行政院國家科學委員會專題研究計畫　成果報告

蛋白質精胺酸甲基化之蛋白體分析（3/3）

計畫類別：個別型計畫
計畫編號：NSC93-3112-B-040-001-
執行期間：93 年 05 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學生物醫學科學學系

計畫主持人：李娟

報告類型：完整報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中　華　民　國 94 年 10 月 27 日

目錄

1. Lin, C-H, Huang, H.-M., Hsieh, M,. and **Li, C.** (2002) Arginine methylation of recombinant murine fibrillarin by protein arginine methyltransferase. **J. Prot. Chem.** 21:443-445.
2. Huang, H.-M., Tam, MF, Tam, T-C S, Chen, D-H, Hsieh, M,. and **Li C.** (2002) Proteomic analysis of stable protein methylation in lymphoblastoid cells. **J. Biochem.** 132: 813-818.
3. Chen, D-H, Wu, KT, Hung, CJ, Hsieh, M, and **Li C.** (2004) Effects of Adenosine Dialdehyde Treatment on *in vitro* and *in vivo* Stable Protein Methylation in HeLa Cells. **J. Biochem.** 136, 371-376.
4. Hung, CM and **Li, C**. (2004) Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians. **GENE**. 340, 179-187.

中文摘要

後基因體時代的一個主要的挑戰就是研究細胞中全部表達出來的蛋白質—也就是蛋白體。轉譯後之修飾使蛋白質的複雜度提昇，不只僅是二十個氨基酸之排列組合而已。本計劃就是要用蛋白體的研究方法來分析一種轉譯後的修飾—蛋白質精胺酸甲基化，主要為使用蛋白質體的方法二維電泳後以質譜來分析。我們也已成功的找到一些已知的甲基接受蛋白和某些可能的新甲基接受蛋白。此計劃我們致力於以下四個方向：（一）蛋白質體法整體性的分析甲基接受蛋白。（二）分析所有蛋白質精胺酸甲基脢在生物界中存在情形。（三）探討透過上述的方法找出之新甲基接受蛋白質的特性。（四）我們將藉由蛋白質體的研究方法法來分析不同生物醫學樣本的精胺酸甲基接受蛋白，以找出蛋白質精胺酸甲基化和人類特殊疾病主要為自體免疫疾病之間的相關性。透過這些蛋白質體的研究我們將可明確得知甲基接受蛋白精胺酸甲基化的功能，使我們能更加瞭解此種轉譯後修飾所扮演的的角色。

關鍵詞: 蛋白體，蛋白質精胺酸甲基化，蛋白質精胺酸甲基轉移脢，轉譯後修飾

Abstract

In the post-genomic era the major challenge is to investigate the complete expressed proteins in cells, the proteome. Posttranslational modifications increase the complexity of proteins beyond the combination of twenty amino acids. The project will investigate one type of protein posttranslational modification— protein arginine methylation using the proteomic tools, basically two dimensional gel electrophoresis followed by mass spectrometry to identify specific protein spot. We have started the investigation of the methylaccepting proteins using proteomic approach and successfully identified some known and novel putative methylaccepting proteins. In this project we focus on four areas. Firstly, we perform global analysis of protein methylaccepting proteins by the proteomic approach. Secondly, we analyze protein arginine methylacceptors globally in subcellular localization. Thirdly, we characterize the novel protein methyl- acceptors identified through the above approaches. At last, we analyze the relationship of protein arginine methyl- -ation with specific human diseases by proteomic analyses of the arginine methylaccepting proteins in different biomedical samples. Through these studies we will identify, and specify the function of arginine methylation of the methylaccepting proteins through the proteomic approach, to help to better understand the roles of this posttranslational modification.

Keywords: proteomics, arginine methylation, protein arginine methyltransferase, posttranslational modification

Introduction

Protein arginine methylation is an irreversible posttranslational modification involved in various cellular functions such as signal transduction, protein subcellular localization, transcriptional regulation and protein–protein interactions (Bedford and Richard, 2005; McBride and Silver, 2001). Since the identification of the first protein *N*-arginine methyltransferase (PRMT) gene PRMT1 (Lin et al., 1996), by now at least eight PRMT genes have been identified in the mammalian system and seven of them has direct or indirect evidences to be able to catalyze the transfer of methyl groups from AdoMet to the side chain ω–guanido nitrogens of arginine residues in protein substrates. The PRMT activity can be further divided into type I and type II, depending on the catalyses of formation of asymmetric N^G,N^G –dimethylarginines (aDMA) or symmetric N^G,N'^G–dimethylarginine (sDMA) residues respectively (Gary and Clarke, 1998).

Even though the protein N-arginine methyltransferase activities have been described in the 1960s, the first PRMT gene was not identified until 1996 as an immediate-early gene TIS21-interacting rat protein by a yeast two hybrid screen (Lin et al., 1996). The human counterpart of the PRMT1 gene was later identified as an interacting protein with the cytoplasmic domain of interferone α/β receptor (IFNR1), that has been shown to be the docking site of a few signaling proteins including proteins in the Jak-Stat pathway. Since cells rendered deficient in PRMT1by antisense oligonucleotides become more resistant to growth inhibition by IFN, methylation of proteins by PRMT1 may be a signaling mechanism complementing protein phosphorylation (Abramovich et al., 1997). PRMT1 appears to be the major protein N-arginine methyltransferase in cells that preferred the arginine and glycine rich domain (GAR or RGG box) in numerous RNA binding proteins including hnRNP and

nucleolar proteins fibrillarin and nucleolin. The methyltransferase modifies arginine residues, in appropriate protein substrates, to form NG-monomethyl and NG,NG-dimethylarginine (asymmetric). The concensus PRMT1 methylation sequence has been suggested. However, other sites have been identified as the GXR motifs and more recently, some without no known sequence (Smith et al., 1999).

PRMT1 knock mice model was reported by Pawlak et al., 2000 in their screen for developmentally regulated genes. Homozygous mutant embryos failed to develop beyond E6.5, a phenotype consistent with a fundamental role in cellular metabolism. Expression of the Prmt1-betageo fusion gene was greatest along the midline of the neural plate and in the forming head fold from embryonic day 7.5 (E7.5) to E8.5 and in the developing central nervous system from E8.5 to E13.5. However, Prmt1 was not required for cell viability, embryonic stem (ES) cell lines established from mutant blastocysts were viable. Low levels of Prmt1 transcripts (approximately 1% of the wild-type level) were detected as assessed by a quantitative reverse transcription-PCR assay. Total levels of arginine N-methyltransferase activity and asymmetric N(G), N(G)-dimethylarginine were reduced by 85 and 54%, respectively, while levels of hypomethylated substrates were increased 15-fold. Prmt1 appears to be a major type I enzyme in ES cells, and in wild-type cells, most substrates of the enzyme appear to be maintained in a fully methylated state.

Besides the TIS21 and IFNR1, the original bait proteins that fish out PRMT1, PRMT1 has been reported to be interacting with many other proteins. Using PRMT1 as a bait, PRMT3, another PRMT gene with substantial sequence similarity to PRMT1 (Tang et al., 1998), and ILF3 (interleukin-enhancer-binding factor 3) were identified to be putative PRMT1-interacting proteins. Interestingly, recent investigations shown that an transcription activator YY1 recruit PRMT1 through DRBP76 (ILF3) to activate transcription (Rezai-Zadeh et al., 2003).

Three alternatively spliced HRMT1L2 transcripts with variable 5'-ends were observed, encoding proteins of 343, 347, and 361 amino acids, respectively. HRMT1L2 maps to human chromosome 19q. (Scott et al, 1998). Scorilas (2000) described the genomic structure of PRMT1 with 12 coding exons. Three PRMT1 isoforms exist as a result of alternative mRNA splicing. Amino acid sequence comparison of the splicing variants indicates that they are all enzymatically active methyl transferases, but with different N-terminal hydrophobic regions. but alternative splicing in the N-terminus. PRMT1 is not only the PRMT1 gene that is first identified, it is the most predominant as well as most versatile PRMT involved in many different aspect of cell activities reported.

PRMT3 was first identified by yeast two hybrid screen of PRMT1-interacting proteins. Recombinant GST- PRMT3 fusion asymmetrically dimethylates arginine residues present both in the designed substrate GST-GAR, thus is a functional type I protein arginine N-methyltransferase. However, rat PRMT1 and PRMT3 glutathione S-transferase fusion proteins have distinct enzyme specificities. PRMT3 is present as a monomer in RAT1 cell extracts, in contrast to PRMT1 is present in an oligomeric complex. Immunofluorescence analysis localized PRMT1 predominantly to the nucleus of RAT1cells. In contrast, PRMT3 is predominantly cytoplasmic. Frankel and Clarke (2000) further showed that the zinc-liganded form of a zinc-finger domain in amino terminus of PRMT3 is required for the enzyme to recognize RNA-associated substrates in RAT1 cell extracts. The recombinant form of PRMT3 is inhibited by high concentrations of ZnCl(2) as well as N-ethylmaleimide, reagents that can modify cysteine sulfhydryl groups.

CARM1/PRMT4 is a coactivator-associated arginine methyltransferase that binds to the p160 family of steroid receptor coactivatorsand has been shown to enhance the transcriptional activation of nuclear receptors (Chen et al., 2000). Even

though a few substrates have been identified, no consensus substrate specific sequence can be suggested.

PRMT6 was first report by Frankel et al., 2002. The novel gene has been found on chromosome 1 and the polypeptide chain of PRMT6 is 41.9 kDa consisting of a catalytic core sequence common to other PRMT enzymes. Glutathione S-transferase fusion PRMT6 demonstrates type I PRMT activity, capable of forming both omega-N(G)-monomethylarginine and asymmetric omega-N(G),N(G)-dimethylarginine derivatives on the recombinant glycine- and arginine-rich substrate. A comparison of substrate specificity reveals that PRMT6 is functionally distinct from two previously characterized type I enzymes, PRMT1 and PRMT4. In addition, PRMT6 displays automethylation activity; it is the first PRMT to do so. HIV Tat 1 protein and HMGA proteins have been reported to be specifically methylated by PRMT6.

Other :PRMT2/HRMT1L1 and HRMT1L3: PRMT2/HRMT1L1 was the first identified PRMT1 homologue by Katsanis et al. (1997) and by Scott el al. (1998) probably due to its localization on the telomere of chromosome 21q, the chromosome with high resolution map and sequence available early. It contains an Src homology 2 domain at its N-terminus. However, no methyltransferase activity can be detected in the recombinant protein (Scott el al., 1998). A mammalian hnRNP E1B-AP5 has been reported to be methylated in vivo in its Arg-Gly-Gly (RGG)-box domain, but HRMT1L2 (hPRMT1) did not detectably methylate endogenous E1B-AP5. However, HRMT1L1 (PRMT2) was identified as one of the proteins interacting with E1B-AP5. It is thus suggested that HRMT1L1 is responsible for specific E1B-AP5 methylation in vivo (Kzhyshkowska et al., 2001, Biochem.J). Interestingly, PRMT2 was identified as a estrogen receptor α binding protein and can activate EraAP-1 and AP-2 as well as PR, PPARγ, and RARα-mediated transcriptional activity (Chao et al., 2002). The

potential methyltransferase activity appeared to be important for the coactivator function.

No detailed description of HRMT1L3 has been published. It is on human 12p13 highly homologous with PRMT1 with 80% amino acid sequence identity and even conserved genomic structure (Zhang and Cheng, 2003). Its mouse ortholog was recently identified in a neural gene screen. Its expression in developing and mature nervous system is reported (Aubert et al., 2003, PNAS).

For the Type II PRMT, the PRMT5 gene was first identified as a Shk1 protein kinase binding protein in Schizosaccharomyces pombe as skb1 (Gilbreth et al., 1996). It can form ternary complex with Shk1 and cdc42/Rac-activated kinase. The skb1null mutant sre less elongate than wild-type cells and moderate growth defect. Overexpression of Shk1 can suppress skb1 delation mutation and overexression of skb1 cause wild-type S. pombe cells to become hyperelongated. The human skb1 homolog, SKB1Hs cab functionally replace skb1 in S. pombe. The Skb1 negatively regulate mitosis partially dependent on Shk1 (Gilbreth et al., 1998). Until 1999 the protein methyltransferase activity was suggested of the human homologue (Pollack et al., 1999) due to its conserved methyltransferase domain and in vitro methylation of histone H2A, H4 and myelin basic protein. It is located on chromosome 14q11.2-21 and was found to be interacting with Jak 2 and was designated as JBP1. The yeast homologue of human JBP1, Hsl7p methylate similar protein substrates (Lee et al., 2000). However, the type II activity of PRMT5 was not confirmed until 2001 by Branscombe et al., 2001). The skb1 protein was also identified as a pICln interacting protein (Krapivinsky et al., 1998). The complex was later found to interact with snRNP core proteins SmD1, D2, D3, X5 and SmB/B' (Schmarda, A). The 20S complex containing JBP1 and pICln has later been designated as "methylsome" by Friesen et al. (2001, MCB) to produce dimethylarginine modified Sm proteins. It was

reported previously that the survival moter neuron (SMN) protein, the spinal muscular atrophy disease gene product, binds preferentially to the sDMA modified SmD1 and D3. The 20S methyltransferase complex contains the unmodified but not sDMA modified SmD1 and D3. Methylation of Sm proteins by the methylosome directs Sm proteins to the SMN complex for assembly into snRNP core particles. PRMT5 was reported to negatively regulate transcription (Fabbrizio et al., 2002). Recently, PRMT5 is association with Brg1 and hBrm-based hSWI/SNF complexes (Pal et al., 2003). hSWI/SNF-associated PRMT5 can methylate hypoacetylated histones H3 and H4 more efficiently than hyperacetylated histones H3 and H4. Protein-protein interaction studies indicate that PRMT5 and mSin3A interact with the same hSWI/SNF subunits as those targeted by c-Myc. Brg1, mSin3A, HDAC2, and PRMT5 are directly recruited to the cad (a c-Myc target gene) promoter. These results suggest that hSWI/SNF complexes, through their ability to interact with activator and repressor proteins, control expression of genes involved in cell growth and proliferation.

Recently Miranda *et al*. demonstrated that PRMT7 catalyzed the formation of ω–N^G monomethylarginine in an arginine/glycine containing peptide but not in known protein substrates (Miranda et al., 2004). However, Lee at al. showed that PRMT7 is a new type II protein arginine methyltransferase capable for sDMA modification (Lee at al., 2004).

Different mammalian protein arginine methyltransferases are localized in different subcellular compartments and appear to have their own substrate sets and physiological roles. For example, the predominant PRMT1 appears to be located mainly in the nucleus (Tang et al., 1998). Its physiological substrates include histone H2A, many hnRNPs, fibrillarin and Sam 68 (Lin et al., 1996; Tang et al., 1998; Mowen et al., 2001; Lee and Bedford, 2002; Wada et al., 2002). The other widely

studied CARM1/PRMT4 were first identified as a coactivator-associated arginine methyltransferase (CARM) localized in the nucleus (Chen et al., 1999). Identified PRMT4 substrates include histone H3 (Chen et al., 1999), p300/CBP (Xu et al., 2001), PABP1 (Lee and Bedford, 2002) and an mRNA –stabilizing protein HuR (Brahms et al., 2000). The JBP1/PRMT5 substrates include myelin basic protein (Pollack et al., 1999; Branscombe et al., 2001; Rho et al., 2001), SmD1, D3 (Brahms et al., 2000), Sm B/B', Lsm4 (Brahms et al., 2001) and coilin (Hebert et al., 2002). Even though cytoplasmic localization of PRMT5 has been suggested (Rho et al., 2001; Frankel et al., 2002), the interactions of PRMT5 with complexes involved in transcriptional regulations (Fabbrizio et al., 2002; Kwak et al., 2003; Pal et al., 2003) indicate that certain portion of the protein should be present in the nucleus also.

In comparison with eight members in mammalian PRMT family, there are only two related protein arginine methyltransferase genes in yeast *S. cerevisiae.* RMT1/HMT1 corresponds to the type I methyltransferase activity (Gary et al., 1996; Henry and Silver, 1996) while Hsl7p is responsible for the type II activity (Lee and Bedford, 2002). The survival of yeast HMT1 or Hsl7 mutants indicates that they are not essential genes (Gary et al., 1996; Gilbreth et al., 1996; Henry and Silver, 1996). On the contrary, mice null for PRMT1 die early during embryogenesis (Pawlak et al., 2000) and mice deficient in PRMT4/CARM1 show neonatal lethality (Yadav et al., 2003), indicating that these genes play critical roles in mammals.

