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Increased methylation of the cytosolic 20-kD protein is accompanied by liver regeneration in a hepatectomized rat

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Abbreviations: ADMA, N^GN^G-dimethyl(asymmetric)arginine; AdoMet, S-adenosyl-L-methionine; CARM1, co-activator associated arginine methyltransferase 1; JBP1, Janus kinase binding protein 1; MBP, myelin basic protein; MMA, N^G-monomethylarginine, PRMT, protein arginine methyltransferase; SDMA, $N^GN'^G$-dimethyl(symmetric)arginine; UP1, unwinding protein 1

Abstract

Arginine methylation has been implicated in the signal transduction pathway leading to cell growth. Here we show that a regenerating rat liver following partial hepatectomy exhibited elevated methyltransferase activity as shown by increased methylation of a subset of endogenous proteins *in vitro*. The 20-kDa protein was shown to be a major cytosolic protein undergoing methylation in regenerating hepatocytes. Methylation of the 20-kDa protein peaked at 1 d following partial hepatectomy, which gradually declined to a basal level within the next 14 d. Likewise, methylation of exogenously added bulk histones followed the similar time kinetics as the 20-kDa protein, reflecting time-dependent changes in methyltransferase activity in regenerating hepatocytes. Presence of exogenously added bulk histone in the *in vitro* methylation assay resulted in dose-dependent inhibition of methylation of the 20-kDa protein. All the histone subtypes tested, histone 1, 2A, 2B, 3 or 4, were able to inhibit methylation of the 20-kDa protein while addition of cytochrome C, a-lactalbumin, carbonic anhydrase, bovine serum albumin, and g globulin minimally affected methylation of the 20-kDa protein. Since methylation of the 20-kDa protein preceded proliferation of hepatocytes upon partial hepatectomy, it is tempting to speculate that the methylated 20-kDa protein by activated histone- specific methyltransferase may be involved in an early signal critical for liver regeneration.

Keywords: arginine N-methylation; cytosolic 20-kDa protein; histone; regenerating rat liver

Introduction

N-methylation on arginine residues 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 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). Enzymes that mediate the transfer of methyl group, protein arginine methyltransferases (PRMT), have been cloned and named as PRMT1 (Lin *et al*., 1996; Tang *et al*., 2000), PRMT2 (Katsanis *et al*., 1997; Scott *et al*., 1998), PRMT3 (Tang *et al*., 1998), PRMT4 (co- activator associated arginine methyltransferase 1, CARM1) (Chen *et al*., 1999), PRMT5 (Janus kinase binding protein 1) (Pollack *et al*., 1999), and PRMT6 (Frankel *et al*., 2002). RNA-binding proteins, myelin basic protein, and histones are well known substrates for PRMTs. RNA-binding proteins which generate MMA and ADMA include hnRNP-A1 (Kumar *et al*., 1986; Kim *et al*., 1997), nucleolin (Lischwe et al., 1982), fibrillarin (Lischwe *et al*., 1985a; 1985b), the Sam68 Src-associated substrate (Bedford *et al*., 2000), poly(A)-binding protein II (Smith *et al*., 1999), the NF90 nuclear-binding factor (Tang *et al*., 2000), interleukin enhancer binding factor 3 (Tang *et al*., 2000) and the yeast Npl3 protein (Lee *et al*., 1996; Siebel and Guthrie, 1996). Myelin basic protein (Stoner, 1984) and proteins SmD1 and SmD3 (Frie-

sen *et al*., 2001) are substrates known to produce SDMA. Once methylated, histones can induce transcriptional silencing at the heterochromatin sites while they can initiate transcription at the euchromatic loci (Bannister *et al*., 2002).

The PRMT activity was shown to be directly correlated with the degree of proliferation and thus, found to be elevated in highly proliferating tissues such as regenerating liver, developing fetal brain, rapidly growing hepatomas and continuously dividing HeLa cells (Paik *et al.,* 1975; Paik and Kim, 1980). Recently, the 20-kDa cytosolic protein was found to be a major endogenous substrate undergoing *in vitro* arginine methylation in proliferating mammalian cells. Human cancer cell lines (HCT-48, HeLa cells, A549 and Hep G2) showed increased level of methylation in this 20-kDa protein as compared to their normal counterparts, suggesting that increased methylation is associated with cellular proliferation and transformation (Gu *et al*., 1999). The identity of this 20-kDa protein is still not known, but addition of bulk histones completely suppressed methylation of 20-kDa protein while addition of hnRNP-A1 partially inhibited the 20-kDa methylation, indicating that these three proteins compete for the same protein methyltransferase (Park *et al*., 1997; Gu *et al*., 1999).

