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Increased lysine N-methylation of a 23-kDa protein during hepatic regeneration

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Abbreviations: AdoMet, S-adenoxyl-L-methionine; aDMA, N^GN^G-dimethyl arginine; DML, dimethyl lysine; MBP, myelin basic protein; MMA; monomethyl arginine; MML, monomethyl lysine; PRMT, protein arginine N-methyltransferase; sDMA, $N^GN'^G$-dimethyl arginine; TML, trimethyl lysine

Abstract

The methylation of a 23-kDa nuclear protein increased after partial hepatectomy and methylation returned to basal levels after the initial stage of regeneration. The methylating enzyme was partially purified from rat liver by ammonium sulfate precipitation, DEAE-anion exchange chromatography and Butyl-Sepharose chromatography. The 23-kDa protein was purified from a nuclear fraction of liver tissue with SP-Sepharose. When the 23-kDa protein was methylated with the partially purified methyltransferase and analyzed on C_{18} high performance liquid chromatography (HPLC), the methylated acceptor amino acid was monomethyl lysine (MML). Previously, only arginine N-methylation of specific substrate proteins has been reported during liver regeneration. However, in this report, we found that lysine N-methylation increased during early hepatic regeneration, suggesting that lysine N-methylation of the 23-kDa nuclear protein may play a functional role in hepatic regeneration. The methyltransferase did not methylate other proteins such as histones, hnRNPA1, or cytochrome C, suggesting the enzyme is a 23-kDa nuclear protein-specific lysine N-methyltransferase.

Keywords: lysine N-methylation; lysine N-methyltransferase; methylated amino acid analysis; nuclear 23-kDa protein; regenerating rat liver

Introduction

Protein methylation is one of the post-translational modifications that occurs in a wide variety of cell types in eukaryotes, as well as in prokaryotes. Protein methylation is catalyzed by methyltransferases that transfer the methyl group from S-adenonosyl-L-methionine (AdoMet) to a variety of nucleophilic oxygen and nitrogen atoms on polypeptides (Paik and Kim, 1980). The protein methyltransferase reaction is classified into carboxyl O-methylation or N-methylation depending on the methyl acceptor molecule. In carboxyl O-methylation, the methyltransferase modifies the oxygen molecules of glutamate or aspartate residues (Paik and Kim, 1990). Several N-methyl modifications have been reported depending on the methyl acceptor amino acid. Protein asparagine N-methyltransferase modifies asparagine residues on phycocyanins of photosynthetic bacteria (Swanson and Glazer, 1990; Thomas *et al.,* 1993). Protein arginine N-methyltransferases (PRMTs) transfer a methyl group to arginine residues forming MMA (ω-NG monomethyl arginine) and asymmetric DMA (ω-NG, NG-dimethyl arginine) or symmetric DMA (ω-NG,N ´ G dimethyl arginine) depending on the specific PRMT (Paik and Kim, 1968; Nakajima et al,1971). Protein lysine N-methylation modifies the ε-amino-group of lysine residues.

Lysine N-methylation occurs in many proteins including bacterial flagellin, ribosomal proteins, myosin, rhodopsin, cytochrome c, tooth matrix proteins, citrate synthase and calmodulin. The protein lysine N-methyltransferase transfers methyl groups to ε-N-lysine residues from AdoMet forming ε-N-MML (monomethyllysine), ε-N-DML (dimethyllysine) and ε-N-TML (trimethyllysine). Lysine N-methylation of histones has been well studied in chromatin function. Early work on histone methylation suggests that H3 and H4 are the primary histones to be modified and sequencing studies have shown that several lysines (4, 9, 27 and 36 of H3 and 20 of H4) are preferred sites of methylation (van Holde 1988; von Holt *et al.,* 1989; Duerre and Buttz 1990).