Nine PRMT genes (DART1-9) have been identified in *Drosophila* (Boulanger et al., 2004). However, only DART1, 4, 5, and 7 are the putative homologues of mammalian PRMT1, 4, 5, and 7. Other DART family members have closer resemblances to PRMT1 but do not have identifiable mammalian homologous. In our recent study, orthologues of the eight human PRMT genes in two fish model systems pufferfish *Fugu rubripes* and zebrafish *Danio rerio* were identified based on their

amino acid sequence homology (Hung and Li, 2004). In addition, the PRMT genes are found in a simplified form with two family members (PRMT2 and HRMT1L3/PRMT8) missing in *Ciona intestinalis*, a plausible approximation to the ancestral chordates for the origin of vertebrates and chordate. Paralogous vertebrate genes such as gene families for cell signaling (such as the fibroblast growth factor) and transcription factors (such as SMAD) often match to a single gene in *C. intestinalis* (*Ci*, Dehal et al., 2002). Interestingly, the missing PRMT1 (HRMT1L2) homologue HRMT1L3/PRMT8 has identical exon junctions and share high sequence identity with PRMT1 in human and fish. It is likely that PRMT8 occurred by gene duplication of PRMT1 after the divergence of the vertebrate ancestors from other chordates and can be viewed as a paralogue of PRMT1 in the vertebrates. It is interesting that we could not identify PRMT2 in *Ciona* also. We found that phylogenetically PRMT6 and PRMT2 converged in one lineage parallel to the PRMT1/3 lineage. The two PRMTs are the least conserved PRMT from fish to human, with about 50% sequence identity. Whether the two PRMT genes are evolved at a higher rate is an interesting issue. However, the *N*-terminal SH3 domain before the catalytic core of PRMT2 and the characteristic one coding exon in PRMT6 is conserved from fish to human. Surprisingly, we found that *Ci* PRMT6 contains six exons, indicating that the one coding exon character of PRMT6 is specific only in the vertebrates. It is attractive to propose that PRMT2 is evolved from a duplication of a common ancestor of both PRMT6 and PRMT2 in the early vertebrates but are diverged with faster rates than other PRMT genes. By now no direct methyltransferase activity has been demonstrated for PRMT2 and no physiological importance of PRMT6 is known. Whether they are of less physiologically importance thus accumulate mutations much faster than other PRMT genes is an interesting question.

In view that this type of posttranslational modification plays important roles in cells, identification of arginine methylaccepting proteins ought to be an important task. The arginine methylaccepting substrates were identified and studied through different approaches. Proteins such as fibrillarin (Lischwe et al., 1985), nucleolin (Lapeyre et al., 1986), and hnRNPA1 (Rajpurohit *et al*. 1994a) were identified to contain asymmetric dimethylarginines through direct biochemical analyses. Other proteins with similar arginine and glycine rich motifs, mostly RNA binding proteins such as herpes simplex virus ICP27 protein (Mears and Rice, 1996), FGF2 (Klein et al., 2000) and EWS (Belyanskaya et al., 2001), have also been identified as methylaccepting substrates. Recently, proteins without typical arginine and glycine rich sequences have been found to contain methylarginines. The methyl group on the PABPII protein was identified on a RXR motif (Smith et al., 1999) while the methylarginine in STAT1 is located in the EIRQY context (Mowen et al., 2001).

Alternatively, cells were made deficient in methyltransferase activity either by genetic approaches (Pawlak et al., 2000; Frankel and Clarke, 1999) or by chemical treatment (Najbauer et al., 1993). The methylaccepting sites would not be occupied in vivo and can be detected by further in vitro methylation reactions and analyzed by gel electrophoresis. Although previous studies had utilized two-dimensional (2-D) gel electrophoresis for product analyzes, the methylated proteins revealed after fluorography could not be identified unambiguously. Candidate proteins were only inferred through their molecular mass and pI (Najbauer and Aswad, 1990; Najbauer et al., 1993; Frankel and Clarke, 1999).

In this study, we treated lymphoblastoid cells with an indirect methyltransferase inhibitor adenosine dialdehyde (AdOx). After in vivo or in vitro methylation reactions, cell extracts were separated by 2-D electrophoresis. Proteins of interest were excised from the gel, digested with specific protease and subjected to

MALDI (matrix assisted laser desorption ionization)-TOF (time of flight) mass spectroscopy for protein identification. With this approach, we have confirmed some previously known methylaccepting proteins and identified some novel putative methyl-accepting proteins. Furthermore, we performed global analysis of protein methylaccepting proteins by the proteomic approach. Basically, the pattern of western blot detected by methylarginine specific antibody was compared with that of total protein stain. Specific protein spots were picked, digested and analyzed by mass spectrometry. Interesting putative methylaccepting proteins were under investigation. We also fractionated mouse brain extract proteins by a chromatophocusing Mono P column with a pH 9-6 gradient in view to resolve basic proteins with the methyl-accepting arginine and glycine rich (GAR) sequences.

Materials and Methods

Culture and fractionation of cells

Lymphoblastoid cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37 ^{o}C in a 5% CO_2 incubator. Treatment of cells with 20 M of adenosine dialdehyde (AdOx) for 72 hours was performed as described (Li *et al.*, 1998). The cells were harvested and subcellular fractionations were prepared as described by Lin *et al* (2000). Basically, cells were washed with PBS and lysed in buffer containing 10 mM Tris-HCl, pH=7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 100 mM NaF, 10 ng/ml aprotinine, 1 mM PMSF, 1 mM dithiothreitol, 10 U/ml RNasin and 0.5% NP-40. The cells were disrupted by passages through hypodermic needles (22 G) for about 10 times. The pellet obtained by the centrifugation at 1000 g for 5 min was the nuclear fraction. The supernatant was further centrifuged at 100,000 g for 60 min to pellet the ribosomal fraction and to obtain the supernatant cytosolic fraction. Alternatively, cells collected from a 75 cm^2 culture flask were resuspended in 600 μl of 2-D extraction buffer (100 mM ammonium carbonate, pH 8.0; 0.5 mM PMSF; 2 mM EDTA; 2% NP-40). Cells were incubation on ice for ten minutes then lysed by sonication. Cell debris was removed by centrifugation at 12,000xg for 20 min. Proteins in the extracts were quantified by BCA assay (Pierce) with bovine serum albumin as standard. Extracts thus prepared gave poor methylaccepting signals after the *in vitro* methylation reaction. We thus followed the protocol of Li *at el*. (2) to prepare the extracts for methylation reaction using the extraction buffer (phosphate-buffered saline [10 mM dibasic sodium phosphate, 1.8 mM monobasic potassium phosphate, 140 mM NaCl, 2.7 mM KCl, pH7.4], 5 % glycerol, 1 mM disodium EDTA, 1 mM EGTA, 40 μg/ml leupeptin and aprotinin, 20 μg/ml pepstatin, 1 mM PMSF, 0.5% of Triton X-100).

For studies using HeLa cells (ATCC CCL-2), cells were grown in MEM medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37 ^{o}C in a 5% CO_2 incubator. Treatment of cells with AdOx for various time periods was performed as indicated. The cells were harvested and washed with phosphate-buffered saline, resuspended in buffer A (phosphate-buffered saline with 5% glycerol, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.5% Triton X-100 and Complete protease inhibitor cocktail [Roche]). Adenosine dialdehyde (AdOx, Sigma) was added to the medium at the indicated final concentration. Cycloheximide (CHX, Sigma) and N-acetyl-leu-leu-norleucinal (ALLN, Sigma) were added to the culture medium at the final concentration of 100 μg/ml and 15 μM respectively.

Preparation of Recombinant Proteins

Recombinant glutathione S-transferase-yeast RMT1 or rat PRMT1 fusion enzyme was prepared from IPTG-induced, pGEX-RMT1 or pGEX-PRMT1 transformed *E. coli* DH5 cells. The expression and purification of the GST-RMT1 and GST-PRMT1 fusion protein were performed as described (Gary *et al.*, 1996; Lin *et al.*, 1996). Recombinant mouse fibrillarin with six histidine tag was expressed in pET28-fibrillarin transformed JM109 (DE3) cells induced with IPTG at 25 ^{o}C. Recombinant protein was purified under denaturing conditions through a Ni-NTA column (Qiagen) according to the procedures described by Pearson *et al.* (1999). For other putative arginine methylaccepting proteins, the cDNA clones were obtained from RZPD (Germany) and subcloned into pGEX4 vector. Recombinant GST-fusions were then purified in similar ways.

In vitro protein methylation and fluorography.

Total extracts (100μg proteins) were incubated with 10 μCi of [*methyl*-^{3}H]-AdoMet

(60 Ci/mmol, Amersham) in the presence or absence of recombinant yeast GST-RMT1 arginine methyltransferase in methylation reaction buffer (50 mM sodium phosphate, pH7.5, 1 mM EDTA, 1 mM EGTA). The final volume was 70 µl and reaction was carried out for two hours at 30 ^{o}C. The reaction was stopped by incubating on ice. If the reaction products were going to be analyzed by 2D-electrophoresis, they were then subjected to dialysis using a mini dialysis unit (MW cut off 3,500; Pierce) for salt removal. If recombinant fibrillarin was sued as the methylaccepting substrate, it was incubated with *S*-adenosyl [*methyl*-^{3}H] L-methionine (60 Ci/mmol, Amersham) for a final concentration of 1µCi/µl reaction in 25 mM of Tris (pH=7.5), 1 mM of EDTA and 1 mM of EGTA in the presence of arginine methyltransferase sources. After indicated time of incubation at 30 ^{o}C, the reaction was terminated by the addition of one third of the volume of 3X SDS sample buffer. After gel electrophoresis, the polyacrylamide gel was stained with Coomassie brilliant blue, destained, and soaked in EN^{3}HANCE (Du Pont NEN) for an hour and then in water for 30 minutes as suggested by the manufacturer. The gel was dried and exposed to X-ray film (Kodak, MS) at -75 ^{o}C for 7 days if not indicated otherwise.

In Vivo Methylation

HeLa cells grown to near confluency were treated with AdOx for the indicated times. Cycloheximide (100 µg/ml) and chloramphenicol (40 µg/ml) were added 30 min prior to the labeling. For *in vivo* methylation, the medium was replaced with DMEM medium without methionine (GIBCO), 10% FBS (GIBCO, dialyzed), [*methyl*-^{3}H] methionine (10 µCi/ml) and translation inhibitors for the indicated labeling time. The cells were then collected, lysed by SDS-PAGE sample buffer and analyzed by SDS-PAGE and fluorography.

Two-dimensional gel electrophoresis.

Samples (1 mg) were loaded onto immobilized pH gradient (IPG) strips (pH3-10, 13 cm) through overnight rehydration at room temperature in a reswelling tray. Samples were prepared by lyophilization then resuspension in 250 µl of 8 M urea, 2% (w/v) CHAPS, 2% IPG buffer and 2.8 mg/ml dithiothreitol. Isoelectrophocusings (IEF) were carried out in a Multiphor II system as instructed by the manufacturer (Amersham Biosciences). Alternatively, the IPG strips were resolved by IPGphore (Amersham Biosciences) as instructed by the manufacturer. Upon completion of IEF (17,000 Volt-hours), the IPG strips were equilibrated in 50 mM Tris-HCl, pH8.8, 6 M urea, 30% glycerol, 2 % (w/v) SDS, 15.4 mg/ml dithiothreitol and 0.0125 % bromophenol blue for 15 min. The strips were then stored frozen at –80 ^{o}C or used immediately. Gradient SDS polyacrylamide gels (5-20 %, 17x20x1 mm) were employed for the second dimensional separation in a Bio-Rad PROTEAN II xi system. Proteins were visualized by colloidal blue staining for 2 days and destaining was carried out in 25% methanol (32). Z3 (Compugen) and Melanie 3 (Genebio) softwares were used to analyze the gel images. Gels containing isotopically labeled proteins were treated with EN^{3}HANCE (Du Pont NEN), dried and exposed to X-ray film (Kodak, MS) at -75 ^{o}C. Since silver-stained gels gave poor fluorographic results and large gels tended to crack after drying, we used colloidal blue staining and 13 cm IPG strips for the analyses of radioactive samples.

In gel protein digestion.

Protein spots were excised from the polyacrylamide gel with pipette tips and the gel plugs were washed extensively in 10% acetic acid and 50% methanol in water. They were then incubated in 200 mM Tris, pH 8.8, 50% acetonitrile for 30 min. The buffer was removed and the gel plugs were dehydrated by adding acetonitrile followed by

vacuum centrifugation. Lys-C protease solution (Wako, 0.033 mg/ml in 100 mM ammonium bicarbonate buffer, pH 9.2) or trypsin solution (Promega, 0.033mg/ml in 50 mM Tris-HCl pH 8.0, 2 mM $CaCl_2$) was then added to restore the gel plugs to their original volume. Digestion was carried out at 42 and 25 ^{o}C overnight for Lys-C and trypsin proteases, respectively. The digests were then acidified by adding 2% TFA. Peptides were extracted from the gel plugs sequentially with 0.1% TFA in water, 30% acetonitrile in water with 0.1%TFA and finally 60% acetonitrile in water and 0.1%TFA. The solutions were combined and dried to less than 50 l then desalted with a Ziptip (Millipore) pipette tip following the manufacturer's instructions.

Endoprotease Lys-C digestion

Methylated fibrillarin protein (5 μg in a 20 μl reaction volume) was separated from the enzyme source by the adsorption to the Ni-NTA resin (Qiagen , 10 μl). The washed pellet was then resuspended in 100 mM $(NH_4)_2 CO_3$ (pH=9.2). Lys-C (Wako) was added in the final concentration of 0.033 mg/ml. Digestion was performed at 42 ^{o}C overnight.

MALDI-TOF MS analysis.

Peptide mass mapping was performed on a Bruker (Bruker-Daltonics, Bremen, Germany) REFLEX III time-of-flight mass spectrometer equipped with a SCOUT source and delayed extraction. Detection was set in positive ion reflector mode with each mass determination being an average of 100 spectra. Samples for mass measurement were prepared using the solution-phase nitrocellulose method (33) with α-cyano-4-hydroxycinnamic acid as matrix. Alternatively, samples were deposited on a Bruker 600 micron AnchorChip according to the manufacturer's instructions. Angiotensin II (1045.54 amu), adrenocorticotropic hormone fragment 18-39 (2465.73 amu) and somatostatin (3146.47) were used as external calibration standards. The

peptide mass spectra obtained by the MALDI-TOF analyses were analyzed using PeptidIdent (http://tw.expasy.org) and Profound (http:// 129.85.19.192/profound/).

Western Blotting.

Equal amounts of protein (30 μg) were separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Gelman Science). The membranes were blocked in 5% skimmed dry milk in TTBS (10 mM Tris-HCl, pH=7.5; 100 mM NaCl; 0.1% tween 20) for 30 min, incubated with primary antibodies (1:200 dilution for 7E6 antibody from Abcam; 1:550 dilution for SYM10, 1:900 dilution for SYM11 and 1:900 dilution for ASYM24 from Upstate) for 1 h, washed three times in TTBS, then incubated with secondary antibody (anti-mouse or rabbit IgG horse radish peroxidase conjugate from Sigma) for 1 h. Chemiluminescent detection was performed using the Supersignal kit according to the manufacturer's instructions (Pierce).

Database searches and analyses

Searches for the homologues of human PRMT genes (*PRMT1v3*: NP_938075; *PRMT2*: NP_001526; *PRMT3*: XP_058460; *PRMT4/CARM1*: XP_032719; *PRMT5/SKB1*: NP_006100; *PRMT6*: AAK85733; *PRMT7*: NP_061896;*HRMT1L3*: NP_062828) were performed by tblastn against *Fugu rubripes* or *Ciona intestinalis* genome databases. Putative *Fugu* or *Ciona* PRMT homologues were identified by GeneScan (http://genes.mit.edu/GENSCAN.html) and GenomeScan (http://genes.mit.edu/GENSCAN.html) for gene prediction with human PRMT protein sequences as the templates. The predicted *Ciona* PRMT was further subjected to blastn against est_others database of NCBI. The matched *Ciona* EST sequences were then retrieved and assembled. Searches for the zebrafish PRMT genes were performed

by tblastn against both zebrafish EST database and HTGS database using human PRMT protein sequences as the templates. The putative PRMT genes were predicted by similar approaches used above.