Since previous data showing increased methylation of the 20-kDa protein were obtained from transformed cancer cell lines (Gu *et al*., 1999), it was of interest to determine if methylation events occur in more physiological settings. Our particular interest was to investigate signaling events involved in the process of liver regeneration. We now found that methylation of the 20-kDa protein was increased in proliferating hepatocytes due to elevated methyltransferase activity, and that histones may play some regulatory roles in this process. Therefore, methylation events are commonly associated with cell proliferation under physiologic conditions.

Materials and Methods

Reagents

S-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]AdoMet; 60 Ci/mmol) was purchased from Amersham Pharmacia Biotech Inc. (NJ). Calf thymus histone II-AS (prepared by extraction in 1 M NaCl, precipitation in water, followed by acid extraction, dialysis and lyophilization), myelin basic protein, cytochrome C, a-lactalbumin, carbonic anhydrase, alcohol dehydrogenase, bovine serum albumin, g-globulin and S-adenosyl-L-homocysteine were purchased from Sigma Chemical Co. (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 (CA).

Partial hepatectomy

Median and left lateral lobes (about 70% of liver) of 6 week-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, 1 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonylfluoride. 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).

In vitro methylation assay

Methylation reaction was carried out by adding 5 mCi of [^{3}H]AdoMet into 50 mg of crude extracts of rat liver (100,000 *g* supernatant) in a total volume of 50 ml. Where indicated, 20 mM 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 mM of myelin basic protein, cytochrome C, a-lactalbumin, carbonic anhydrase, alcohol dehydrogenase, bovine serum albumin, or bovine g-globulin, were 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).

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 were measured using Fujix-Bio-Imaging Analyzer BAS2500 (Fuji Photo Film).

Results

Increased methylation activity in a regenerating rat liver as revealed by *in vitro* methylation assay

Liver regeneration following partial hepatectomy in a rat involves initiation of proliferation and differentiation of the residual hepatic parenchymal stem cells. As a result, hepatectomized liver can restore the liver mass to its original size within 10-20 d (Waynforth and Flecknell, 1992). To determine the time course of liver regeneration, we measured the weight of the remaining liver at different days following partial hepatectomy. As shown in Figure 1, weight of the remaining liver (right lateral and caudate lobes) increased following partial hepatectomy (day 0) and reached the normal mass by 28 d. By comparison, weight of right lateral and caudate lobes from age-matched non-

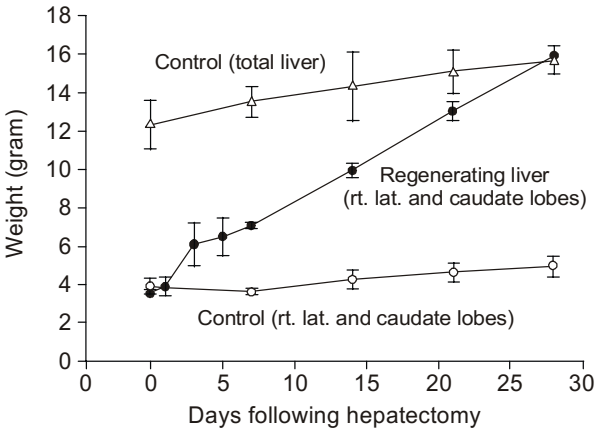

Figure 1. Liver regeneration following partial hepatectomy. Median and left lateral lobes of 6 week-old Sprague Dawley rats were removed at day 0 as described in Materials and Methods. Remaining right lateral and caudate lobes were removed at 0, 1, 3, 5, 7, 14, 21, 28 days following partial hepatectomy and their weight was measured (closed circle). As a control, the right lateral and caudate lobes were removed from age-matched normal rats and their weight was measured (open circle). Total liver mass was also measured from the control age-matched rats (open triangle), showing that partially hepatectomized liver restored its original mass within 28 days. At each time point, three rats were used for analysis.

