bars indicate \pm SD. (B) 20 μ M of histones were added to the methylation assay and autoradiography was performed.

ylating a 16-kD protein in the nuclear fraction can also bind to histones. However, unlike cytosolic fraction which can methylate exogenously added histones (Kwon *et al.*, 2004), nuclear methyltransferases did not appear to methylate histones as no bands were present around 16 kD (Figure 1, day 1 N+H).

To further study the pattern of methylation during the regeneration process, we obtained nuclear extracts from regenerating rat liver at various time points post hepatectomy and subjected to the *in vitro* methylation assay. Interestingly, as shown in Figure 2A, methylation of a 16-kD protein only occurred 1 day following hepatectomy. After 3 days, methylation of this band was not detected. To see if methylation events occurring during the course of regenerating period were affected by histones, bulk histone were added to the reaction mixture and the methylation events were monitored (Figure 2B). However, even at later time points, methylation of nuclear proteins did not appear to be affected by added histones. Variations in the methylation levels of nuclear proteins other than a 16-kD protein including 70-kD, 34-kD, 24-kD, 18-kD and 7-kD proteins in these particular gels were found to arise from individual variations after three independent experiments.

The transient nature of methylation in the 16-kD protein following hepatectomy suggests that a specific nuclear methyltransferase activity is highest in the early stage of liver regeneration where DNA replication and gene transcription leading to cell proliferation and differentiation begin to start. Alternatively, the 16-kD protein would be expressed in increased amounts in the early stage of liver regeneration and need methylation for its optimal function. Either way, activation of the 16-kD methylation appeared to precede cell proliferation leading to liver regeneration. Therefore, the characterization and identification of the methyltransferases and the 16-kD protein will aid our understanding of the role of methylation in the signaling process resulting in the liver regeneration.

Methylation of a 16-kD protein is inhibited by histone subtypes tested

Since bulk histone used as an inhibitor of methylation in our assay are mixtures of various subtypes, we next determined the identity of histones responsible for inhibiting methylation of a 16-kD protein. Figure 3 shows that methylation of a 16-kD protein was completely inhibited by S-adenosyl homocysteine (SAH) (Figure 3A, lane 2), a well known inhibitor of methyltransferases (Gu *et al.*, 1999). Addition of bovine serum albumin (BSA) seemed not to affect the methylation (Figure 3A, lane 3). Addition of bulk histone (Figure 3A, lane 4, H2AS) completely inhibited the methylation of a 16-kD protein similar to that shown in Figure 1 and 2. When individual isoforms