Alignment and Phylogenetic analyses of the protein arginine methyltransferase

Multiple Sequence alignments were performed with clustalX (1.83) (Thompson et al., 1997) and the output of the alignments was formatted by CHROMA (Goodstadt and Ponting, 2001). The phylogenetic tree was calculated using Neighbor-Joining method (Saitou and Nei, 1987) integrated in clustalX, then visualized with Treeview (v1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results and Discussion

The effects of adenosine dialdehyde treatment on in vitro and in vivo Stable Protein Methylation in HeLa Cells

Treating cells with a methyltransferase indirect inhibitor AdOx that would accumulate methylaccepting proteins in the hypomethylation states was one of the most important tools we would use. However, this approach was limited to *in vitro* methylation. We used lymphoblastoid cells as our study system previously. When we were studying the subcellular localization of the methylarginine containing polypeptides, we used HeLa cells since they were easier to handle for microscopic analyses. We thus determined the suitable treatment conditions of AdOx to HeLa cell, and performed more experiments to demonstrate that AdOx can be applied to *in vivo* mehtylation studies. We described our interesting results in a manuscript titled "The effects of adenosine dialdehyde treatment on *in vitro* and *in vivo* Stable Protein Methylation in HeLa Cells", which is published in Journal of Biochemistry (2004). In this paper we also suggested that the methylaccepting proteins accumulated upon AdOx treatment are most likely to be from the newly synthesized proteins in the incubation period. Our results also indicated that protein methylation, mostly arginine methylation, occurred soon after its synthesis, and the methylated proteins were rather stable. This investigation also provides us with a good experimental system for future studying of *in vivo* methylated proteins proteomically.

Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians

We found that there are eight PRMT genes in human, although only six of them had been designated as PRMT1-6 previously. We searched for the human, mouse, as

well two other fish model system puffer fish *Fugu rubripes* and zebra fish *Danino rerio* and an invertebrate chordate ascidian *Ciona intestinnalis*. The results of the data mining and the analyses of the putative PRMT genes in these systems are in a paper "*Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians*", now published by GENE (2004). The PRMT genes we identified in both fish model system are conserved not only in the amino acid sequences, but also in the genomic structure. We also constructed a phylogenetic tree with these data and it correlated perfectly with the type I and type II activity divisions of PRMT. Besides, We cloned the human PRMT6 as well as the zebrafish and *Fugu* PRMT6 gene, both differ from other vertebrate PRMT genes, contain only one coding exon. We were able to express both PRMT6 as GST fusion in *E. coli* and detected the methyltransferase activity.

Proteomic analyses of arginine methylaccepting proteins and basic proteins

Previous investigations indicated that most of the methylaccepting proteins are basic RNA binding proteins containing arginine and glycine rich (GAR) motifs. In order to analyze the arginine methylaccepting proteins, in this study we used proteomic approaches including two-dimensional (2-D) electrophoresis, chromatofocusing chromatography (Mono P column) and MALDI-TOF-MS analysis to identify these proteins. In the lst part, HeLa cell extracts were separated by the 2-D electrophoresis and then detected by mono- and di-methylarginine specific antibody 7E6. We compared the patterns of the protein stain and the western signals and found some matched spots (Fig. 1). The spots were cut, endoprotease in-gel digested, the fragments were analyzed by MALDI-TOF-MS and MS/MS. We identified the spots by the help of online programs (Mascot). The identification of the previously reported methylarginine-contains protein such as hnRNP A2/B1, hnRNP A1, hnRNP G, Sam68 and FUS gycline rich protein indicated our approach is feasible. In addition, our data

suggested hnRNP M and 54 kDa nuclear RNA- and DNA-binding protein (p54nrb**)** are putative methylaccepting proteins (Table 1). We got further evidence of the methylation of recombinant hnRNP M and the peptide with the RGG sequence in the hnRNP M protein. In the 2nd part, we fractionated mouse brain extracts by a chromatofocusing Mono P column with a pH 9-6 gradient. As few proteins were eluted, we further treated the brain extract with nuclease before chromatofocusing to release basic nucleic acid binding proteins. Distinct patterns of the elutted proteins in Mono P column fractions with nuclease or not were detected and the proteins in the flowthrough fractions of the column increased significantly upon nuclease digestion (Fig. 2). The highly basic proteins released by nuclease treatment were identified by MALDI-TOF spectrometry. Surprisinly, the majority of the proteins are enzymes in important metabolic pathways such as glyceraldehydes-3-phosphate dehydrogenase, malate dehydrogenase and transketolase. However, no GAR containing nucleic acid binding proteins were identified in the basic proteins (Table 2). This part of work were in the master thesis of Chien-Jen Hung (Proteomic studies of methylaccepting proteins in HeLa cells and mouse brains, Master thesis, Institute of Medicine, Chung Shan medical University).

Protein arginine methylation in autoimmune disease

Many methylarginine containing proteins such as fibrillarin, several hnRNP, myelin basic protein and SmD1 and D3 are known to be autoantigens of different autoimmune diseases. Patients suffering from the autoimmune disease systemic lupus erythematosus (SLE) spontaneously produce autoantibodies against a multitude of cellular components. Antibodies against Sm proteins (anti-Sm autoantibodies) are SLE-specific and react predominantly with the Sm proteins B/B’, D1, D3, E, F, G and,

to a lesser extent, D2. Sm protein D1 and D3 were reported to contain symmetric di-methylarginines（sDMA）and a few different anti-Sm autoantisera recognized only the sDMA peptide of SmD1 and D3 but not unmethylated or asymmetric dimethylarginine peptides. The results indicate the importance of methylarginine modification for autoantibody recognition. We thus would like to know if the anti-Sm sera from local SLE patients also preferentially recognize the methyl-modified Sm D proteins and if there are other proteins that can be differentially recognized by the anti-Sm sera due to their methylation states. We obtained some antisera including anti-Sm and anti-RNP from SLE patients through collaboration with Dr. Gregory Tsai in Institute of Immunology in our school. We performed preliminary studies using one anti-Sm serum for immunoprecipitation. We then use the anti-Sm to immunoprecipitate in vitro methylated proteins from lymphoblastoid cell extracts (Fig. 7). We used radioactive [*methyl*-^{3}H]-S-adenosylmethionine (AdoMet) to in vitro methylate AdOx-treated lymphoblastoid cell extracts. The labeled reaction mixture was then used for the immunoprecipitation and analyzed by gel electrophoresis and fluorography. The result indicated that anti-Sm recognize specific methylaccepting proteins or proteins that specifically interacted with the methyl-modified proteins. We were planning to analyze the immunoprecipitants for methylarginine containing polypeptides. However, the signals from the immunoglobulin proteins from the antisera were too strong thus covered the signals detected by methylarginine specific antibodies. We thus immobilized the autoantibodies to gel supports to prepare the autoantigens by immunoaffinity. However, no protein signals could be obtained from the eluted fractions of the immobilized antibodies. Awe thus changed our strategy by using anti-Sm autosera from three different SLE patients in western blot analyses of AdOx-treated (proteins presumably at hypomethylation state) and untreated (proteins at normal methylation states) HeLa cell extracts. There were no significant differences

between the signals corresponding to SmD1 in samples of different methylation status. However, in one-dimensional (Fig. 3) and 2D-dimensional gel electrophoresis (Fig. 4), weaker signals between molecular mass of 18 to 21 kDa and about 40 kDa were both consistently detected from cell extracts treated with AdOx compared to the ones without AdOx treatment . We identified these proteins by mass spectrometry. We were interested in two putative autoantigens CArG binding factor and ZNF9 proteins, they contain GAR domain and are likely to be methylarginine containing proteins. We inferred that CArG binding factor and ZNF9 protein might contain methyl-arginines thus lead to the differential recognition by anti-Sm autoantibodies due to their mehtylation status. This part of work were in the master thesis of Hong-How Chang (Differential recognition of proteins due to methylation status by anti-Sm autoantibodies from SLE patients And Proteomic analysis of autoantigens of antineutrophil cytoplasmic autoantibodies (c-ANCA) from vasculitis patients; mater thesis, Institue of Stamatology, Chung Shan Medical University).

.

Modulation of stable protein methylation by KCl

1. Addition of 0.5 M KCl to the total extract of LCL or HeLa cells activate the methylation of a typical type II substrate fibrillarin

In our previous studies of type I protein arginine methyltransferase in porcine brain (Hung et al., manuscript in preparation), an interesting result triggered our investigation in this study. Inclusion of 0.5 M KCl in the buffer to resuspend the precipitants of an ultracentrifugation (100,000 x g) of the brain extract released type I methyltransferase of high specific activity. Similarly, addition of 0.5 M KCl to HeLa or lymphoblastoid cell total extracts and preincubation of the extract on ice for 30 min before the methyltransferase assay elevated the activity significantly. However, inclusion of 0.5 M KCl with recombinant PRMT1 did not activate the

methyltransferase activity. The results exclude the possibility that addition of 0.5 M KCl might affect the methylaccepting substrate, the recombinant mouse fibrillarin, to become a better methylaccepting substrate for the enzyme. Other monovalent salts such as NaCl and NaF produce similar effects as KCl indicate that it is the high ionic strength produced by the salts that activate the methyltransferase in the total extract.

2. Disruption of cytoskeleton and elevation of type I PRMT activity

The high ionic strength most likely interrupted non-covalent association of proteins. We proposed that the certain fraction of the type I methyltransferase activity might be associated with components that are linked with the cell membrane or other large cellular complexes that can be precipitated by ultracentrifugation. Release of the methyltransferase from the association increased the methyltransferase activity. To test if the methyltransferase attached to cytoskeleton and thus modulate its activity, we treat HeLa cells with cyclostatin which can disrupt the cytoskeleton structure. Small but consistent increased type I methyltransferase activity could be observed upon cyclostatin treatment. Addition of 0.5 M KCl to cyclostatin-treated or untreated cells resulted in the same level of activation. It is thus possible that part of the PRMTs were associated with cytoskeleton and the interruption of the association can activate the methyltransferase activity. This activation can explain partly the activation of the PRMT activity in cell total extracts by high ionic strength.

3. The effect of KCl on methylation of endogenous methylaccepting proteins.

The previous in vitro methylation experiments were performed using an exogenous type I methyltransferase substrate-recombinant mouse fibrillarin. AdOx is an indirect methyltransferase inhibitor that has been widely used to treat cells to accumulate methylaccepting proteins at hypomethylation state. In vitro methylation reaction can be performed with [*methyl*-^{3}H]-AdoMet to probe for the methylaccepting proteins that can be recognized by endogenous protein methyltransferases. Normally many

methylaccepting polypeptides can be detected in this way. Interestingly, inclusion of 0.5M KCl in the methylation reaction of AdOx-treated cell extracts showed totally different methylation patterns compared with that of the no KCl ones. (Fig. 3) The methylation of certain polypeptides such as polypeptide at 100, 115, 34 and 30 are highly elevated. One heavily methylated polypeptide at the molecular mass at 33 kD under the high KCl reaction condition was not detected under the normal methylation reaction. However, the methylation of many polypeptides between molecular mass about 36 to 85 KD was decreased.

4. Identification of the proteins with by proteomic approach.

The total extract from HeLa cells treated with AdOx were methylated in the presence or absence of KCl treatment and then analyzed by two-dimensional electrophoresis. To avoid the high salt included in the methylation reaction to interfere with the isoelectrofocusing electrophoresis, the reaction product were dialyzed before the 1st dimensional electrophoresis. Most of the methylation signals from the 2-D electrogram were horizontal streaks. The electrogram of the protein-staining pattern showed that the majority of protein spots were separated well except the more basic and high molecular weight regions. The methylated protein signals around 90 kDa upon the addition of KCl were of the pH range from 4-7. We have performed the 2-D gel electrophoresis to separate the methylaccepting proteins and are going to identify the proteins by mass spectrometry soon.

The preliminary results were presented as a poster titled “Modulation of type I protein arginine methyltransferase activity and protein methylation pattern by high ionic strength” in the ASBMB annual meeting (2005) at San Diego, CA, USA. Manuscript of this part of work is under preparation.

Protein arginine methylation analyses of PAI-RBP1and other putataive

methylaccepting proteins

We are also analyzing putative novel methylaccepting proteins. We finally subcloned and expressed GST-fused recombinant protein of prohibitin, a putative methylaccepting protein identified through our previous investigation. We have troubles in cloning and expression of recombinant proteins for our studies that slowed down our progress. We are glad that the Core facilities now can provide the service for recombinant proteins constructs. Even though we have successfully subcloned a novel RGG containing protein PAI-RBP1 (plasminogen activator inhibitor RNA binding protein) into expressing vector, we had struggled for obtaining the proteins and thus cannot get convincing results (Fig. 6). We have obtained the PAI-RBP1 expression construct from the Core and are reanalyzing the putative methylaccepting protein. It can be recognized by porcine brain extract and we are reconfirming the identity of the recombinant protein by MALDI-MS and are purifying this recombinant protein for antibody induction and other analyses.

References

Ai, L.S., Lin, C.H., Hsieh, M. and Li, C. 1999 Arginine methylation of a glycine and arginine rich peptide derived from sequences of human FMRP and fibrillarin. Proc Natl Sci Counc Repub China B 23,175-80.

Bedford MT, Frankel A, Yaffe MB, Clarke S, Leder P, Richard S. Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. J Biol Chem. 2000 May 26;275(21):16030-6.

Bedford MT, Richard S. Arginine methylation an emerging regulator of protein function.Mol Cell. 2005 Apr 29;18(3):263-72. Belyanskaya LL, Gehrig PM, Gehring H. Exposure on cell surface and extensive arginine methylation of ewing sarcoma (EWS) protein. J. Biol Chem. 2001 Jun 1;276(22):18681-7.

Boulanger, M.C., Branscombe Miranda, T., Clarke, S., DiFruscio, M., Suter, B., Lasko, P. and Richard, S. 2004 Characterization of Drosophila protein arginine methyltransferases DART1 and DART4. Biochem J Pt.

Boulanger, M.C., Liang, C., Russell, R.S., Lin, R., Bedford, M.T., Wainberg, M.A. and Richard, S. 2005 Methylation of Tat by PRMT6 Regulates Human Immunodeficiency Virus Type 1 Gene Expression. J Virol 79,124-31.

Brahms H, Meheus L, de Brabandere V, Fischer U, Luhrmann R. Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein. RNA. 2001 Nov;7(11):1531-42.

Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L. and Luhrmann, R. 2000 The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. J Biol Chem 275,17122-9.

Branscombe TL, Frankel A, Lee JH, Cook JR, Yang Z, Pestka S, Clarke S. PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins.J Biol Chem. 2001 Aug 31;276(35):32971-6.

Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S. and Clarke, S. 2001 PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J Biol Chem 276,32971-6.

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R. 1999 Regulation of transcription by a protein methyltransferase. Science 284,2174-7.

Chen, D.H., Wu, K.T., Hung, C.J., Hsieh, M. and Li, C. 2004 Effects of Adenosine Dialdehyde Treatment on In Vitro and In Vivo Stable Protein Methylation in

HeLa Cells. J Biochem (Tokyo) 136,371-6.

Cheng X, Collins RE, Zhang X. 2005 Structural and sequence motifs of protein (histone) methylation enzymes. Annu Rev Biophys Biomol Struct.;34:267-94.

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., Harafuji, N., Hastings, K.E., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H.G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-Bow, S., DeSantis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S., Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B.I., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-i, T., Spagnuolo, A., Stainier, D., Suzuki, M.M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N. and Rokhsar, D.S. 2002 The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Science 298,2157-67.

Fabbrizio, E., El Messaoudi, S., Polanowska, J., Paul, C., Cook, J.R., Lee, J.H., Negre, V., Rousset, M., Pestka, S., Le Cam, A. and Sardet, C. 2002 Negative regulation of transcription by the type II arginine methyltransferase PRMT5. EMBO Rep 3,641-5.

Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S. and Bedford, M.T. 2002 The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. J Biol Chem 277,3537-43.

Gary, J.D. and Clarke, S. 1998 RNA and protein interactions modulated by protein arginine methylation. Prog Nucleic Acid Res Mol Biol 61,65-131.