Several protein lysine N-methyltransferase enzymes have been characterized and shown to methylate specific lysine residues or histone types. SUV39H1 is an H3-lysine 9-specific methyltransferase (Rea *et al.,* 2000) and SET7 from HeLa cells is an H3-lysine 4-specific methyltransferase (Wang *et al.,* 2001). Lysine N-methylation of histones at different residues seems to play distinctive roles in transcriptional regulation (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001); for example, H3K9 methylation initiates a gene repression pathway (Snowden *et al.,* 2002) and H3K4 methylation activates transcription (Wang *et al.,* 2001; Nishioka *et al.,* 2002). With the exception of histone methylation, little is known about the functional role of methylated lysine residues on other proteins. Lysine methylation is widespread and has been conserved during evolution, suggesting that protein lysine methylation is an important regulatory mechanism in cells.

During liver regeneration, protein arginine N-methyltransferase activity was correlated with the degree of proliferation and is elevated in highly proliferating tissues (Paik and Kim, 1980). We have identified specific changes in protein methylation during hepatic regeneration, and previously reported that the arginine methylation of a 23 kDa protein is decreased during early hepatic regeneration (Lee *et al.,* 1994). In this study, we found that the lysine N-methylation of this 23-kDa nuclear protein increased during early hepatic regeneration, and we have characterized the protein lysine N-methyltransferase from rat liver.

Materials and Methods

Materials

Rat livers from Sprague-Dawley rats were purchased from Pel Freeze Co. (USA). S-Adenosyl-L-[*methyl*-^{3}H] methionine (specific activity, 73 Ci/mmol) was obtained from Amersham Pharmacia Bioscience International. N^G-Monomethyl arginine, $CuCl_2$, myelin basic protein (MBP), polylysine, calmodulin, bovine serum albumin, histone type II-AS (a mixture of various subtypes of calf thymus histones), CNBr-activated Sepharose, 2-mercaptoethanol (2-ME), ethylenediamine-tetraacetate (EDTA), phenylmethylsulfonyl fluoride (PMSF), dimethylsulfoxide (DMSO) and 2, 5-diphenyloxazol (PPO) were purchased from Sigma Chemical Co. Histone H3, H4 and core histone were purchased from Upstate Biotechnology Co. DEAE-Sepharose was purchased from Pharmacia Chemical Co. Acrylamide, Tris, PVDF membrane and glycine were obtained from Bio-Rad Laboratories. AFC, N^G, NG-DMA and NG, N'G-DMA were kindly provided by Dr. WK Paik (Fels Institute for Cancer Research & Molecular Biology, Temple University School of Medicine, Philadelphia, PA.).

Partial hepatectomy

Partial hepatectomy was performed by the procedure of Higgins and Anderson (1931) on rats under ether anesthesia. Hepatectomy was carried out after a midline laparotomy by aseptic extirpation of the median and left lateral lobes.

Preparation of nuclei

Nuclei from adult rat livers were prepared by the method of Kay and Johnson (1977). Liver tissue (30 g) was homogenized in 3 volumes of homogenization buffer (0.32 M sucrose, 3 mM $MgCl_2$, 0.5 mM PMSF, 10 mM Tris-HCl buffer, pH 7.4) and the homogenate was centrifuged at 700 *g* for 10 min. The pellet was resuspended in homogenization buffer and the suspension was centrifuged at 700 *g* for 10 min. The pellet containing crude nuclei was suspended in homogenization buffer with 2.4 M sucrose, and the suspension was centrifuged at 50,000 *g* for 60 min. The precipitate was again suspended in homogenization buffer with 0.25 M sucrose, and purified nuclei were obtained by centrifugation at 700 *g* for 10 min.