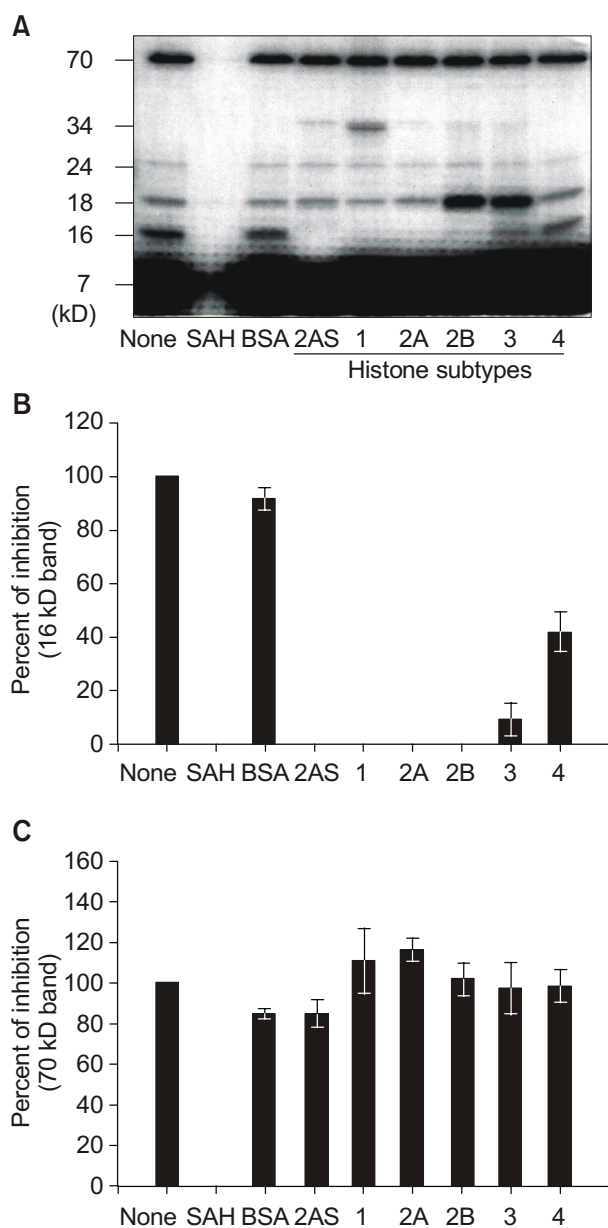


Figure 3. Effects of individual histone subtypes on the methylation of a 16-kD protein. (A) Nuclear extracts prepared from day 1 liver following hepatectomy were used for methylation assay in the presence of 20 μ M of bulk histone (H2AS), or individual subtypes of histones, H1, H2A, H2B, H3, or H4 (lane 4-9). Lane 1, none, indicates no exogenously added proteins. Addition of 0.8 mM SAH, an inhibitor of methyltransferases, completely inhibited methylation of a 16 kD protein (lane 2). 20 μ M BSA (bovine serum albumin), added as a negative control (lane 3), resulted in slight reduction of methylation in the 16-kD protein in this blot, however, it was not statistically significant when data are averaged from three individual experiments as shown in a bar graph in (B). (B) The bands of a 16-kD protein or a 70-kD protein from three different experiments were measured using densitometer and their averages were calculated as a percentage as compared to the control value. Error bars indicate \pm SD.

of histones were added to the reaction mixture, the histone subclasses 1, 2A or 2B showed near complete inhibition of the 16-kD protein methylation while histones 3 and 4 showed partial inhibition of the methylation (Figure 3A, lane 5-9). Inhibition of the 16-kD protein methylation by histones was more noticeable when the percent density change of a 16-kD protein was averaged from three different experiments and plotted as a bar graph (Figure 3B). Among the subtypes of histones tested, histone H4 was the weakest inhibitor for competing with a 16-kD protein (showing up to 60% inhibition). H3 showed slightly better inhibition than H4, but failed to inhibit methylation of a 16-kD protein completely (up to 90%). In contrast, methylation of a 70-kD, 24-kD, 18-kD or 7-kD protein was not affected significantly by addition of any histone subtypes tested (Figure 3A and C). These data demonstrate that the 16-kD protein and all the subclasses of histones are recognized by a same methyltransferase, but do not share a common methylation motif or a significant homology. Rather, histones are likely to act as allosteric inhibitors for the methyltransferase. Of note, addition of histones, particularly H1, resulted in methylation of a new band running around 34 kD. Likewise, addition of histones 2B and 3 generated more intense 18-kD methylation (3-4 fold higher than when its absence). Identity of the band is not clear at present, however, given the molecular weight of the 18-kD proteins, it can be speculated to be one of the histone subtypes tested.

To further determine the specificity of methyltransferase reaction, we added known methyl donors to prevent methylation reaction between nuclear methyltransferases and a 16-kD protein (Figure 4). Among the proteins tested, cytochrome C (cyt), and alcohol dehydrogenase (ADH) specifically inhibited the methylation of a 16-kD protein, and, interestingly, alcohol dehydrogenase inhibited the 70-kD methylation as well. By contrast, myelin basic protein (MBP), carbonic anhydrase (Car), χ globulin (Glo), and α -lactalbumin (Lac) minimally affected the methylation of a 16-kD protein (Figure 4, lane 3, 6, 7, 8). Taken together, these data suggest that methyltransferases responsible for methylating a 16-kD protein possess distinct substrate specificity.

A methylated 16-kD protein is resistant to alkali/acid-induced demethylation but sensitive to GTP

We next determined if methylation of a 16-kD protein occurs through carboxyl O-methylesterification on aspartate or glutamate residues, or C-terminal farnesylated cysteine carboxyl methylation. Methyl-incorporation through ester linkages is alkali-labile (Tan *et al.*, 1991), and methylation at the carboxyl group of C-terminal farnesylated cysteine carboxyl methylation should be stimulated by GTP analogs such as