Gary, J.D., Lin, W.J., Yang, M.C., Herschman, H.R. and Clarke, S. 1996 The predominant protein-arginine methyltransferase from Saccharomyces cerevisiae. J Biol Chem 271,12585-94.

Gilbreth, M., Yang, P., Wang, D., Frost, J., Polverino, A., Cobb, M.H. and Marcus, S. 1996 The highly conserved skb1 gene encodes a protein that interacts with Shk1, a fission yeast Ste20/PAK homolog. Proc Natl Acad Sci U S A 93,13802-7.

Hebert, M.D., Shpargel, K.B., Ospina, J.K., Tucker, K.E. and Matera, A.G. 2002 Coilin methylation regulates nuclear body formation. Dev Cell 3,329-37.

Henry, M.F. and Silver, P.A. 1996 A novel methyltransferase (Hmt1p) modifies poly(A)+-RNA-binding proteins. Mol Cell Biol 16,3668-78.

Huang, H.M., Tam, M.F., Tam, T.C., Chen, D.H., Hsieh, M. and Li, C. 2002 Proteomic analysis of stable protein methylation in lymphoblastoid cells. J Biochem (Tokyo) 132,813-8.

Hung, C.M. and Li, C. 2004 Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians. Gene 340,179-87.

Katsanis, N., Yaspo, M.L. and Fisher, E.M. 1997 Identification and mapping of a novel human gene, HRMT1L1, homologous to the rat protein arginine N-methyltransferase 1 (PRMT1) gene. Mamm Genome 8,526-9.

Kwak, Y.T., Guo, J., Prajapati, S., Park, K.J., Surabhi, R.M., Miller, B., Gehrig, P. and Gaynor, R.B. 2003 Methylation of SPT5 regulates its interaction with RNA polymerase II and transcriptional elongation properties. Mol Cell 11,1055-66.

Kzhyshkowska, J., Schutt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H. and Dobner, T. 2001 Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1. Biochem J 358,305-14.

Lee, J. and Bedford, M.T. 2002 PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. EMBO Rep 3,268-73.

Lee, J.H., Cook, J.R., Yang, Z.H., Mirochnitchenko, O., Gunderson, S., Felix, A.M., Herth, N., Hoffmann, R. and Pestka, S. 2004 PRMT7: A new protein arginine methyltransferase that synthesizes symmetric dimethylarginine. J Biol Chem.

Lee J, Sayegh J, Daniel J, Clarke S, Bedford MT. 2005 PRMT8, a new membrane-bound tissue-specific member of the protein argininemethyltransferase family. J Biol Chem. 280(38):32890-6.

Li, C., Ai, L.S., Lin, C.H., Hsieh, M., Li, Y.C. and Li, S.Y. 1998 Protein N-arginine methylation in adenosine dialdehyde-treated lymphoblastoid cells. Arch Biochem Biophys 351,53-9.

Lin, C.H., Hsieh, M., Li, Y.C., Li, S.Y., Pearson, D.L., Pollard, K.M. and Li, C. 2000 Protein N-arginine methylation in subcellular fractions of lymphoblastoid cells. J Biochem (Tokyo) 128,493-8.

Lin, C.H., Huang, H.M., Hsieh, M., Pollard, K.M. and Li, C. 2002 Arginine methylation of recombinant murine fibrillarin by protein arginine methyltransferase. J Protein Chem 21,447-53.

Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S. and Herschman, H.R. 1996 The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. J Biol

Chem 271,15034-44.

Ma, X.J., Lu, Q. and Grunstein, M. 1996 A search for proteins that interact genetically with histone H3 and H4 amino termini uncovers novel regulators of the Swe1 kinase in Saccharomyces cerevisiae. Genes Dev 10,1327-40.

McBride, A.E. and Silver, P.A. 2001 State of the arg: protein methylation at arginine comes of age. Cell 106,5-8.

Miranda TB, Webb KJ, Edberg DD, Reeves R, Clarke S. 2005 Protein arginine methyltransferase 6 specifically methylates the nonhistonechromatin protein HMGA1a.Biochem Biophys Res Commun. 336(3):831-5.

Miranda, T.B., Miranda, M., Frankel, A. and Clarke, S. 2004 PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. J Biol Chem.

Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R. and David, M. 2001 Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. Cell 104,731-41.

Najbauer J, Johnson BA, Young AL, Aswad DW. Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. J Biol Chem. 1993 May 15;268(14):10501-9.

Pal, S., Yun, R., Datta, A., Lacomis, L., Erdjument-Bromage, H., Kumar, J., Tempst, P. and Sif, S. 2003 mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the Myc target gene cad. Mol Cell Biol 23,7475-87.

Pan, H., Dung, H.N., Hsu, H.M., Hsiao, K.M. and Chen, L.Y. 2003 Cloning and developmental expression of p73 cDNA in zebrafish. Biochem Biophys Res Commun 307,395-400.

Pawlak, M.R., Scherer, C.A., Chen, J., Roshon, M.J. and Ruley, H.E. 2000 Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. Mol Cell Biol 20,4859-69.

Pollack, B.P., Kotenko, S.V., He, W., Izotova, L.S., Barnoski, B.L. and Pestka, S. 1999 The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. J Biol Chem 274,31531-42.

Qi, C., Chang, J., Zhu, Y., Yeldandi, A.V., Rao, S.M. and Zhu, Y.J. 2002 Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. J Biol Chem 277,28624-30.

Rho, J., Choi, S., Seong, Y.R., Cho, W.K., Kim, S.H. and Im, D.S. 2001 Prmt5, which

forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. J Biol Chem 276,11393-401.

Scott, H.S., Antonarakis, S.E., Lalioti, M.D., Rossier, C., Silver, P.A. and Henry, M.F. 1998 Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). Genomics 48,330-40.

Singh V, Miranda TB, Jiang W, Frankel A, Roemer ME, Robb VA, Gutmann DH, Herschman HR, Clarke S, Newsham IF. DAL-1/4.1B tumor suppressor interacts with protein arginine N-methyltransferase3 (PRMT3) and inhibits its ability to methylate substrates in vitro and in vivo.Oncogene. 2004 Oct 14;23(47):7761-71.

Smith JJ, Rucknagel KP, Schierhorn A, Tang J, Nemeth A, Linder M, HerschmanHR, Wahle E. Unusual sites of arginine methylation in Poly(A)-binding protein II and in vitro methylation by protein arginine methyltransferases PRMT1 and PRMT3. J Biol Chem. 1999 May 7;274(19):13229-34.

Tang, J., Frankel, A., Cook, R.J., Kim, S., Paik, W.K., Williams, K.R., Clarke, S. and Herschman, H.R. 2000 PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. J Biol Chem 275,7723-30.

Tang, J., Gary, J.D., Clarke, S. and Herschman, H.R. 1998 PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. J Biol Chem 273,16935-45.

Wada, K., Inoue, K. and Hagiwara, M. 2002 Identification of methylated proteins by protein arginine N-methyltransferase 1, PRMT1, with a new expression cloning strategy. Biochim Biophys Acta 1591,1-10.

Wen, F.C., Li, Y.H., Tsai, H.F., Lin, C.H., Li, C., Liu, C.S., Lii, C.K., Nukina, N. and Hsieh, M. 2003 Down-regulation of heat shock protein 27 in neuronal cells and non-neuronal cells expressing mutant ataxin-3. FEBS Lett 546,307-14.

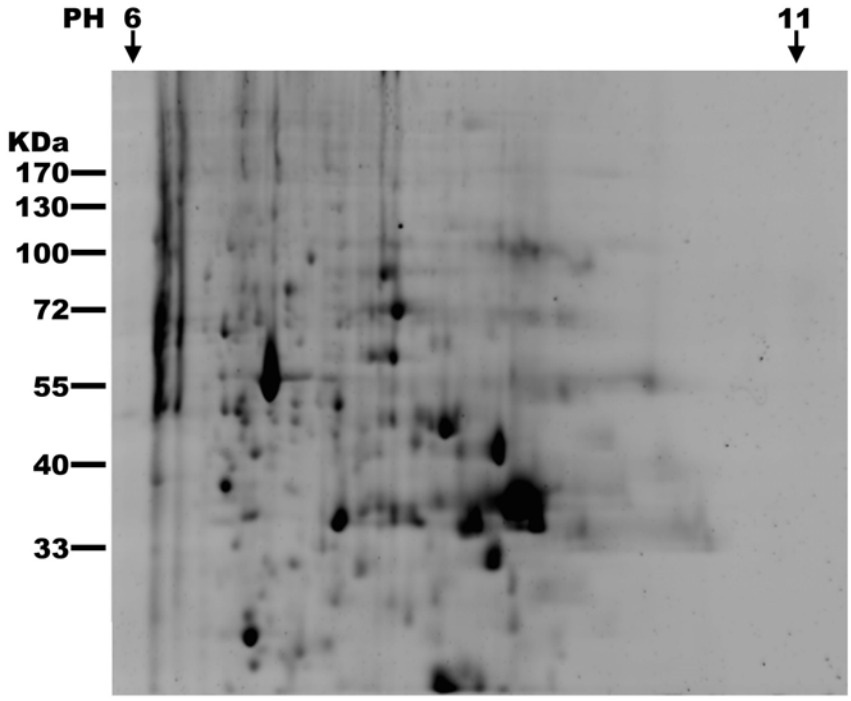
Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B.M., Montminy, M. and Evans, R.M. 2001 A transcriptional switch mediated by cofactor methylation. Science 294,2507-11.

Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M.C., Aldaz, C.M. and Bedford, M.T. 2003 Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. Proc Natl Acad Sci U S A 100,6464-8.

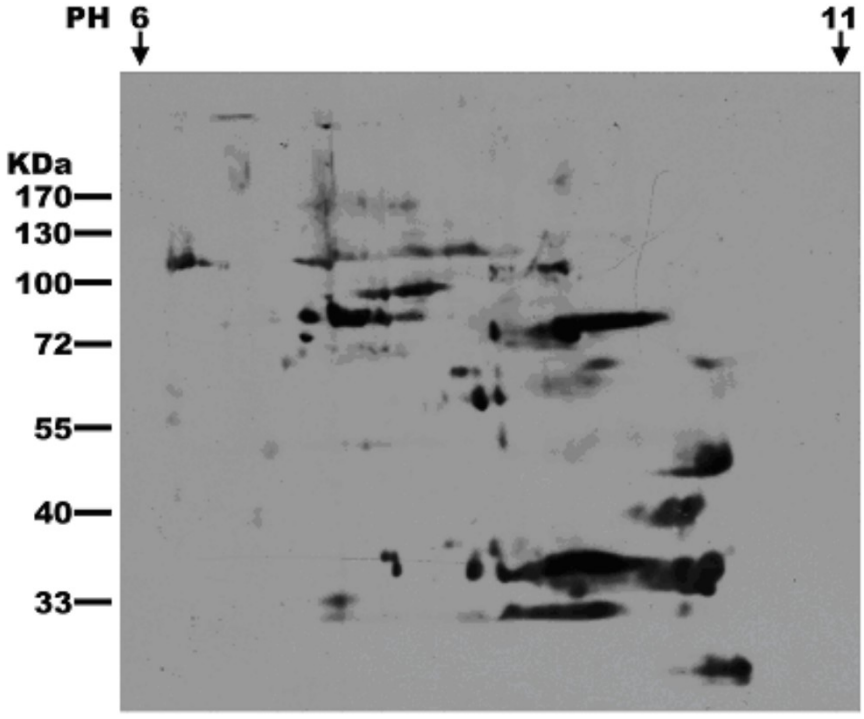
Zhang, X. and Cheng, X. 2003 Structure of the predominant protein arginine methyltransferase PRMT1 and analysis of its binding to substrate peptides. Structure (Camb) 11,509-20.

Figures and Tables

(A)



(B)



(C)

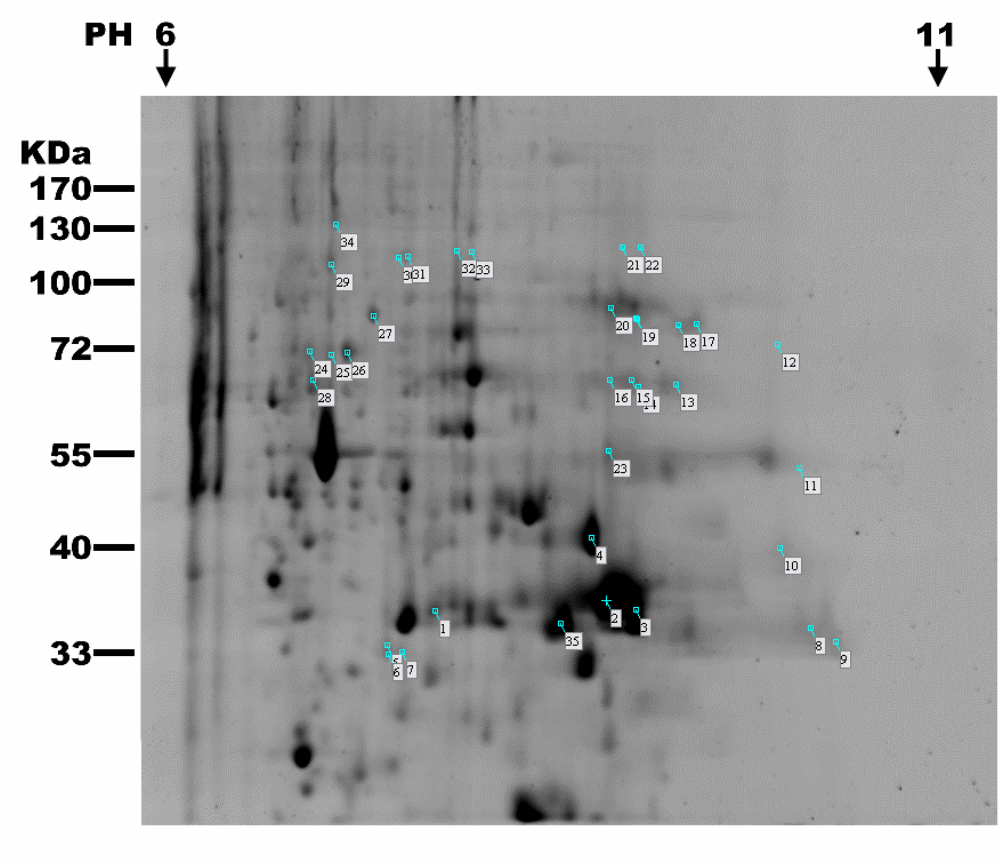


Fig. 1 HeLa 細胞萃取蛋白經由二維膠體電泳分離後，以 Coomassie blue 染色及西方點墨法分析之結果

取 250 μg 的細胞萃取蛋白進行二維膠體電泳，電泳分離後的膠，分別以 coomassie blue 染色的結果 (A)，及西方點墨法 (7E6 1:200)壓片的結果 (B)。(C) 是將上述二者進行比對的結果，將符合的點以數字標記起來。