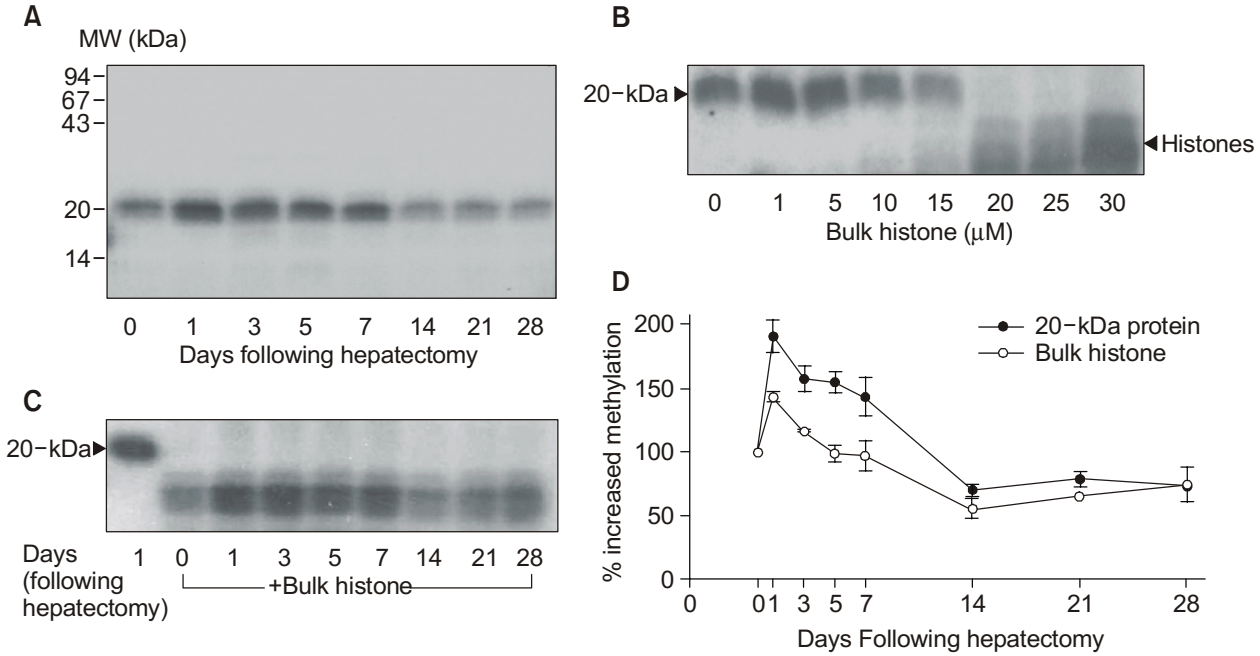

Figure 2. A 20-kDa protein is a major substrate methylated by regenerating rat liver extracts. (A) Cytosolic extracts (100,000 *g* for 60 min supernatants of whole cell extracts) were prepared from rat liver at 0, 1, 3, 5, 7, 14, 21, 28 days following partial hepatectomy. Equal amounts of protein (50 mg) were incubated with Ado [methyl-^{3}H]Met for 30 min, and analyzed by SDS-PAGE and autoradiography. (B) Cytosolic extracts prepared from day 1 liver following hepatectomy were incubated with various doses of bulk histone and subjected to methylation assay. Numbers at the bottom indicate the concentration of bulk histone added to the reaction mixtures. (C) Twenty mM Bulk histone was added to the methylation assay mixture with the liver extracts prepared as in (A). (D) Changes of methylation on a 20-kDa protein (A) and bulk histone (C) obtained from three individual experiments were plotted over time. The level of methylation was measured by densitometry and the degree of methylation was normalized to that observed in cell extracts prepared from non-hepatectomized liver (day 0). Error bars indicate±SD.

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 extracts were prepared at different days post-hepatectomy and subjected to an *in vitro* methylation assay. As shown in Figure 2A, the major protein appearing in the gel following *in vitro* methylation assay was a band migrating at 20-kDa. Methylation of this 20-kDa protein peaked at day 1, which then subsequently declined to a basal level by 14 d post-hepatectomy. Basal level of methylation was maintained after 14 d till 28 d. Interestingly, when bulk histone (histone II-AS) prepared from calf thymus was added as an exogenous substrate to the reaction mixture containing day 1 liver extracts, methylation of a 20-kDa protein was dose-dependently inhibited (Figure 2B). Maximal inhibition occurred at 20 mM of bulk histone. Interestingly, while methylation of a 20-kDa protein is inhibited, methylation of bulk histone becomes evident, suggesting that histones can function as a competitive inhibitor for methyltransferases responsible for methylation of a 20-kDa protein. Inhibition of the 20-kDa protein methylation by histones was evident with all samples prepared from different days post hepatectomy (Figure 2C). Similar to a 20-kDa protein, methylation of exogenously added bulk histone followed the same kinetic during the course of liver regeneration, which reflects time-dependent changes in methyltransferase activity post hepatectomy (Figure 2C and D). The fact that methylation of a 20-kDa protein was completely inhibited by histones and that histones themselves became methylated by liver extracts strongly suggests that a 20-kDa protein might have been methylated by histone-specific methyltransferases in the cytosolic extracts of regenerating hepatocytes. Since increased methylation of the 20-kDa protein was also observed in proliferating cancer cell lines (Gu *et al*., 1999), it is tempting to speculate that methylation of a 20-kDa protein is associated with signaling events leading to the proliferation of hepatocytes.