Enzyme activity assay and analysis of [methyl-^{3}H]-labeled proteins by SDS-PAGE and autofluorography

For the detection of [methyl-^{3}H]-labeled protein, a mixture of purified liver nuclei (6×10^5), or the partially purified 23-kDa protein, Ado[methyl-3H]Met (0.125 nmoles, 20 μCi), 75 mM Tris buffer (pH 8.0), 600 mM NaCl, 0.1 μM leupeptin, 0.1 μM E-64 and the partially purified rat liver enzyme fraction (or 6×10^5 rat liver nuclei), in a final volume of 20 μl, was incubated at 37°C for 40 min. The reaction mixture was combined with 6.7 μl of 4× sample buffer (62 mM Tris-HCl buffer, pH 6.8, 2% SDS, 5% 2-ME, 10% glycerol, 0.002% bromophenol blue) and heated for 5 min. Proteins were separated on a 15% SDS-PAGE gel followed by autofluorography for 1-7 days to detect [methyl-^{3}H]-labeled proteins (Kyounghwa Lee *et al.,* 2004).

Purification of the 23-kDa methyl acceptor protein

The nucleoli were prepared from nuclei by a slightly modified version of the method of Rothblum *et al.* (1977). Briefly, nuclei were re-suspended in sucrose buffer (0.88 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.5 M PMSF, 0.1 μM leupeptin, 12 mM $MgCl_2$ and 0.1 μM E-64) and sonicated for 15 s at 30 s intervals until no intact nuclei remained. The sonicated samples were centrifuged at 1,100 *g* for 20 min. The nucleolar pellet was resuspended in the above sucrose buffer and sonicated again to disperse the chromatin.

The nucleolar pellet was resuspended in HUDE buffer (20 mM HEPES, pH 7.3, 4 M urea, 1 mM DTT, 10 mM EDTA) containing 3 M $LiCl_2$, and the suspension was homogenized with a glass/Teflon ho-

mogenizer and incubated at 4°C for 12 h, followed by centrifugation at 27,000 *g* for 30 min. The extracted protein was dialyzed in HUDE buffer containing 0.26 M KCl. The dialyzed protein was centrifuged at 30,000 *g* for 30 min. This partially purified nucleolar substrate fraction was used for purification of the 23-kDa protein.

The extracted protein (150 mg) was applied to an SP-Sepharose cation exchange column (2.6/23 cm) in HUDE buffer containing 0.26 M KCl. The proteins were eluted by a linear salt gradient from 0.26 M to 0.6 M and then by a second salt gradient from 0.6 M to 1 M KCl in HUDE buffer. Part of the eluate (0.5 ml out of 6 ml each fraction) was desalted using Centricon 15 filters (Millipore) in desalting buffer (10 mM Tris, pH 7.4, 1 mM DTT, 1 mM EDTA, 1 mM benzamidine-HCl, 0.5 mM PMSF, 0.1 μM TPCK). Desalting required 3 centrifugations at 5,000 *g* for 30 min each. Each desalted fraction was concentrated using a speed vac and used in a methyltransferase enzyme activity assay using the partially purified liver enzyme fraction, purified as described below. The active fractions were pooled.

Partial purification of the 23-kDa nuclear protein-specific protein lysine N-methyltransferase from rat liver

Rat liver tissue (150 g) was homogenized in 300 mL of TED buffer (25 mM Tris HCl, pH 8.3, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 mM PMSF, 10 nM TPCK, 10 nM E-64) with a polytron homogenizer for 3 min, and the homogenate was centrifuged at 39,000 *g* for 30 min. The supernatant was collected and centrifuged at 105,000 *g* for 1 h. The supernatant was treated with finely powdered ammonium sulfate to obtain proteins precipitating at 30-60% saturation. The precipitate was dissolved and dialyzed in 25 ml TED buffer, followed by centrifugation of the supernatant at 105,000 *g* for 1 h. The supernatant was applied to a DEAE-Sepharose (2.6/30) column equilibrated with TED buffer. The column was eluted with a NaCl gradient (0 to 0.5 M) in TED buffer. The enzyme activity was assayed with 10 μl of each fraction using the nucleolar fraction as a substrate. The fractions with methyltransferase activity were pooled and suspended in TED buffer containing 1 M ammonium sulfate. The solution was filtered through Whatman filter paper No. 6 and loaded onto a Butyl-Sepharose column (2.6 × 25 cm) equilibrated in TED buffer containing 1 M ammonium sulfate. The protein was eluted with an ammonium sulfate reverse gradient from 1 M to 0 M at a flow rate of 0.3 ml/min and 6 ml fractions were collected. The enzyme activity was assayed using 10 μl of each fraction, as described earlier, using the nucleolar protein as a substrate. The fractions with enzyme activity were pooled, glycerol (20%) was added to preserve the enzyme activity, and the fractions were stored at -70°C until use.