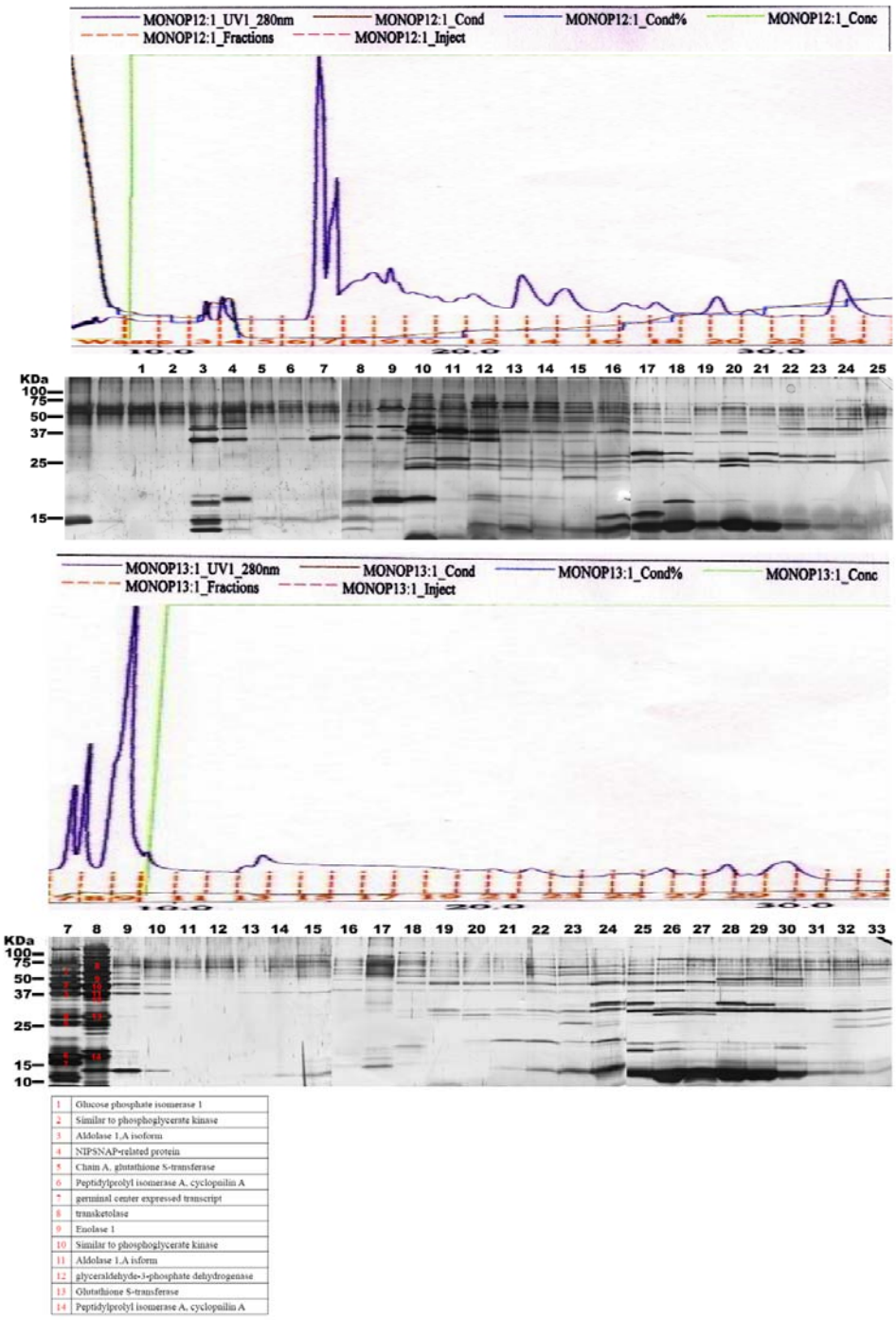


Fig. 2. Mouse brain extract treated with nuclease (upper panel) or not (lower panel) was separated by mono P chromatofocusing chromatography. The column was washed with 2 column volume (CV, 4 ml) of buffer A (0.075 M Tris-acetate, pH9.3), then eluted with the gradient of 100% buffer A to buffer 100% buffer B (poly buffer 96, pH 6.0) in 11 CV. The flow rate was 1 ml/min and the fractions were collected every one min. The proteins in each fraction were examined by SDS-PAGE and silver stain. The polypeptides identified by MALDY-TOF mass spectrometry were indicated below.

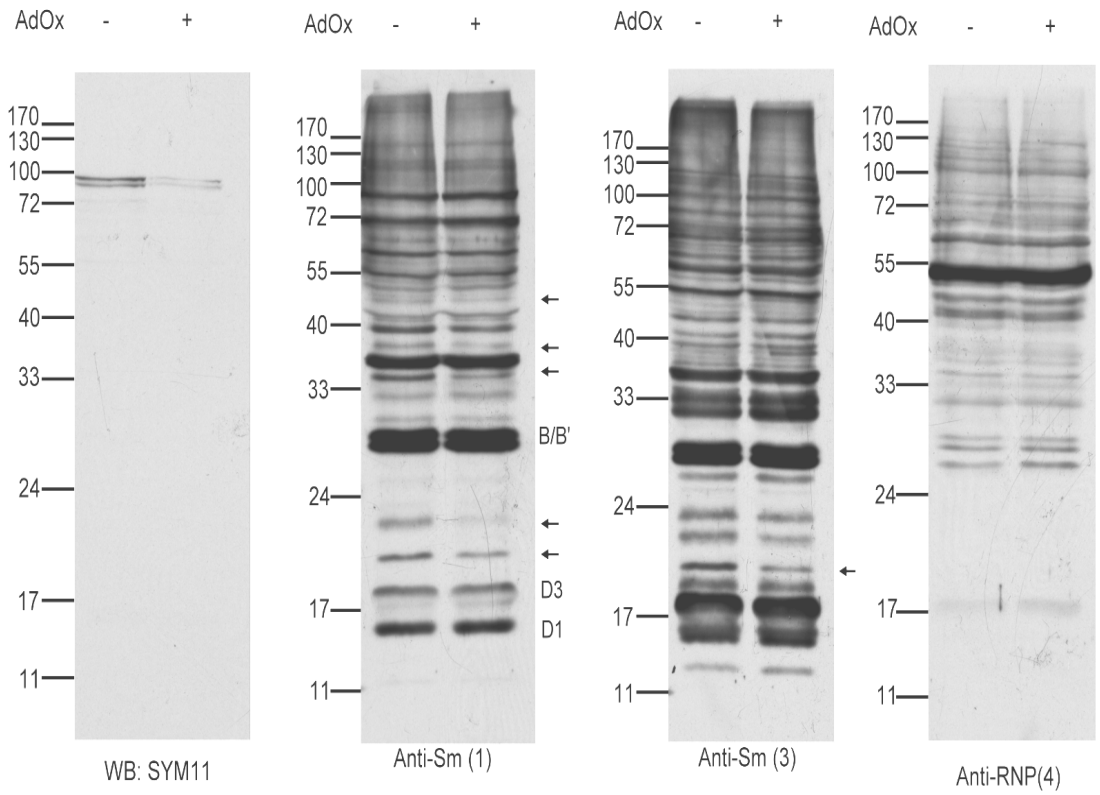


Fig.3 因蛋白質甲基化程度不同而影響 anti-Sm autoantisera 辨識上的不同

HeLa cells 分別培養在有加（20 μM）或沒加 AdOx 的細胞培養液中 24 小時，再取 cell extracts 20 μg 蛋白量跑 SDS-PAGE，並將蛋白轉移至 NC 膜上做 western blot。（SYM11：會辨識對稱型雙甲基精胺酸，當作控制組；來自 SLE 病人（1 號和 3 號）的 anti-Sm 自體免疫抗體；來自 4 號 SLE 病人的 anti-RNP 自體免疫抗體）

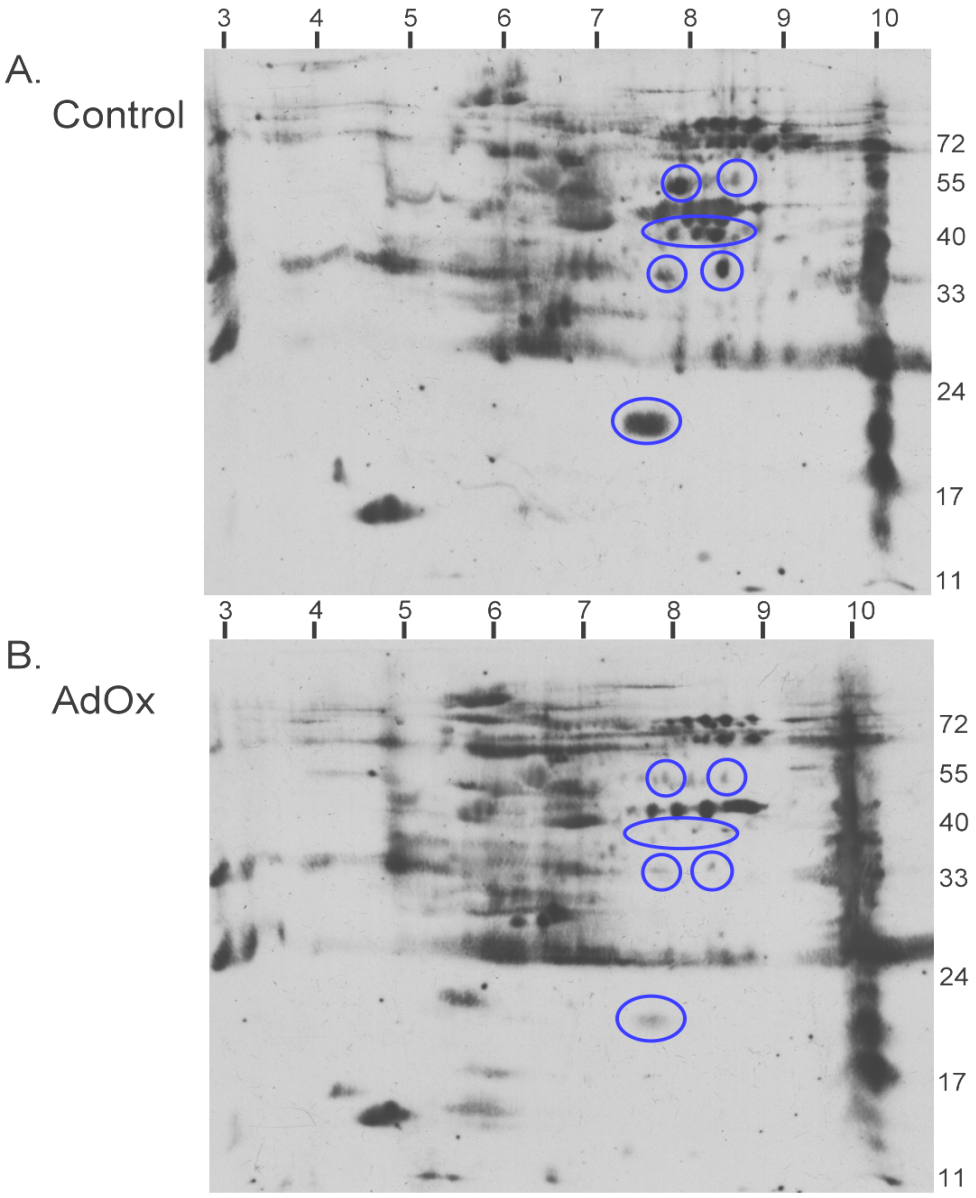


Fig.4 Anti-Sm 在蛋白質二維電泳 pattern 上辨識的不同

有加 AdOx 處理和無加 AdOx 處理(control)的細胞萃取液(250 μg)，我們將這不同的樣品跑二維電泳並以 SLE 1 號病人的 anti-Sm autoantibody 做 western blot 比對。(打藍圈處：加 AdOx 處理後，anti-Sm (1)辨識變弱（與控制組比較））

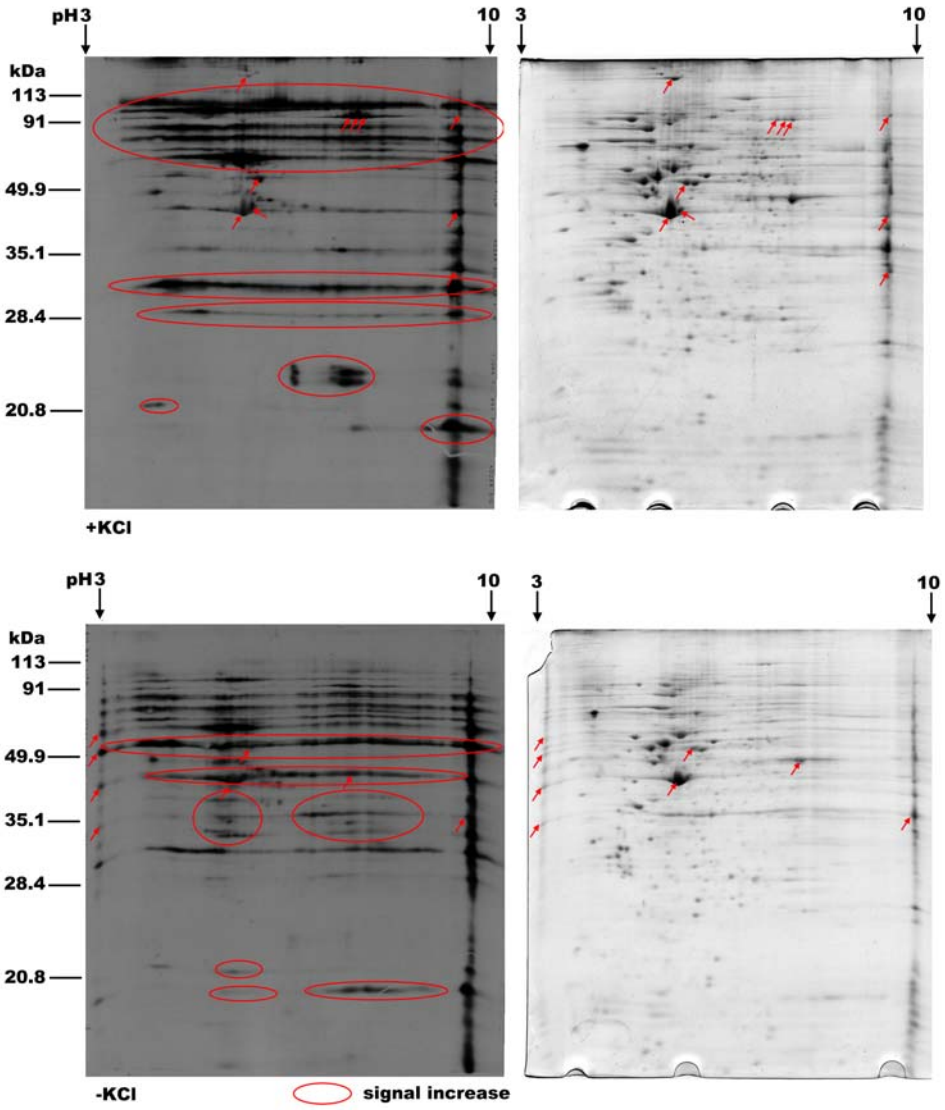


Fig. 5. High KCl concentration changed the pattern of in vitro protein methylation in HaLa cell extracts. HeLa cell extract treated with a methylation inhibitor AdOx were prepared and subjected to in vitro methylation in the presence of [*methyl*-3H]-S-adenosylmethionine. The upper panel is the protein pattern of 2-D separation of HeLa cell extract in vitro methylated with the addition of 0.5 M KCl (right) and the fluorography (left). The lower panel is the protein pattern of 2-D separation of HeLa cell extract in vitro methylated without 0.5 M KCl (right) and the fluorography (left).

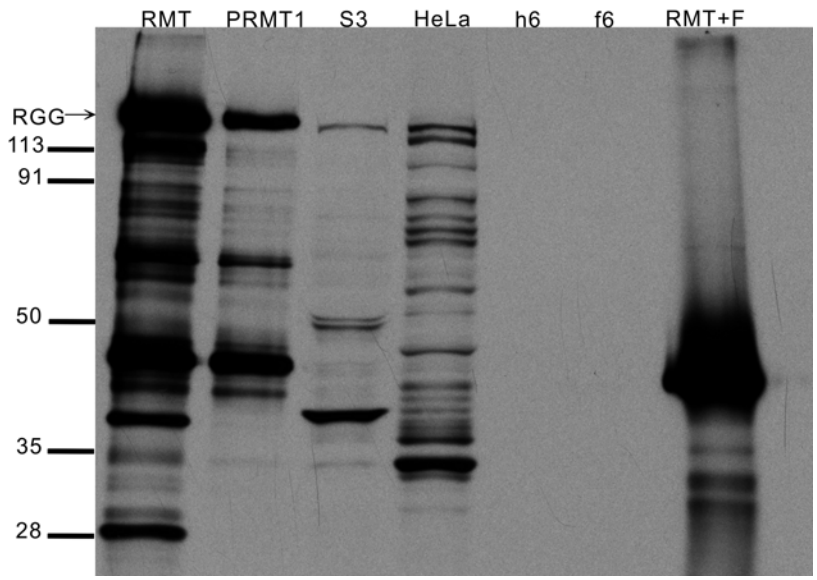


Fig. 6 *In vitro* methylation of PAI-RBP1 by porcine brain extract. PAI-RBP1 is a putative methylarginine containing protein with typical RGG boxes. Recombinant PAI-RBP1 (RGG) was used as the methylacceptor and recombinant yeast RMT1, rat PRMT1and porcine brain extract (S3) and HeLa cell extract were used the methyltransferase source for in vitro methylation. PAI-RBP1 protein (mw. About 119 kDa) appeared to be specifically methylated by certain methyltransferase.