Individual histone subtypes, H1, H2A, H2B, H3, or H4, can all suppress methylation of a 20-kDa protein

Since bulk histone used as an inhibitor of methylation in our assay contained various subtypes of histones, we next attempted to determine the identity of histones responsible for inhibiting methylation of a 20-kDa protein. Figure 3 shows that methylation of a 20-kDa protein was completely inhibited by S-adenosyl homocysteine (SAH), a well known inhibitor of methyltransferases (Figure 3A, lane 2) (Gu *et al*., 1999). Interestingly, addition of bovine serum albumin (BSA) also partially inhibited methylation of a 20-kDa protein in this particular blot (Figure 3A, lane 3). However, this inhibition was not statistically significant when data were averaged from three individual experiments as shown in a bar graph in Figure 3B. Addition of bulk histone (Figure 3A, lane 4, H2AS) completely inhibited methylation of a 20-kDa protein, similar to that observed in Figure 2 (B and C). When individual isoforms of histones were added to the reaction mixture, all the histone subclasses tested, histone 1, 2A, 2B, 3 or 4, showed almost complete inhibition of the 20-kDa protein methylation, indicating that the 20-kDa protein and all the subclasses of histones share a common methylation motif recog-

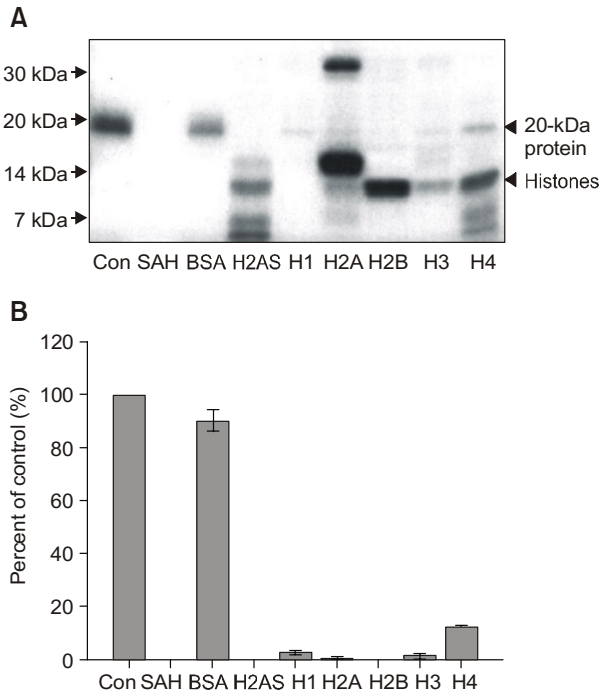

Figure 3. Effects of individual histone isotypes on methylation of the 20-kDa protein. (A) Cytosolic extracts prepared from day 1 liver following hepatectomy were incubated with Ado[methyl-^{3}H]Met in the presence of 20 mM of bulk histone (H2AS), or individual subtypes of histones, H1, H2A, H2B, H3, or H4 (lane 4-9). Lane 1, Con, indicates control without any exogenously added proteins. Addition of 0.8 mM SAH, an inhibitor of methyltransferases, completely inhibited methylation of a 20 kDa protein (lane2). Twenty mM BSA (bovine serum albumin), added as a negative control (lane 3), resulted in slight reduction of methylation in the 20-kDa 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 20 kDa-protein from three independent experiments were measured using densitometer and their averages were calculated as a percentage as compared to the control value. Error bars indicate±SD.