Amino acid analysis of ^{3}H-methyl labeled 23-kDa protein

After incubation of the reaction mixture containing the purified enzyme, Ado[methyl-^{3}H]Met and the partially purified 23-kDa protein for 1 h, the mixture was subjected to SDS-PAGE. The protein bands containing the [methyl-^{3}H]-labeled 23-kDa protein were cut from the gel and subjected to hydrolysis in 6 N HCl at 105°C for 24 h. The hydrolyzed protein was converted to PITC (phenylisothiocyanate) derivatives and mixed with PITC-amino acid standards-MMA, aDMA, sDMA, DML, TML, lysine and arginine. The PITC-amino acid mixtures were analyzed by HPLC on a μBondapak C_{18} 125 mm column, with a Waters HPLC system utilizing the Amersham Pharmacia AKTA explorer system 10. The column was maintained at 43°C and pre-equilibrated with 5% B buffer. PITC-amino acids were eluted from the column with a linear gradient at a flow rate of 1.0 ml/min. Absorbance was monitored at 254 nm. Fractions of 1.0 ml were collected and mixed with 5 ml of scintillation cocktail to quantify radioactivity.

Results and Discussion

We have previously identified specific changes in protein methylation during hepatic regeneration, and reported that the arginine methylation of a 23-kDa nuclear protein decreases during hepatic regeneration (Lee *et al.,* 1994). During the purification of the enzyme, we found another methyltransferase activity from rat liver which modified the 23-kDa nuclear protein. A rat liver homogenate was precipitated with ammonium sulfate and the enzyme was purified with DEAE-Sepharose and Butyl-Sepharose (Figure 1A and 1B). The partially purified enzyme was used for the methylation of nuclear fractions from regenerating rat liver. The methylation of a 23-kDa nuclear protein increased on the 2nd day of the partial hepatectomy, and decreased thereafter to the basal level (Figure 2).

Previous reports have shown an increase in protein arginine methyltransferase activity in regenerating rat liver (Paik *et al.,* 1975; Paik and Kim, 1980) and arginine methylation of a 20-kDa protein is increased in cancer cell lines (Gu *et al.,* 1999) and regenerating rat liver (Kwon *et al.,* 2004). These reports indicated that protein methylation related to cell proliferation or liver regeneration was on arginine residues.

Here, we determined what type of methylation is occurring in the methylation of a 23-kDa nuclear protein. When the partially purified 23-kDa nuclear protein was used as a substrate, the specific inhibitor of carboxyl O-methyltransferase, AFC (N-acetyl-S-farnesyl-cysteine), did not inhibit the activity of the partially purified enzyme and the specific activator of carboxyl O-methyltransferase, GTPγS, did not enhance the methylation, suggesting that methylation of the 23-kDa protein is not a carboxyl O-methylation (Figure 3A). This was confirmed by vapor phase