表一 **Spots** 進行鑑定的結果

Spot	Identilied protein	NCBI prot	Theor. MW	EXP. MW	Theor. PI	EXP. PI	Score	Reported methylaccepting protein	Containing RGG RG/GR RXR
2	glyceraldehyde-3-phosph ate dehydrogenase	CAA25833	36	37	8.4	8.9	148	N/A	
3	hnRNP A2/B1	P22626	37	36	9.0	9.1	113	Sun et al., 2003 Huang et al., 2002	grggnfgfg dsrgggnfg pgpgsnfrgg (202-230)
4	aldolase A	CAA30979	39	41	8.3	8.7	133	N/A	
8	hnRNP A1	AAH71945	34	35	9.3	10.2	268	Nichols et al., 2000	qrgrsgsg nfgggrgggf ggndnfgrgg nfsgrggfgg srgg (193-234)
11	hnRNP G	S41766	47	52	9.6	10.1	60	Liu and Dreyfuss,1995 Ong et al., 2004	grrgppppp rsrgpprglr ggrggsggtr g (92-121)

13	hnRNP I (polypyrimidine tract binding protein)	NP_114367	59	65	9.2	9.3	138	N/A	lrgqpiyiqf (121-130)
15	54 kDa nuclear RNA- and DNA-binding protein	Q15233	54	65	9.1	9.0	134	N/A	mplrgkqlrv (131-140) ddrgrpsgkg (181-190) amginnrga (391-400)
16	54 kDa nuclear RNA- and DNA-binding protein	Q15233	54	65	9.1	8.9	134	N/A	
18	FUS gycline rich protein	CAA50559	53	79	9.4	9.3	68	Wada et al.,2002	rgggn rggrgrggp mgrggygggg sggggrggf (376-409)
19	Sam68	NP_006550	48	82	8.7	9.0	37	Cote et al., 2003	phrsrgggggg srggaraspa (41-60) srgrgvpvrg rgaapppppv prgrgvgppr galvrg (281-316)
20	hnRNP M4	S35532	78	86	8.9	8.9	77	N/A	rgsgpkgege

									rpaqnekrkv knikrggnrf (31-60)
35	lactate dehydrogenase A	NP_005557	37	35	8.4	8.5	129	N/A	

表二 MonoP 分離之 basic proteins 經由 **MALDI-Q-TOF massspectrometry** 鑑定的結果

Numbers	**Identilied protein**	**NCBI protein**	**Theor. MW**	**EXP. MW**	**Theor. PI**	**EXP. PI**	**Score**
1	Aspartate transaminase	S01174	48	40	9.1	9.0	40
2	Malate dehydrogenase	AAA39509	36	35	8.9	9.0	91
3	Glucose phosphate isomerase 1	NP_032181	63	65	7.8	8.5	43
4	Peptidylprolyl isomerase A	NP_032933	18	18	7.7	8.5	124
5	Aldolase A	AAA37210	40	37	8.6	8.4	63
6	Peptidylprolyl isomerase A	NP_032933	18	18	7.7	8.4	233
7	Aldolase A	AAA37210	40	37	8.6	8.3	68
8	Glutathione transferase	AAA37748	25	26	7.6	8.3	71
9	Transketolase	NP_033414	68	72	7.2	8.2	270
10	Carbonic anhydrase 2	AAH55291	29	30	6.5	7.8	86
11	Malate dehydrogenase	P08249	36	65	8.8	9.3	175
12	Similar to phospoglycerate kinase	XP_130894	42	45	8.4	9.3	73
13	Aldolase l, A isoform	NP_031464	39	40	8.3	9.3	77
14	Malate dehydrogenase	AAA39509	36	35	8.9	9.3	47
16	ChainA, glutathione s-transferase	1GLP_A	23	23	8.1	9.3	76

17	Peptidylprolyl isomerase A	NP_032933	18	16	7.7	9.3	155
19	Aconitase 2	NP_542364	85	90	8.1	9.3	166
20	Transketolase	NP_033414	68	75	7.2	9.3	85
21	Enolase 1	NP_075608	47	50	6.4	9.3	62
22	Similar to phospoglycerate kinase	XP_130894	42	45	8.4	9.3	42
23	Aldolase l, A isoform	NP_031464	39	40	8.3	9.3	68
24	Glyceraldehydes-3-phosphate dehydrogenase	CAA25833	36	35	8.4	9.3	149
25	Glutathione s-transferase	NP_034488	26	27	7.7	9.3	65
26	Peptidylprolyl isomerase A	NP_032933	18	16	7.7	9.3	177
27	Carbonic anhydrase 2	AAH55291	29	31	6.5	8.0	69
28	Aldolase 3, C isoform	NP_033787	39	40	6.7	7.9	65
29	Carbonic anhydrase 2	AAH55291	29	31	6.5	7.9	63
31	Glutathione S-transferase , alpha 4	NP_034487	26	25	6.8	7.8	124
32	Glutathione S-transferase , alpha 4	NP_034487	26	25	6.8	7.6	117
33	Aspartate aminotransferase	CAA30275	46	43	6.7	7.5	242
34	Carbonic anhydrase 2	AAH55291	29	31	6.5	7.5	92
35	Aspartate aminotransferase	CAA30275	46	43	6.7	7.3	114

Table.1 anti-Sm辨識上降低的點挖下作質譜儀分析的結果

Score分別為MALDI-TOF及MS/MS分析結果，score為粗體字指在統計上有可信度。

Spots	**Protein name**	**NCBI prot**	**Measured MW / pI**	**Theoretical MW / pI**	**Score** (peptide mass fingerprint / MS/MS ion search)
1	Hypothetical protein	CAD97642	40 / 7.7	47.063 / 7.57	none / **72**
2	Hypothetical protein	CAD97642	40 / 8.1	47.063 / 7.57	41 / **111**
3	Hypothetical protein	CAD97642	39 / 8.3	47.063 / 7.57	**73** / **180**
4	CArG binding factor	AAR17782	34 / 8.3	33.193 / 8.16	57 / **94**
5	ZNF9 protein	AAH00288	19 / 7.9	18.729 / 8.00	none / **62**
6	OXCT	CAG33330	53 / 7.8	56.156 / 7.13	**95** / **198**
7	LASP1 protein	AAH07560	33 / 7.8	35.991 / 8.92	37 / **164**

藍色點：因甲基化程度降低而anti-Sm辨識減弱的點

Journal of Protein Chemistry, Vol. 21, No. 7, October 2002 (© 2002)

Arginine Methylation of Recombinant Murine Fibrillarin by Protein Arginine Methyltransferase

Chia-Hui Lin,[1] Hung-Ming Huang,[1] Mingli Hsieh,[2] K. Michael Pollard,[3] and Chuan Li[2,4]

Received July 3, 2002

Fibrillarin is a conserved nucleolar SnoRNP with a diverse N-terminal glycine- and arginine-rich (GAR) domain in most eukaryotes. This region in human fibrillarin is known to contain modified dimethylarginines. In this report we demonstrate that recombinant murine fibrillarin is a substrate for protein arginine methyltransferase, including the purified recombinant enzyme (rat PRMT1 and yeast RMT1) and the protein methyltransferases present in lymphoblastoid cell extracts. Our results of protease digestion, methylation competition reactions, and immunoblotting with a methylarginine-specific antibody all indicate that the methylation of fibrillarin is in the N-terminal GAR domain and arginyl residues are modified. Finally, amino acid analyses revealed that the modification of recombinant murine fibrillarin forms methylarginines, mostly as dimethylarginines.

KEY WORDS: Fibrillarin; arginine methylation; GAR domain; methylarginine.

1. INTRODUCTION

Protein *N*-arginine methylation is an irreversible post-translational modification on the guanidino nitrogens of the arginyl residues. This reaction accounts for the majority of stable cellular protein methylation (Li *et al.*, 1998; Najbauer *et al.*, 1993). Most of the methylarginines identified on proteins appear to be N^G-monomethylarginine (MMA) and asymmetric N^G, N^G-dimethylarginines (aDMA)[5] occurring in RNA binding proteins within the Arg-Gly-Gly (RGG) context (Li *et al.*, 1998, 2000; Najbauer *et al.*, 1993). These modifications were catalyzed by the type I arginine methyltransferases (Gary and Clarke, 1998). On the other hand, the type II methyltransferases modify proteins such as myelin basic protein (Baldwin and Carnegie, 1971), the core snRNP SmD1, D3 (Brahms *et al.*, 2000), B/B′, and one of the Sm-like proteins, LSm4 (Brahms *et al.*, 2001) to form MMA and symmetric N^G, N'^G-dimethylarginine (sDMA).

Why the arginine residues in the RGG box are specifically modified is still equivocal. Protein arginine methylation has been proposed to be involved in various cellular processes such as RNA or protein interaction (Friesen *et al.*, 2001; Mowen *et al.*, 2001; Rajpurohit *et al.*, 1994b), signal transduction (Abramovich *et al.*, 1997; Lin *et al.*, 1996), transcriptional regulation (Chen *et al.*, 1999; Wang *et al.*, 2001), and subcellular localization (Nichols *et al.*, 2000; Shen *et al.*, 1998). To date, six different protein arginine methyltransferase (PRMT) genes have been identified in mammals (Chen *et al.*, 1999; Frankel *et al.*, 2002; Katsanis *et al.*, 1997; Lin *et al.*, 1996; Rho *et al.*, 2001; Scott *et al.*, 1998; Tang *et al.*, 1998). Among these, PRMT1 is the predominant

[1] Institute of Medicine, Chung Shan Medical and Dental University, Taichung, Taiwan, R.O.C.

[2] Department of Life Science, Chung Shan Medical and Dental University, Taichung, Taiwan, R.O.C.

[3] W. M. Keck Autoimmune Disease Center, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037.

[4] To whom correspondence should be addressed. E-mail: cli@mercury.csmu.edu.tw

[5] Abbreviations: aDMA, asymmetric N^G, N^G-dimethylarginine; sDMA, symmetric N^G, N'^G-dimethylarginine; MMA, N^G-monomethylarginine; AdOx, adenosine dialdehyde; RMT, arginine methyltransferase; PRMT, protein arginine methyltransferase; GST, glutathion-*S*-transferase; AdoMet, *S*-adenosylmethionine; hnRNP, heterogeneous nuclear ribonucleoproteins; snoRNP, small nucleolar ribonucleoproteins; GAR domain, glycine- and arginine-rich domain; SMN, survival of motor neurons.

0277-8033/02/1000-0447/0 © 2002 Plenum Publishing Corporation

one (Tang *et al.,* 1998), and all of the methyltransferase appear to be the type I enzyme except PRMT5, which catalyzes type II reactions (Branscombe *et al.,* 2001).

Fibrillarin is a nucleolar protein widely present in small nucleolar ribonucleoprotein particles (snoRNP). Fibrillarin appears to participate in major posttranscriptional activities in ribosome synthesis, pre-rRNA processing, pre-rRNA modification, and ribosome assembly (Tollervey *et al.,* 1993). Fibrillarin, along with many other RNA binding proteins involved in ribosome biogenesis and RNA processing, such as nucleolin, and heterogeneous nuclear ribonucleoproteins (hnRNPs), contain glycine- and arginine-rich motifs (GAR or RGG box) with modified arginine residues as N^G-monomethylarginine or N^G,N^G-dimethylarginine (asymmetric; Lapeyre *et al.,* 1986; Lischwe *et al.,* 1985; Liu and Dreyfuss, 1995). Human fibrillarin contains 4.1 mol% N^G, N^G-dimethylarginine, and the N-terminal sequence of the first 31 residues of the 34-kDa protein determined by Lischwe *et al.,* (1985) revealed clusters of glycine and dimethylarginines. An R9 peptide, GGRGRGGGF, derived from the sequence of residues 32–40 of fibrillarin beyond the region that has been determined by Lischwe *et al.* (1985) could also be specifically methylated (Ai *et al.,* 1999). Besides, a GST-fused recombinant with the N-terminal 182 amino acid residues of human fibrillarin has been shown to be an excellent substrate for PRMT1 (Tang *et al.,* 1998). The murine fibrillarin protein sequence displays 94.2% identities with human fibrillarin. The N-terminal GAR domain, even though is not as strongly conserved as other regions of the protein, is still mostly composed of glycines and arginines (Turley *et al.,* 1993). We had shown that the cytosolic, ribosomal, and nuclear subcellular fractions of lymphoblastoid cells all contain methyltransferase activity to modify the recombinant fibrillarin (Lin *et al.,* 2000). In this report we showed the evidences that the recombinant murine fibrillarin can be modified on the arginine residues in the RGG region.

2. MATERIALS AND METHODS

2.1. Culture and Fractionation of Lymphoblastoid Cells

Lymphoblastoid cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C in a 5% CO_2 incubator. Treatment of cells with 20 μM of adenosine dialdehyde (AdOx) for 72 hr was performed as described by Li *et al.* (1998). The cells were harvested and subcellular fractionations were prepared as described by Lin *et al.* (2000). Basically, cells were washed with PBS and lysed in buffer containing 10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1.5 mM $MgCl_2$; 100 mM NaF; 10 ng/ml aprotinine; 1 mM PMSF; 1 mM dithiothreitol; 10 U/ml Rnasin; and 0.5% NP-40. The cells were disrupted by passages through hypodermic needles (22 G) for ~10 times. The pellet obtained by the centrifugation at 1000 *g* for 5 min was the nuclear fraction. The supernatant was further centrifuged at 100,000 *g* for 60 min to pellet the ribosomal fraction and to obtain the supernatant cytosolic fraction.

2.2. Western Blotting

Protein samples separated by 12.5% SDS-PAGE were subsequently transferred to nitrocellulose membranes (Gelman Science). The membrane was then blocked in 5% skimmed dry milk in TTBS (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.05% tween 20) for 30 min, incubated with primary Ab (1:500 dilution for 7E6 monoclone antibody from Abcam) for 1 hr, washed two times in TTBS, and then incubated with secondary antibody (anti–mouse IgG horseradish peroxidase conjugate from Sigma) for 1 hr. Chemiluminescence detection was performed using the WestPico Supersignal kit according to the instructions of the manufacturer (Pierce).

2.3. Preparation of Recombinant Proteins

Recombinant glutathione-*S*-transferase-yeast RMT1 or rat PRMT1 fusion enzyme was prepared from IPTG-induced, pGEX-RMT1 or pGEX-PRMT1 transformed *Escherichia coli* DH5α cells. The expression and purification of the GST-RMT1 and GST-PRMT1 fusion protein were performed as described by Gary *et al.* (1996) and Lin *et al.* (1996). Recombinant mouse fibrillarin with a six histidine tag was expressed in pET28-fibrillarin transformed JM109 (DE3) cells induced with IPTG at 25°C. Recombinant protein was purified under denaturing conditions through a Ni-NTA column (Qiagen) according to the procedures described by Pearson *et al.* (1999).

2.4. *In Vitro* Protein Methylation

Recombinant fibrillarin was incubated with *S*-adenosyl [*methyl*-^{3}H] *l*-methionine (60 Ci/mmol, Amersham) for a final concentration of 1μCi/μl reaction in 25 mM of Tris (pH 7.5), 1 mM of EDTA, and 1 mM of EGTA in the presence of arginine methyltransferase sources. After the indicated time of incubation at 30°C, the reaction was terminated by the addition of one third

of the volume of 3X SDS sample buffer. After gel electrophoresis, the polyacrylamide gel was stained with Coomassie brilliant blue, destained, and then soaked in EN^{3}HANCE (Du Pont NEN) for 1 hr and then in water for 30 min, as suggested by the manufacturer. The gel was dried and exposed to X-ray film (Kodak, MS) at −75°C for 7 days, if not indicated otherwise.

2.5. Endoprotease Lys-C Digestion

Methylated fibrillarin protein (5 μg in a 20 μl reaction volume) was separated from the enzyme source by the adsorption to the Ni-NTA resin (Qiagen, 10 μl). The washed pellet was then resuspended in 100 mM $(NH_4)_2CO_3$ (pH 9.2). Lys-C (Wako) was added in the final concentration of 0.033 mg/ml. Digestion was performed at 42°C overnight.