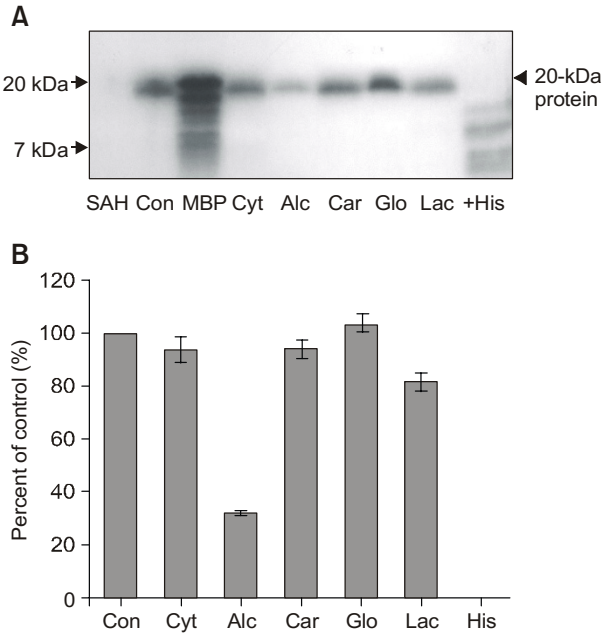

Figure 4. Effects of various proteins on methylation of a 20-kDa protein. (A) Day 1 liver cytosolic extracts were incubated with Ado[methyl-^{3}H]Met in the presence of 20 mM myelin basic protein (MBP, lane3), cytochrome C (Cyt, lane 4), alcohol dehydrogenase (Alc, lane 5), carbonic anhydrase (Car, lane 6), g globulin (Glo, lane 7), a-lactalbumin (Lac, lane 8), or bulk histone (His, lane 9). S-adenosyl homocysteine (SAH, lane 1) was added as a transmethylation inhibitor. Control (Con, lane 2) indicates no addition of exogenous proteins or agents. Reactions were subjected to SDS-PAGE and autoradiography. (B) The bands of a 20 kDa-protein from three independent experiments were measured using densitometer and their averages were calculated as a percentage as compared to the control value. Error bars indicate±SD.

nized by a methyltransferase (Figure 3A, lane 5-9). Inhibition of a 20-kDa protein methylation by histones was more noticeable when the percent density change of a 20-kDa 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 20-kDa protein. By contrast, cytochrome C, carbonic anhydrase, g-globulin, and a-lactalbumin had no significant effect on the methylation of a 20-kDa protein (Figure 4A, lane 4, 6, 7, 8). Interestingly, alcohol dehydrogenase partially inhibited methylation of the 20-kDa protein (Figure 4A, lane 5), indicating that the protein is a weak substrate for the 20-kDa protein-specific methyltransferase. Myelin basic protein, a well known methyl acceptor (Chou *et al*., 1976), became methylated by endogenous methyltransferases present in the cytosolic extracts, however, it was difficult to conclude if MBP inhibited methylation of a 20-kDa protein since MBP co-migrated with a 20-kDa protein on the gel (Figure 4A, lane 3). These data suggest that a methyltransferase responsible for methylating a 20-kDa protein possesses distinct substrate specificity. Taken together, these data demonstrate that a protein arginine methyltransferase present in the cytosol becomes activated upon partial hepatectomy and may participate in the early signaling pathway leading to liver regeneration.

Discussion

Post-translational protein methylation induced by increased methyltransferase activity has been observed in proliferating tissues and cancer cell lines (Paik *et al*., 1975; Paik and Kim, 1980). Recently, a 20-kDa protein present in the cytosol of various human transformed cell lines was found to be the major substrate for activated methyltransferases (Park *et al*., 1997; Gu *et al*., 1999). In this study, we examined if proliferating hepatocytes following partial hepatectomy showed increased methylation of the 20-kDa protein. We found that the 20-kDa protein was the major substrates that underwent methylation by activated methyltransferases in regenerating liver hepatocytes. Whether the changes in methylation of a 20-kDa protein contributes to the liver regeneration is not clear at present. However, the transient increase in protein methylation associated with proliferation of hepatocytes allows us to speculate that activation of methyltransferases and subsequent methylation may play a critical role in transducing its signal to downstream events leading to DNA synthesis and cell proliferation.