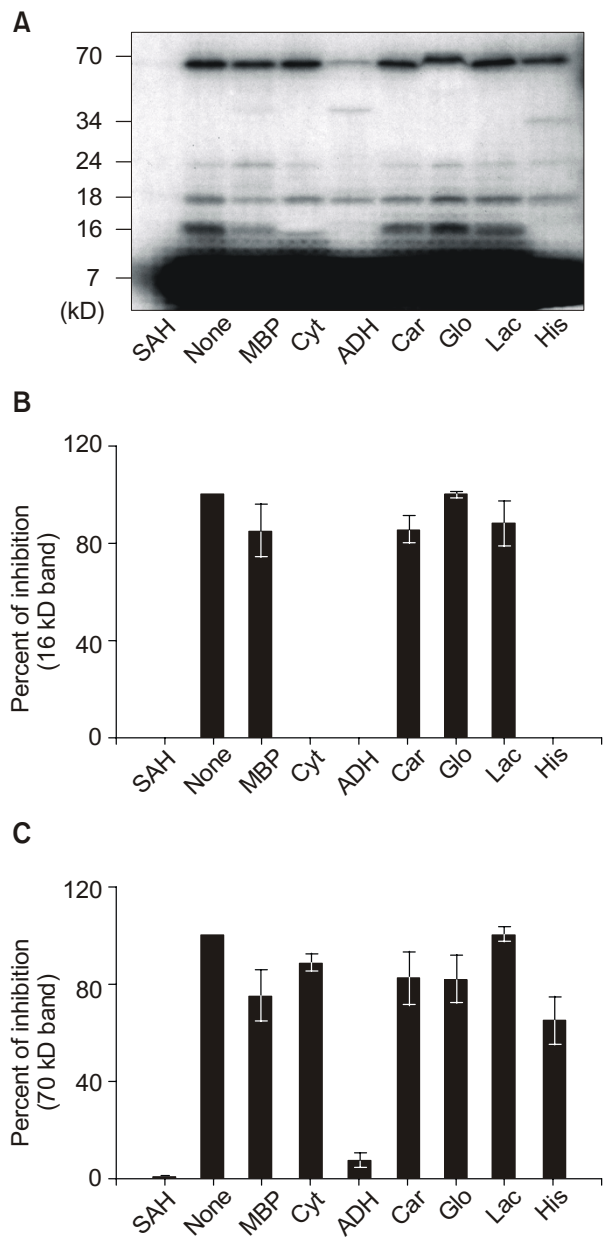


Figure 4. Effects of various proteins on the methylation of a 16-kD protein. (A) Day 1 liver nuclear extracts were subjected to the methylation assay in the presence of 20 μ M myelin basic protein (MBP, lane 3), cytochrome C (Cyt, lane 4), alcohol dehydrogenase (ADH, lane 5), carbonic anhydrase (Car, lane 6), χ globulin (Glo, lane 7), α -lactalbumin (Lac, lane 8), or bulk histones (His, lane 9). S-adenosyl homocysteine (SAH, lane 1) was added as a trans-methylation inhibitor. Reactions were subjected to SDS-PAGE and autoradiography. (B) The intensities of a 16-kD protein and 70-kD protein were measured from three different experiments and their averages were calculated as a percentage as compared to the control value. Error bars indicate \pm SD.

GTP γ S. Proteins containing C-terminal farnesylated cysteine may indeed belong to the family of small

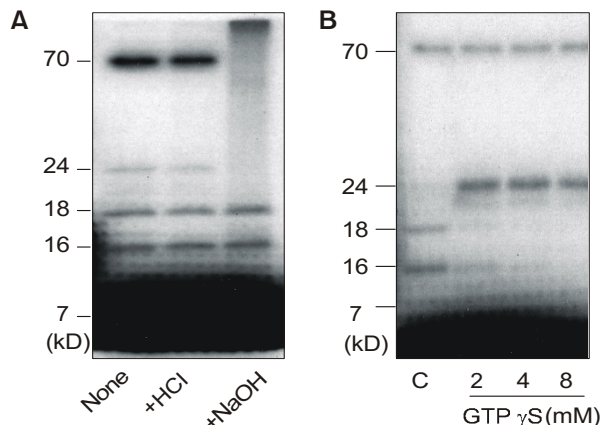


Figure 5. Effects of acid, alkali, and GTP γ S in the methylation of nuclear proteins. (A) Nuclear extracts subjected to methylation assay were treated with either 1 N HCl or NaOH. In (B) 2-8 mM of GTP γ S was added to the methylation assay. The resulting samples were subjected to SDS-PAGE and autoradiography.

molecular G proteins (Backlund, 1992). Thus, if methylation of a protein increases by GTP γ S, it will be likely to function as a G protein itself or an intermediate. When nuclear extracts were treated with HCl (pH 3-4), no significant changes were detected in any of the methylated proteins (Figure 5A lane 2). Intriguingly, when pH was lowered further below 2, only the 18-kD methylation was affected and disappeared completely, strongly indicating the presence of potential acid lability in strong acid conditions in certain methylations (data not shown). In contrast, treatment of nuclear extracts with NaOH resulted in disappearance of bands running at 70 kD and 24 kD while left no changes in the rest of bands including a 16-kD protein. These data suggest that the methyl linkage of 70-kD and 24-kD proteins were likely formed via carboxyl O-methylesterification while that of 16-kD and 18-kD proteins do not involve ester linkage.