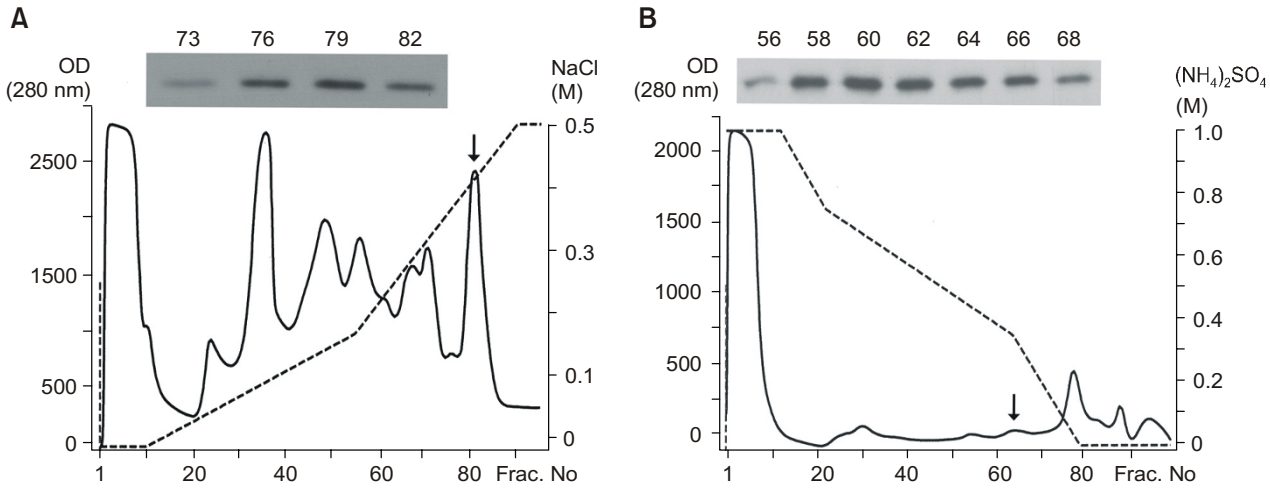

Figure 1. Purification of a methyltransferase from rat liver. The proteins in the 30-60% ammonium sulfate precipitation of the liver cytosolic fraction were fractionated by DEAE sepharose (A), and Butyl-Sepharose (B) column chromatography. The fractions containing methyltransferase activity are indicated by the arrow. The inserts show the methyltransferase activity of the fractions using the 23-kDa nuclear protein as substrate.

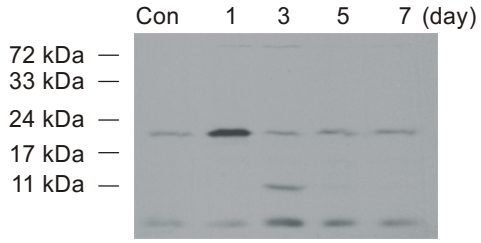

Figure 2. Changes in the 23-kDa Protein Methylation during Hepatic Regeneration. Nuclei (6×10^5) from rat liver after the partial hepatectomy were subfractionated and the proteins in the nuclei were methylated with methyl-^{3}H using the pooled enzyme fraction. Samples were separated by SDS-PAGE and the figure shows an autofluorogram of samples from control liver (Con) and regenerating liver 1 day (1), 3 days (3), 5 days (5) and 7 days (7) after the partial hepatectomy.

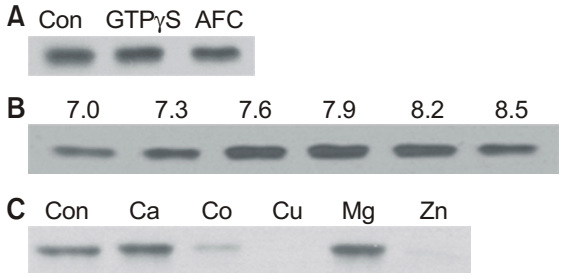

Figure 3. General properties of the partially purified methyltransferase from rat liver. (A) The effect of GTPγS (4 μM) and AFC (200 μM) was tested with the partially purified enzyme and the partially purified 23-kDa nuclear protein, and the amount of methylation was compared to controls (Con). (B) Optimum pH was determined by examining the extent of methylation of the 23-kDa nuclear protein at various pHs. The pH is indicated above each lane. (C) Effect of divalent cations, (Ca), cobalt (Co), copper (Cu), magnesium (Mg) and zinc (Zn), on the 23 kDa protein methylation was tested. Five mM of each divalent cation was included in the enzyme assay.