Arginine methylation has been shown to be involved in various signaling pathways. At present, 6 forms of arginine methyltransferases are cloned; PRMT1-6. PRMT1 has been shown to be involved in the early signaling pathway *via* associating with TIS21 immediate-early gene, leukemia-associated BTG1 gene (Lin *et al*., 1996), the interleukin enhancer binding factor 3 (Tang *et al*., 2000), and the interferon a/b receptor (Abramovich *et al*., 1997; Kim *et al*., 2003). PRMT1 was also shown to be involved in the signaling of nerve growth factor and regulate neurite outgrowth (Cimato *et al*., 1997). PRMT4/CARM1 has been shown to interact with p160 family of nuclear hormone receptor coactivators and enhance transcriptional activation, presumably *via* methylation of histone H3 (Chou *et al*., 1999). PRMT1, like PRMT4, can also bind p160, and these two PRMTs have been shown to act synergistically to enhance reporter gene activation by nuclear receptors (Koh *et al*., 2000). PRMT5/JBP1 was shown to be required for signaling of the interferon receptor to downstream Stat activation (Pollack *et al*., 1999).

Our data show that bulk histone as well as histone

1, 2A, 2B, 3 and 4 were potent inhibitors of the cytosolic 20-kDaa protein methylation. IC_{50} of bulk histones was about 10 mM and those of each subclass of histones appeared to have similar values. These data suggests that the 20-kDa protein and histones compete for the same methyltransferase. This can be possible if both histones and the 20-kDa protein possess the common methylation motif, such as GAR domain containing the multiple potential RGG motifs in hnRNP proteins (Dreyfuss *et al*., 1993). To support this, the major arginine methylation products of both 20-kDa protein and histones were found to be ADMA and MMA (Rajpurohit *et al*., 1992; Park *et al*., 1997). Indeed, PRMT1, PRMT4 and PRMT6 have been demonstrated to have histone methyltransferase activity (McBride and Silver, 2001). PRMT1 and PRMT4 were shown to function as transcriptional coactivators *in vivo* by methylating histone H4 and histone H3, respectively (Chen *et al*., 1999; Wang *et al*., 2001), while PRMT6 was able to methylate histones H2A and H4 in an *in vitro* methylation reaction (Pollack *et al*., 1999). However, PRMT1, PRMT4/CARM1 and PRMT6, when tagged with green fluorescence protein (GFP), were all shown to be localized in the nucleus. Thus, the PRMT responsible for methylation of both 20-kDa protein and histones in the cytoplasm of hepatocytes may be distinct from those listed above. Therefore, it is tempting to speculate that a novel PRMT may exist in the cytosol, which regulate methylation of a 20-kDa protein. In support of this idea, the 20-kDa proteins were neither found to be present nor methylated in the nuclear fraction (Park *et al*., unpublished results) where histones reside. Therefore, although histones can be potent competitor for PRMTs *in vitro*, they are not likely to compete with 20-kDa proteins for binding to nuclear PRMTs. Since both histones and PRMTs responsible for methylating histones are present in the nucleus, it will be interesting to see if 20-kDa proteins become translocated into the nucleus upon methylation, where they can compete with histones for further methylation by PRMTs.

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 methylation was only observed when cells were pretreated with adenosine dialdehyde to obtain hypomethylated state of endogenous substrates. In that study, proteins migrating between 29 and 90 kDa were shown to be methylated by endogenous cytosolic PRMTs (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-kDa, which could be catalyzed by PRMT1 and PRMT3 (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 cytosolic 20-kDa protein became methylated in the absence of pretreatment to enhance *in vitro* substrate methylation, these proteins may represent the true physiologic substrates undergoing methylation and thus, be involved in early signaling events leading to cell proliferation.

At present, the identity of the cytosolic 20-kDa protein is not known. However, our preliminary data show that this 20-kDa protein co-migrates with UP1, a shortened derivative of hnRNP A1 (aa 1-196) (Kumar et al., 1986). Furthermore, its methylation is blocked by oligopeptide spanning amino acids 187-196 of UP1 that contains a methylation site, Arg 193, suggesting that this protein may indeed be UP1. Since hnRNP A1 and histones were previously shown to compete for the same PRMT (Rajpurohit *et al*., 1994), identifying UP1 as another substrate for PRMTs adds more complexity of methylation events. UP1 has been shown to bind to DNA carrying single-stranded telomeric extensions at the 3' terminus and protects telomeric sequences against degradation by endo- and exonucleases (Dallaire *et al*., 2000). Furthermore, recent studies have demonstrated that overexpression of UP1 extends life of primary porcine fetal fibroblasts in culture by preventing telomere shortening (Mir *et al*., 2003). Thus, it is tempting to speculate that UP1 becomes methylated upon proliferating stimuli in a regenerating liver. Further studies are under way to examine this possibility.

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