2.6. Acid Hydrolysis of Methylated Proteins and Analysis of Methylated Amino Acids

To analyze the methylated amino acids in fibrillarin, after *in vitro* methylation described above, the reaction was quenched by the addition of equal volume of 25% of trichloroacetic acid. Insoluble proteins were precipitated by centrifugation and washed by acetone for one time and dried. The polypeptides were hydrolyzed in 6 N of HCl (Pierce) at 105°C under vacuum for 24 hr. The hydrolysates derivatized by phenylisothiocyanate (PITC, Pierce) were analyzed with a C18 column (Waters, PICO-TAC, 5 μm, 3.9 mm × 15 cm) and were monitored at the wavelength of 254 nm. The derivatized amino acids were eluted starting with 100% solvent A (8.225 mM sodium acetate; 0.047% triethylamine, pH 6.4; 6% acetonitrile). Solvent B (60% acetonitrile) was increased by linear gradient to 13% at 5 min, 25% at 10 min, 33% at 15 min, 37% at 20 min, 39% at 25 min, and 40% at 30 min. Fractions were collected every 0.25 min, and the radioactivities in 100 μl of the fractions were determined by liquid scintillation. Methyl amino acid standards were purchased from Sigma.

3. RESULTS

3.1. Methylation of Fibrillarin

Recombinant mouse fibrillarin prepared from *E. coli* has been shown to be methylated by endogenous protein methyltransferase in lymphoblastoid cells. Whether the fibrillarin can be recognized specifically by arginine methyltransferase was determined. *In vitro* methylation reaction in the presence of ^{3}H-AdoMet was performed. Fibrillarin appeared to be methylated upon the addition of recombinant GST-fused rat PRMT1 or yeast RMT1 (Fig. 1); both are known to be a type I methyltransferase. As indicated in Fig. 1, levels of fibrillarin methylation catalyzed by either recombinant rat PRMT1 or yeast RMT1 increased with progressively decreasing rate in the 180-min reaction interval and reached the plateau at 150 min. Thus, for further analysis, we performed the methylation reaction for 150 min to achieve maximum methylation of fibrillarin in our *in vitro* reaction system.

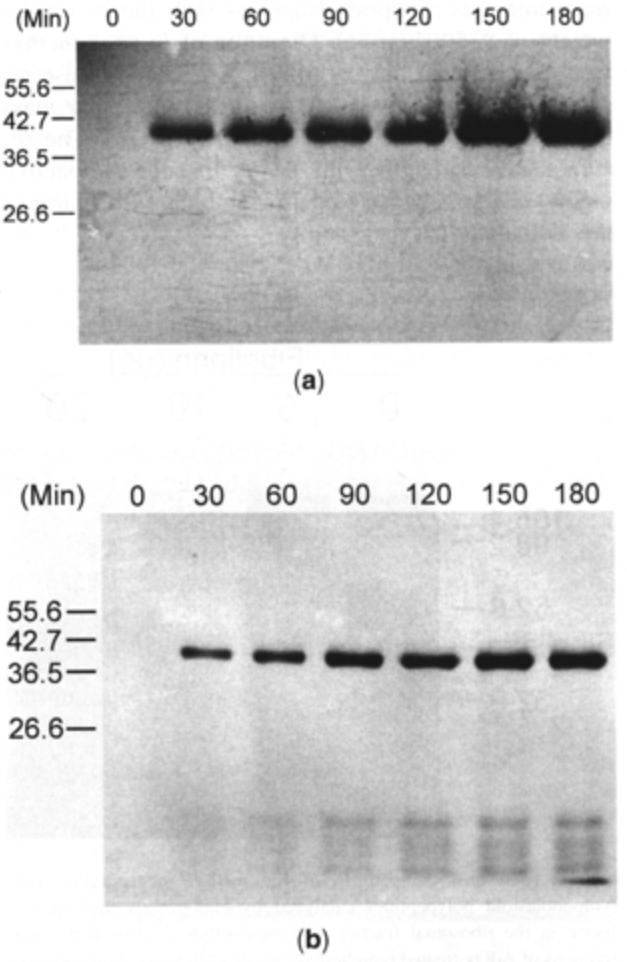


Fig. 1. Methylation of recombinant murine fibrillarin. (a) Methylation of recombinant fibrillarin was performed in the presence of recombinant yeast RMT for different reaction time. Methylation reactions, gel electrophoresis, and fluorography were conducted as described in Materials and Methods. The localization of fibrillarin in the gels is indicated by the *arrow*. (b) Methylation of fibrillarin by rat PRMT1 for different reaction time.

3.2. Fibrillarin Methylation in the GAR Domain

A synthetic peptide R9 with a typical RGG box sequence found in human FMRP and fibrillarin had been shown to be able to compete with the methylation of hypomethylated methylaccepting protein in lymphoblastoid cell extracts (Li *et al.*, 1998). Similarly, addition of fibrillarin can compete with the majority of the protein methylation of lymphoblastoid subcellular fractionation (Fig. 2). These results again suggest that fibrillarin methylation is likely to occur in the RGG box region as arginine methylation.

Lys-C endoprotease cleaving proteins at the C-terminal of lysyl residues predictively will digest the fibrillarin proteins into mostly peptides smaller than the molecular mass of 5,000, while leaving the GAR domain intact as a peptide fragment with the molecular weight of 8759 (Fig. 3a). Digestion of *in vitro* methylated recombinant fibrillarin by Lys-C endoprotease resulted in a radioactive signal with the molecular mass corresponding to the GAR domain (Fig. 3b). The results further strengthen the theory that the radioactive methyl group is added to the recombinant fibrillarin in this domain.

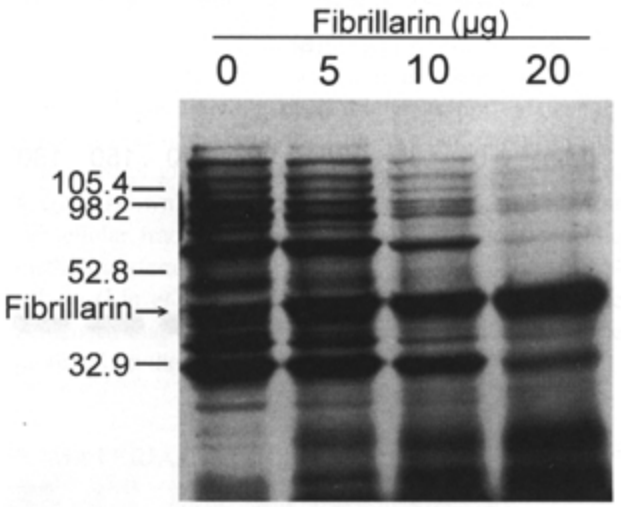
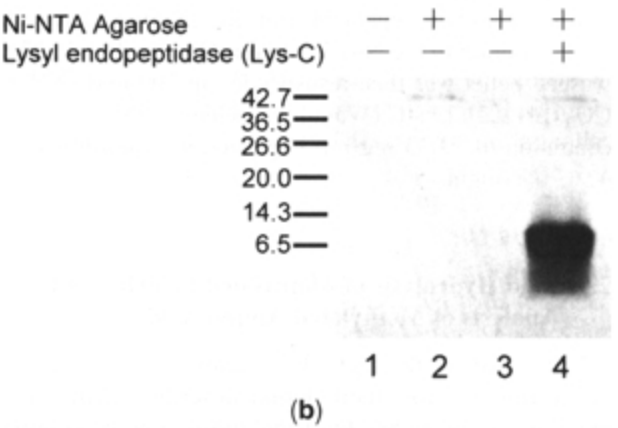


Fig. 2. Fibrillarin competition of the methyl incorporation into lymphoblastoid polypeptides catalyzed by endogenous methyltransferase in the ribosomal fraction of lymphoblastoid cells. Ribosomal fractions of AdOx-treated lymphoblastoid cells (30 μg of proteins) were incubated in the presence of 0.75 μCi of *S*-adenosyl [*methyl*-^{3}H] *l*-methionine and increasing amount of fibrillarin (0, 5, 10, and 20 μg). Methylation reactions, gel electrophoresis, and fluorography were conducted as described in Materials and Methods. The localization of fibrillarin in the gels is indicated by the *arrow*. The strong signals at the bottom of the gel are likely to be due to the methylation of fragments of degraded recombinant fibrillarin

Mass	Position	#MC	Peptide sequence
8759.241	1-96	0	MASMTGGQQMGGTMKPGFRP RGGGFGGRGGFGDRGGRGGG RGGRGGFGGGRGGFGGGGRG RGGGGGGFRGRGGGGVRGGG FQSGGNRGRGGGRGGK
4840.590	242-284	0	YRMLIAMVDVIFADVAQPDQ TRIVALNAHTFLRNGGHFVI SIK
4484.357	182-224	0	VLYLGAASGTTVSHVSDIVG PDGLVYAVEFSHRSGRDLIN LAK
3906.963	305-337	0	MQQENMKPQEQLTLEPYERD HAVVVGVYRPPPK
2626.301	141-162	0	RVSISEGDDTIEYRAWNPFR SK
2108.064	104-121	0	NVMVEPHRHEGVFICRGK
1912.896	285-303	0	ANCIDSTASAEAVFASEVK
1896.056	226-241	0	RTNIIPVIEDARHPHK
1872.106	163-181	0	LAAAILGGVDQIHIKPGAK

(a)



(b)

Fig. 3. Analyses of Lys-C endoprotease digestion of fibrillarin (a) Peptide fragments of Lys-C digestion of recombinant murine fibrillarin with predicted molecular mass larger than 1800. (b) Fluorography of Lys-C digested methylated fibrillarin. One-tenth of the *in vitro* methylation reaction of fibrillarin was reserved and analyzed in lane 1. After Ni-NTA agarose absorption of the recombinant fibrillarin protein, the supernatant and one tenth of the pellet was analyzed in lane 2 and lane 3, respectively. All of the remaining Ni-NTA agarose pellet was resuspended in 100 mM $(NH_4)_2$ CO_3 buffer and digested by Lys-C. The supernatant of the digestion was analyzed in lane 4. The fluorogram is a one-day exposure at −75°C

3.3. Arginine Methylation of Fibrillarin

Fibrillarin methylation on arginyl residues was further confirmed. A mono- and di-methylarginine specific antibody (7E6) was used to detect whether the modified recombinant fibrillarin contain methylarginines. The *in vitro* methylation increased significantly the detection of the fibrillarin signal (Fig. 4). The results indicate that the modified fibrillarin indeed contains methylarginines. Recombinant fibrillarin from *E. coli* can still be detected by the antibody probably because of the presence of the slight amount of arginine methylation on the fibrillarin protein that occurred in the *E. coli* cells or the less specific recognition of the antibody to the RGG region in the protein.

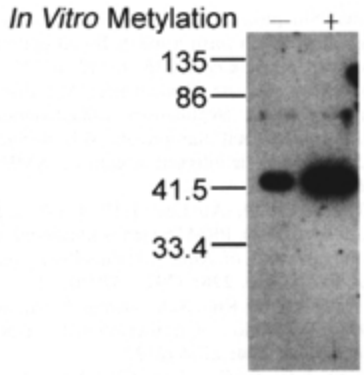


Fig. 4. Arginine methylation of fibrillarin detected by a methylarginine specific antibody. Recombinant fibrillarin (2.5 μg) either unmethylated or methylated by *in vitro* methylation reaction was separated by SDS-PAGE, transferred to nitrocellulose membrane, and then detected by a methylarginine-specific antibody 7E6, as described in Materials and Methods.

To specifically identify the methyl-accepting amino acid(s) in fibrillarin, recombinant fibrillarin that was *in vitro* methylated in the presence of PRMT1 and ^{3}H-AdOMet was acid hydrolyzed and the methylated amino acids were then analyzed (Fig. 5). In comparison, parallel reactions with either fibrillarin or PRMT1 only were incubated with ^{3}H-AdOMet and then subjected to amino acid analyses. The radioactivities eluted about fraction 54 were also detected in two control reaction samples and thus were likely to represent the residual radioactive AdoMet that was carried over from the *in vitro* methylation reaction to the amino acid analysis. For the rest of the radioactivity, the majority (86%) coeluted with dimethylarginine and ~12% coeluted with monomethylarginine. The results confirm that arginine methylation is the sole methylation modification in the recombinant murine fibrillarin.

4. DISCUSSION

We have demonstrated that recombinant fibrillarin can be specifically modified by methylation to form dimethylarginine and monomethylarginines. The methylation is most likely to occur at the N-terminal RGG region of the protein. Fibrillarin is a highly conserved protein that is present in the nucleoli for ribosome biosynthesis. Fibrillarin is a common component in many C/D box small nucleolar ribonucleoprotein (snoRNP) particles, which are involved in rRNA ribose methylation (Maxwell and Fournier, 1995). Fibrillarin is widely conserved in both eukaryotes and archaebacteria. The crystal structure of a fibrillarin homologue from *Methanococcus jannaschii* has been reported (Wang *et al.*, 2000). The fibrillarin structure consists a methyltransferase-like domain in the C-terminus and a novel fold in the N-terminus that are likely to be involved in dimer formation. It is interesting that although the fibrillarin of archae is of high degree of similarity with that of eukaryotes, it is lacking an N-terminus arginine- and glycine (RGG)–rich motif that is present in eukaryotic fibrillarin. Because the archae do not have nucleolus but contain snoRNA containing RNP, it is likely that the RGG motif is involved in nucleolus localization (David *et al.*, 1997).

The N-terminal GAR motifs in mammalian fibrillarin composed of glycines and arginines with a few interrupting phenylalanines last for ~80 amino acids. However, this is the most sequence-diverse region (70.4% homology) between human and mouse fibrillarin. A higher rate of evolution of the GAR domains compared with the rest of the molecules was reflected by phylogenetic constructions (David *et al.*, 1997). Why the fibrillarin protein with highly conserved methyltransferase domain is highly diverse in the N-terminal region is an interesting question. It is likely that the sequence diversity in the eukaryotic N-terminal GAR regions reflects the diverse requirement of modification in this region or the difference in the modification system in different organisms. The arginine residues in the RGG region of human fibrillarin are known to be modified to asymmetric dimethylarginines (Lischwe *et al.*, 1985). Recent studies have shown that the spinal muscular atrophy SMN protein can interact with the sDMA modified SmD1 and D3 but not the unmodified proteins (Friesen *et al.*, 2001).

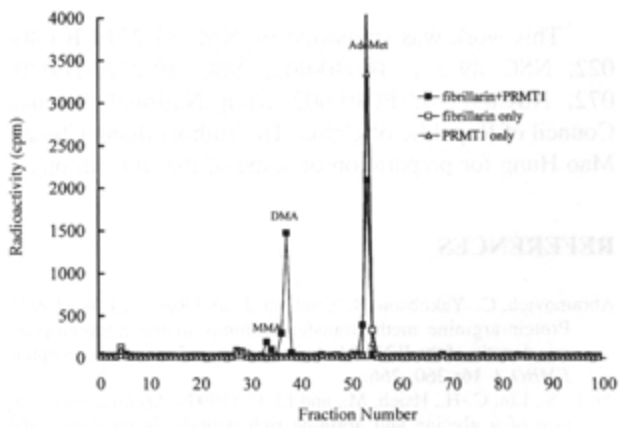


Fig. 5. Amino acid analyses of methylated amino acids in recombinant murine fibrillarin. Methylation reaction, acid hydrolysis, HPLC analyses of the hydrolysates, and scintillation counting were performed as described in Materials and Methods. The positions of the MMA and DMA determined by parallel runs of methylarginine standards were indicated.

Interaction with SMN is important for the assembly of snRNP (Pellizzoni *et al.,* 1999). Besides SmD1 and D3, fibrillarin is known to interact with SMN (Jones *et al.,* 2001; Pellizzoni *et al.,* 2001). Whether, as with SmD1 and D3, the asymmetrically arginine methylation in fibrillarin might be important for SMN interaction and snoRNP assembly is an interesting question.

In this study we demonstrated that recombinant mouse fibrillarin can be modified in a similar way as the human fibrillarin. The ratio of the DMA/MMA for fibrillarin methylated by RMT is close to 7, much higher than the ratio (2 to 4.8) obtained from methyl-accepting proteins in lymphoblastoid subcellular fractions catalyzed by exogenous RMT or endogenous methyltransferases in cells (Lin *et al.,* 2000). The DMA/MMA ratio of recombinant hnRNP A1 modified by partially purified protein arginine methyltransferase from calf brain was 2.4 at most (Rajpurohit *et al.,* 1994a). The high DMA/MMA ratio obtained by our experiment probably suggests the specificity of both the methyl-accepting substrate and the enzyme.