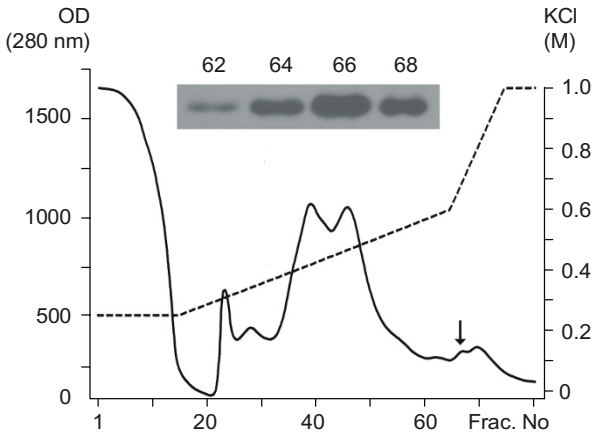

Figure 4. Purification of a 23-kDa nuclear protein by SP-Sepharose column chromatography. After the nuclear extraction from rat liver, the sample was loaded onto SP-Sepharose (2.6 × 25 cm) equilibrated with HUDE buffer containing 0.26 M KCl. A two step gradient elution was performed from 0.26 M to 1 M KCl in HUDE buffer.

analysis, as described earlier (Hong *et al.,* 1999; data not shown). The optimum pH of the purified enzyme, using the 23-kDa nuclear protein as substrate, was around pH 7.9 (Figure 3B), and the activity was inhibited by divalent cations such as Cu^{++}, Zn^{++} and Co^{++}, but not by Ca^{++} or Mg^{++} (Figure 3C).

For further characterization, the 23-kDa nuclear protein was partially purified by subnuclear fractionation and SP-Sepharose column chromatography (Figure 4). The partially purified 23-kDa protein was methylated with the partially purified enzyme and Ado [methyl-^{3}H]Met, and then separated on SDS-PAGE. The 23-kDa methyl-^{3}H-labeled protein was excised from the gel and hydrolyzed with 6N hydrochloric acid. To analyze the methylated amino acids, the