When nuclear extracts were treated with GTP γ S, methylation of both 16-kD and 18-kD proteins was significantly inhibited. As shown in Figure 5B, addition of GTP γ S, which activates small molecular weight G proteins, inhibited methylation of 16-kD and 18-kD proteins in a dose dependent manner (Figure 5B). These data suggest that activation of G protein negatively regulates the activity of methyltransferases responsible for methylation of 16-kD and 18-kD proteins. These data are in contrast to those observed in the case of C-terminal farnesylated cysteine carboxyl methylation stimulated by unhydrolyzable GTP and further indicate that small molecular weight G proteins function as a regulator of methylation. In contrast to 16-kD and 18-kD proteins, a 24-kD protein

showed an enhanced methylation upon addition of GTP γ S, suggesting that methyl group of a 24-kD protein was likely to be incorporated to the C-terminal farnesylated cysteine. By contrast, a 70-kD protein (Figure 5A), whose methylation was significantly labile in alkaline condition, did not show any changes in the level of methylation by GTP (Figure 5B).

Taken together, our data suggest that the nature of methylation process of individual nuclear proteins shows specificity during the course of liver regeneration. These data also demonstrate that more than one methyltransferases function to methylate nuclear proteins and each reaction is likely performed on distinct amino acids, such as arginine, lysine, aspartates, glutamates, or cysteine. The resistance of a 70-kD protein to alkali and GTP suggests that its methylation is most likely to be carboxyl O-methylesters in the side chains of aspartates or glutamates. The 24 kD methylation was alkali-labile and stimulated by GTP, indicating it involves a carboxyl methyl-esterification on C-terminal cysteinyl residues. On the other hand, the methyl groups in the 18-kD, 16-kD and 7-kD proteins were stable at pH 10-11 (37°C for 30 min), indicating that neither carboxyl methyl-esterification on isoaspartyl residues nor C-terminal cysteinyl residues were involved, but rather methylations on arginyl or lysyl residue may be operational. Importantly, the lability of the 18-kD methylation in strong acid condition could provide a new approach to differentiate among different amino acid methylations.

Discussion

Covalent modification of proteins such as phosphorylation, acetylation, ubiquitination, and methylation is central for fundamental cellular processes from DNA replication to cell proliferation and differentiation. The level of modification in a given cell is determined by a balance between forward and reverse reactions carried out by specific enzymes. While protein phosphorylation, acetylation, and ubiquitination events are widely studied, protein methylation events have only recently been acknowledged in spite of the discovery of cellular methyltransferases (PRMTs) and their substrates several decades ago. Histones are one such substrate which can undergo methylation and regulate gene transcription. However, it has been a great question how methylation reaction is terminated. In the case of phosphorylation and acetylation, phosphatases and deacetylases were identified to regulate the level of modification. However, enzymes responsible for demethylation of proteins have not been identified up to now (Bannister *et al.*, 2002). Only recently a nuclear deiminase called "peptidyl arginine deiminase 4 (PADI4)" is identified as an enzyme

capable of preventing methylation by converting arginine residues into citrulline. An example was shown in the regulation of histone H3 where methylation of arginine residues R2, R8, R17, and R26 could be specifically deiminated (Cuthbert *et al.*, 2004). Therefore, methylation events are also subject to tight regulation which can be turned on by cellular stimuli and turned off when it is no longer needed.

Our data shown here together with the previous reports strongly demonstrate that proliferating hepatocytes undergo time-dependent activation of methyltransferase activity. Methyltransferase activity is present in both fractions of cells; cytosol and nucleus, and shown to regulate methylation of cytosolic and nuclear proteins with distinct specificity and time dependency. First of all, methylation of proteins in the cytosol was only detected in a 20-kD protein and persisted up to 7 days following hepatectomy (Kwon *et al.*, 2004). In contrast, nuclear fraction showed basal level of methyltransferase activities to methylate at least four distinct bands (7, 18, 24, 70 kD; Figure 1B). Upon hepatectomy, none of these bands seemed to undergo methylation or demethylation, but a distinct new band running at 16-kD protein appeared to be heavily methylated *in vitro* (Figure 1B). Methylation of the 16-kD protein is only observed 1 day following hepatectomy, indicating that in contrast to the cytosolic methyltransferases, the nuclear methyltransferase responsible for methylating a 16-kD protein in regenerating liver shows transient early activation following hepatectomy, or alternatively level of the nuclear 16-kD methylation increases only in the initial stage of liver regeneration. These data suggest that distinct methyltransferases, showing specificity against their substrates in the specific location, may reside in the cytosol and nucleus. How these enzymes become activated or whether the changes of methylation in the 16-kD protein contribute to the liver regeneration is not clear at present. However, early activation of nuclear methyltransferases immediately after proliferating stimuli allows us to speculate that activation of specific methyltransferases and subsequent methylation may play a role in turning on or off specific genes culminating in cell proliferation as shown for the case of histones (Nielsen *et al.*, 2001).