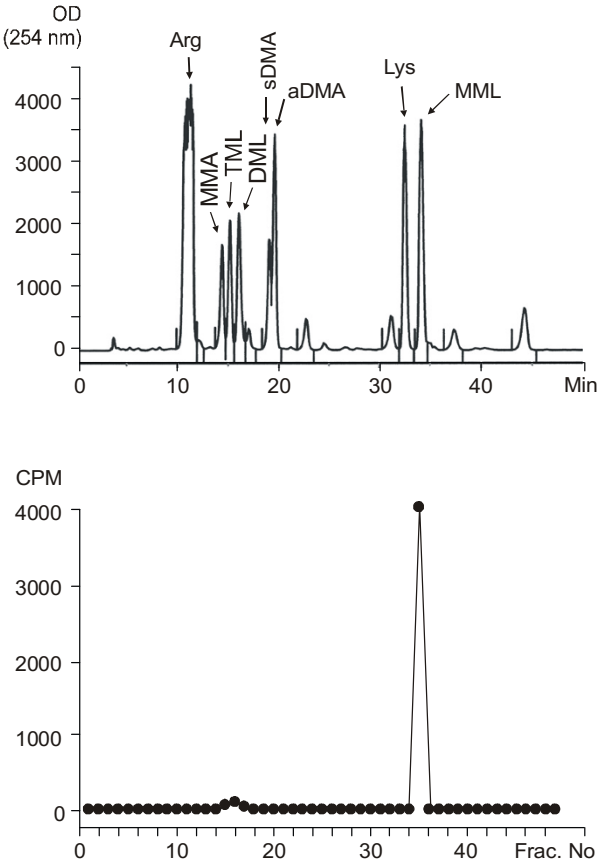

Figure 5. Determination of methylation subtype by amino acid analysis of the methylated 23-kDa protein hydrolysate. The purified 23 kDa protein was methylated with [methyl-^{3}H] by the partially purified methyltransferase. The amino acid hydrolysate of the [methyl-^{3}H]-labeled 23-kDa protein was prepared in 6 N HCl, and was labeled with PITC. The sample was mixed with PITC-labeled methylated lysine or arginine standards (each 10 μg) and analyzed by HPLC. During the HPLC separation, the fractions were collected and counted for radioactivity. Panel A shows the absorbance from the sample hydrolysate and standard amino acids during the HPLC chromatography. Panel B shows the radioactivity from each fraction. Arg, arginine; MMA, monomethyl-arginine; DML, dimethyllysine; sDMA, NG, N'G-dimethylarginine; aDMA, NG, NG-dimethylarginine.

methylated amino acids from the hydrolysate were labeled with PITC and separated by C_{18} column chromatography. The analysis was performed with internal standard methylated amino acids, and the major methylated product in the 23-kDa protein was monomethyl lysine (Figure 5), suggesting that the partially purified enzyme is a lysine N-methyltransferase.

There are several substrate-specific lysine N-methyltransferases, such as calmodulin methyltransferase and cytochrome c methyltransferase. However, the cloned lysine methyltransferases are also known to methylate histones. We tested whether the purified methyltransferase methylates histones and found that various types of histones and cytochrome c are not

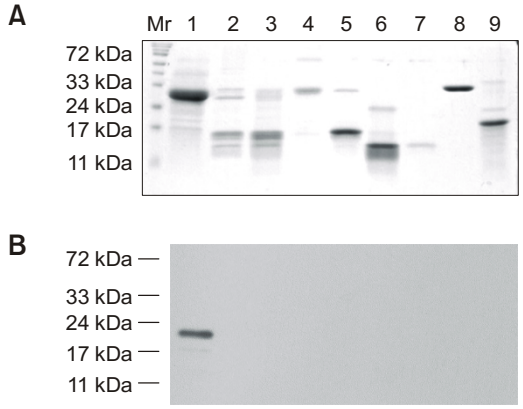

Figure 6. Substrate specificity of lysine methyltransferase from rat liver. The partially purified enzyme was used to methylate various known methyltransferase substrates (5 μg each) such as histones (lane 2, core histones; lane 3, histone type II AS; lane 4, histone type IIIs; lane 5, recombinant H3; lane 6, histone H4), cytochrome C (lane 7), hnRNPA1 (lane 8), MBP (lane 9), and the 23-kDa nuclear protein (lane 1) using Ado[methyl-^{3}H]Met as a methyl donor. After the methylation reaction, the SDS-PAGE gel was stained with Coomassie (A) and was used for autofluorography (B). Molecular weight is indicated on the vertical axis.

methylated by the purified methyltransferase (Figure 6), suggesting that the enzyme is a 23-kDa nuclear protein-specific lysine methyltransferase. Arginine methyltransferase substrates such as hnRNP A1 and MBP were not methylated by the purified enzyme, as expected.

In this study, we found that the lysine N-methylation of a 23-kDa nuclear protein was increased during early hepatic regeneration and the partially purified lysine N-methyltransferase enzyme did not methylate other known lysine methyltransferase-substrate proteins such as histones and cytochrome C, suggesting that the enzyme is a 23-kDa nuclear protein-specific lysine N-methyltransferase. These results suggest that the lysine N-methylation of the 23-kDa nuclear protein by its specific lysine N-methyltransferase plays a role in hepatic regeneration.

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