Interestingly, inhibition of the methylation of a 16-kD protein by histones made us question if this protein may indeed be histones. However, it appeared not to be the case. Rather histones function as an allosteric inhibitor to prevent methylation of a 16-kD protein, since methyltransferases for a 16-kD protein did not cause methylation of exogenously added histones. Interestingly, unlike cytosolic methyltransferases which can be competed out only by alcohol dehydrogenases, nuclear methyltransferases for a 16-kD protein were able to be competed out by both

alcohol dehydrogenases and cytochrome C. And alcohol dehydrogenase also inhibited the 70-kD methylation. These data reinforce the distinct nature of methyltransferases in the cytosol and the nucleus. Addition of alcohol dehydrogenase not only inhibited methylation of a 16-kD protein but also made a new band running at 40 kD (Figure 4A). Since alcohol dehydrogenases themselves migrate around 150 kD, it could possibly be suggested that added alcohol dehydrogenases became methylated by the nuclear methyltransferases and was thereby facilitated to be degraded. More interesting phenomenon is the fact that the methyl group of a 16 kD-protein is neither on the carboxyl group nor on the farnesylated cysteine. And the fact that added GTP γ S, which activates G proteins through preventing dephosphorylation of γ phospho group bound on G proteins (Sasaki and Hasegawa-Sasaki, 1987), resulted in disappearance of the 16-kD methylation suggested that a 16-kD protein is neither methylated on farnesyl cysteines nor GTP-binding proteins. Rather, activation of GTP-binding proteins tends to suppress the 16-kD methylation. Interestingly, addition of GTP γ S enhanced methylation of a 24-kD protein, suggesting that this band may be a GTP-binding protein. Alternatively, activated G proteins in the nucleus may in turn cause activation of methyltransferases to methylate the 24-kD protein. Further study is under way to identify the nature of bands affected by GTP-binding proteins.

Many of the substrates of protein arginine methyltransferases (PRMTs) such as hnRNP A1 (M_r ~34,000) (Williams *et al.*, 1985) and A2 protein (M_r ~34,000) (Nichols *et al.*, 2000), nucleolin (M_r ~100,000) (Srivastava and Pollard, 1999), fibrillarin (M_r ~34,000) (Lischwe *et al.*, 1985), HMG chromosomal protein (M_r ~9,200 kDa) (Boumba *et al.*, 1993) and histones (Wallwork *et al.*, 1977), have been shown to be localized in the nucleoplasm, nucleoli or chromosome (Boffa *et al.*, 1979; Lischwe *et al.*, 1985), indicating their regulatory roles in gene expression (Dreyfuss *et al.*, 1993; Caceres *et al.*, 1994) or DNA replication (Planck and Wilson, 1980; Williams *et al.*, 1985; Kumar *et al.*, 1986; Cobianchi *et al.*, 1988). Studies are in the progress to determine if the methylated proteins shown in our study co-migrate with PRMT substrates described above.

In our study, methylation of both cytosolic and nuclear proteins was detected in the absence of pretreatment of cells with endogenous methyltransferase inhibitors, such as adenosine dialdehyde. However, previous reports using human lymphoblastoid cells showed that methylations of proteins between 29 and 90 kDa were observed only when cells were pretreated with adenosine dialdehyde to obtain hypomethylated state of endogenous substrates (Li *et*

al., 1998). Similarly, RNase treatment of RAT1 cell extracts resulted in methylation of hnRNP A1 and a series of cellular proteins running between 34- and 55-kD (Frankel and Clarke, 1999). These data suggested that substrates were constantly associated with RNAs, which inhibited the access of these proteins to methyltransferases. Since our study indicates that the nuclear 16-kD protein became methylated in the absence of pretreatment, and its *in vitro* methylation was remarkably enhanced by proliferative stimuli only in the beginning of rat liver regeneration, the protein may represent the true physiologic substrates undergoing methylation and thus, be involved in early signaling events leading to cell proliferation.

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