Our results showed that the recombinant murine fibrillarin is a good substrate for *in vitro* methylation reaction to detect the activity of the type I enzyme and can be fully methylated to the dimethylarginines than other reported recombinant methylaccepting proteins such as hnRNPA1. Further investigation of the fibrillarin and methyltransferase from different organisms might provide important clues for the enzyme-substrate recognition evolved in different biological systems.

ACKNOWLEDGMENTS

This work was supported by NSC-87-2314-B-040-022, NSC 89-2316-B-040-003, NSC 89-2320-B-040-072, NSC89-2745-P040-002 from National Science Council of Republic of China. The authors thank Chuan-Mao Hung for preparation of some of the illustrations.

REFERENCES

Abramovich, C., Yakobson, B., Chebath, J., and Revel, M. A. (1997). Protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *EMBO J.* **16:** 260–266.

Ai, L.-S., Lin, C.-H., Hsieh, M., and Li, C. (1999). Arginine methylation of a glycine and arginine rich peptide derived from the sequences of human FMRP and fibrillarin. *Proc. Natl. Sci. Counc. Repub. China B* **23:** 175–180.

Baldwin, G. S., and Carnegie, P. R. (1971). Specific enzymatic methylation of an arginine in the experimental allergic encephalomyelitis protein from human myelin. *Science* **171:** 579–581.

Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L., and Luhrmann, R. (2000). The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. *J. Biol. Chem.* **275:** 17122–17129.

Brahms, H., Meheus, L., de Brabandere, V., Fischer, U., and Luhrmann, R. (2001). Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B'pr and the Sm-like protein LSm4, and their interaction with the SMN protein. *RNA* **7:** 1531–1542.

Branscombe, T. L., Frankel, A., Lee, J. H., Cook, J. R., Yang, Z., Pestka, S., *et al.* (2001). PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. *J. Biol. Chem.* **276:** 32971–32976.

Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., *et al.* (1999). Regulation of transcription by a protein methyltransferase. *Science* **284:** 2174–2177.

David, E., McNeil, J. B., Basile, V., and Pearlman, R. E. (1997). An unusual fibrillarin gene and protein: structure and functional implications. *Mol. Biol. Cell* **8:** 1051–1061.

Frankel, A., Yadav, N., Lee, J., Branscombe, T. L., Clarke, S., and Bedford, M. T. (2002). The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* **277:** 3537–3543.

Friesen, W. J., Massenet, S., Paushkin, S., Wyce, A., and Dreyfuss, G. (2001). SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets. *Molec. Cell* **7:** 1111–1117.

Gary, J. D., Lin, W. J., Yang, M. C., Herschman, H. R., and Clarke, S. (1996). The predominant protein-arginine methyltransferase from *Saccharomyces cerevisiae. J. Biol. Chem.* **271:** 12585–12594.

Gary, J. D., and Clarke, S. (1998). RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.* **61:** 65–131.

Jones, K. W., Gorzynski, K., Hales, C. M., Fischer, U., Badbanchi, F., Terns, R. M., *et al.* (2001). Direct interaction of the spinal muscular atrophy disease protein SMN with the small nucleolar RNA-associated protein fibrillarin. *J. Biol. Chem.* **276:** 38645–38651.

Katsanis, N., Yaspo, M.-L., and Fisher, E. M. C. (1997). Identification and mapping of a novel human gene, HRMT1L1, homologous to the rat protein arginine N-methyltransferase 1 (PRMT1) gene. *Mamm. Genome* **8:** 526–529.

Lapeyre, B., Amalric, F., Ghaffari, S. H., Venkatarama Rao, S. V., Dumbar, T. S., and Olson, M. O. (1986). Protein and cDNA sequence of a glycine-rich, dimethylarginine containing region located near the carboxyl-terminal end of nucleolin (c23 and 100 kDa). *J. Biol. Chem.* **261:** 9167–9173.

Li, C., Ai, L. S., Lin, C. H., Hsieh, M., Li, Y. C., and Li, S. Y. (1998). Protein N-arginine methylation in adenosine dialdehyde-treated lymphoblastoid cells. *Arch. Biochem. Biophys.* **351:** 53–59.

Lin, C. H., Hsieh, M., Li, Y. C., Li, S. Y., Pearson, D. L., Pollard, K. M., *et al.* (2000). Protein N-arginine methylation in subcellular fractions of lymphoblastoid cells. *J. Biochem (Tokyo)* **128,** 493–498.

Lin, W. J., Gary, J. D., Yang, M. C., Clarke, S., and Herschman, H. R. (1996). The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. *J. Biol. Chem.* **271:** 15034–15044.

Lischwe, M. A., Ochs, R. L., Reddy, R., Cook, R. G., Yeoman, L. C., Tan, E. M., *et al.* (1985). Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in N^G, N^G-dimethylarginine. *J. Biol. Chem.* **260:** 14304–14310.

Liu, Q., and Dreyfuss, G. (1995). *In vivo* and *in vitro* arginine methylation of RNA-binding proteins. *Mol. Cell. Biol.* **15:** 2800–2808.

Maxwell, E. S. and Fournier, M. J. (1995). The small nucleolar RNAs. *Annu. Rev. Biochem.* **64:** 897–934.

Mowen, K. A., Tang, J., Zhu, W., Schurter, B. T., Shuai, K., Herschman, H. R., *et al.* (2001). Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. *Cell* **104:** 731–741.

Najbauer, J., Johnson, B. A., Young, A. L., and Aswad, D. W. (1993). Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. *J. Biol. Chem.* **268:** 10501–10509.

Nichols, R. C., Wang, X. W., Tang, J., Hamilton, B. J., High, F. A., Herschman, H. R., *et al.* (2000). The RGG domain in hnRNP A2 affects subcellular localization. *Exp. Cell Res.* **256:** 522–532.

Pearson, D. L., Reimonenq, R. D., and Pollard, K. M. (1999). Expression and purification of recombinant mouse fibrillarin. *Protein Expr. Purif.* **17:** 49–56.

Pellizzoni, L., Charroux, B., and Dreyfuss, G. (1999). SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins. *Proc. Natl. Acad. Sci. USA* **96:** 11167–11172.

Pellizzoni, L., Baccon, J., Charroux, B., and Dreyfuss, G. (2001). The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1. *Curr. Biol.* **11:** 1079–1088.

Rajpurohit, R., Lee, S. O., Park, J. O., Paik, W. K., and Kim, S. (1994a). Enzymatic methylation of recombinant heterogeneous nuclear RNP protein A1: Dual substrate specificity for S-adenosylmethionine:histone-arginine N-methyltransferase. *J. Biol. Chem.* **269:** 1075–1082.

Rajpurohit, R., Paik, W. K., and Kim, S. (1994b). Effect of enzymatic methylation of heterogeneous ribonucleoprotein particle A1 on its nucleic-acid binding and controlled proteolysis. *Biochem. J.* **304:** 903–909.

Rho, J., Choi, S., Seong, Y. R., Cho, W. K., Kim, S. H., and Im, D. S. (2001). Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. *J. Biol. Chem.* **276:** 11393–11401.

Scott, H. S., Antonarakis, S. E., Lalioti, M. D., Rossier, C., Silver, P. M., and Henry, M. F. (1998). Identification and Characterization of two putative human arginine methyltransferase (HRMT1L1 and HRMT1L2). *Genomics* **48:** 330–340.

Shen, E. C., Henry, M. F., Weiss, V. H., Valentini, S. R., Silver, P. A., and Lee, M. S. (1998). Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes Dev.* **12:** 679–691.

Tang, J., Gary, J. D., Clarke, S., and Herschman, H. R. (1998). PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. *J. Biol. Chem.* **273:** 16935–16945.

Tollervey, D., Lehtonen, H., Jansen, R., Kern, H., and Hurt, E. C. (1993). Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell* **72:** 443–457.

Turley, S. J., Tan, E. M., and Pollard, K. M. (1993). Molecular cloning and sequence analysis of U3 snoRNA-associated mouse fibrillarin. *Biochim. Biophys. Acta* **1216:** 119–122.

Wang, H., Boisvert, D., Kim, K. K., Kim, R., and Kim, S. H. (2000). Crystal structure of a fibrillarin homologue from *Methanococcus jannaschii*, a hyperthermophile, at 1.6 A resolution. *EMBO J.* **19:** 317–323.

Wang, H., Huang, Z. Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B. D., *et al.* (2001). Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* **293:** 853–857.

J. Biochem. **132**, 813–818 (2002)

Proteomic Analysis of Stable Protein Methylation in Lymphoblastoid Cells[1]

Hung-Ming Huang,[*] Ming F. Tam,[‡] Tsuey-Chyi S. Tam,[‡] Da-Huang Chen,[*] Mingli Hsieh[*,‡] and Chuan Li[*,†,2]

[*]Institute of Medicine, [†]Department of Life Science, Chung Shan Medical and Dental University, 110 Sec. 1 Chien-Kuo N. Rd, Taichung, Taiwan, ROC; and [‡]Institute of Molecular Biology, Academia Sinica, Nankang, Taiwan, ROC

Received May 14, 2002; accepted September 13, 2002

We investigated the global distribution of methylaccepting proteins in lymphoblastoid cells by two-dimensional (2-D) gel electrophoresis. The 2-D electrophoreograms of normal and hypo-methylation (cells grown with a methyltransferase inhibitor adenosine dialdehyde) protein extracts did not exhibit significant differences. However, *in vitro* methylation of the hypomethylated extracts in the presence of the methyl-group donor *S*-adenosyl-[methyl-^{3}H]-methionine revealed close to a hundred signals. Less than one-fifth of the signals could be correlated with protein stains, indicating that most of the methylaccepting proteins are low abundant ones. We analyzed six of the spots that can be correlated with protein stains and suggested their identities. Among these putative protein methylacceptors, three are heterogeneous nuclear ribonucleoproteins (hnRNPA2/B1 and hnRNP K) that are reportedly methylated in their arginine- and glycine-rich RGG motifs.

Key words: hnRNPA2/B1, posttranslational modification, two-dimensional gel electrophoresis.

One of the major challenges in the post-genomic era is to investigate the expression of the complete set of cellular proteins (*1*). Posttranslational modifications increase the complexity of proteins beyond the combination of twenty amino acids. Protein *N*-arginine methylation is an irreversible modification of the guanidino nitrogens of arginyl residues that accounts for the majority of stable cellular protein methylation (*2, 3*). Most methylarginines identified on proteins appear to be N^G-monomethylarginine (MMA) and asymmetric N^G,N^G-dimethylarginines (aDMA) that occur among RNA binding proteins within the Arg-Gly-Gly context (*2–4*). These modifications were catalyzed by type I arginine methyltransferases (*5*). Type II methyltransferases modify proteins such as myelin basic protein (*6*), the core snRNP SmD1, D3 (*7*), B/B′, and one of the Sm-like proteins, LSm4 (*8*). The methylation products are MMA and symmetric N^G,$N^{G'}$-dimethylarginine (sDMA).

Protein arginine methylation has been proposed to be involved in various cellular processes. Firstly, arginine methylation was suggested to be important for RNA binding, and methylated recombinant heterogeneous nuclear ribonucleoproteins A1 (hnRNP A1) showed reduced RNA binding activity as compared to the unmethylated form (*9*). However, methylation of Hrp1p, a yeast hnRNP, did not affect its specific RNA binding activity (*10*). Secondly, since the predominant protein arginine methyltransferase PRMT1 interacts with proteins involved in signal transduction pathways such as TIS 21 (*11*), the intracellular domain of the interferone α/β receptor (*12*), and ILF3 (*13*), protein arginine methylation was suggested to play roles in signal transduction (*11, 14*). Recently, arginine methylation of certain proteins such as STAT1 was shown to directly affect their binding with other proteins (*15*). The modification may also modulate protein-protein interactions by interfering with other important modifications of neighboring motifs of the same protein, as reported for Np13p (*16*), Sam 68 (*17*), and histone H4 (*18*). Lastly, arginine methylation has been implicated in subcellular localization. Yeast deficient in RMT1/HMT1, a predominant arginine methyltransferase, has problems in hnRNP and mRNA nuclear export (*19*). Furthermore, decreased methylation of hnRNP A2/B1 resulted in accumulation of the protein in the nucleus rather than in the cytoplasm (*20*).

In view of that this type of posttranslational modification plays important roles in cells, identification of arginine methylaccepting proteins ought to be an important task. Arginine methylaccepting substrates have been identified and studied through different approaches. Proteins such as fibrillarin (*21*), nucleolin (*22*), and hnRNPA1 (*23*) were found to contain asymmetric dimethylarginines on direct biochemical analyses. Other proteins with similar arginine-

[1] The work was supported by grants NSC 89-2745-P040-002, 89-2320-B-040-072, and NSC 90-2320-B-040-031 from the National Science Council of Republic of China, grant CSMC 88-OM-A-014 from Chung Shan Medical and Dental University to CL, and grant 90-2311-B-001-092 from the National Science Council of Republic of China to MFT.

[2] To whom correspondence should be addressed. Fax: +886-4-2475-7412, E-mail: cli@mercury.csmu.edu.tw

Abbreviations: aDMA, asymmetric N^G, N^G-dimethylarginine; sDMA, symmetric N^G, $N^{G'}$-dimethylarginine; MMA, N^G-monomethylarginine; AdOx, adenosine dialdehyde; RMT, arginine methyltransferase; PRMT, protein arginine methyltransferase; GST, glutathion-S-transferase; AdoMet, *S*-adenosylmethionine; hnRNP, heterogeneous nuclear ribonucleoproteins; RGG box, arginine and glycine rich motif, MALDI-TOF, matrix assisted laser desorption ionization-time of flight; 2-D, two-dimensional; IPG, immobilized pH gradient.

and glycine-rich motifs, mostly RNA binding proteins such as herpes simplex virus ICP27 protein (*24*), FGF2 (*25*), and EWS (*26*), have also been identified as methylaccepting substrates. Recently, proteins without typical arginine- and glycine-rich sequences have been found to contain methylarginines. The methyl group on the PABPII protein was found on a RXR motif (*27*), while the methylarginine in STAT1 is located in the EIRQY context (*15*).

Alternatively, cells were made deficient in methyltransferase activity by either genetic approaches (*28, 29*) or by chemical treatment (*3*). The methylaccepting sites would not be occupied *in vivo*, and can be detected by further *in vitro* methylation reactions and analyzed by gel electrophoresis. Although previous studies had involved two-dimensional (2-D) gel electrophoresis for product analyses, the methylated proteins revealed on fluorography could not be identified unambiguously. Candidate proteins were only inferred from their molecular masses and pIs (*3, 29, 30*).

In this study, we treated lymphoblastoid cells with an indirect methyltransferase inhibitor, adenosine dialdehyde (AdOx). After *in vivo* or *in vitro* methylation reactions, cell extracts were separated by 2-D electrophoresis. The proteins of interest were excised from the gels, digested with a specific protease and then subjected to MALDI (matrix assisted laser desorption ionization)-TOF (time of flight) mass spectroscopy for protein identification. With this approach, we have confirmed some previously known methylaccepting proteins and identified some novel putative ones.

MATERIALS AND METHODS

Cell Cultures and Protein Extraction—EB-virus transformed lymphoblastoid cell line 003 was grown as previously described (*2*). For methylation reactions, cells were grown in the presence of 20 μM AdOx for 72 h for the accumulation of methylaccepting proteins. Cells collected from a 75 cm^2 culture flask were resuspended in 600 μl of 2-D extraction buffer (100 mM ammonium carbonate, pH 8.0, 0.5 mM PMSF, 2 mM EDTA, 2% NP-40). The cells were incubated on ice for 10 min and then lysed by sonication. Cell debris was removed by centrifugation at 12,000 ×*g* for 20 min. Proteins in the extracts were quantified by means of the BCA assay (Pierce) with bovine serum albumin as the standard. Extracts thus prepared gave poor methylaccepting signals after the *in vitro* methylation reaction. We thus followed the protocol of Li *et al.* (*2*) to prepare the extracts for the methylation reaction using extraction buffer (phosphate-buffered saline [10 mM dibasic sodium phosphate, 1.8 mM monobasic potassium phosphate, 140 mM NaCl, 2.7 mM KCl, pH 7.4], 5% glycerol, 1 mM disodium EDTA, 1 mM EGTA, 40 μg/ml leupeptin and aprotinin, 20 μg/ml pepstatin, 1 mM PMSF, 0.5% Triton X-100).

In Vitro Methylation Reaction and Fluorography—Total extracts (100 μg proteins) were incubated with 10 μCi of [*methyl*-^{3}H]-AdoMet (60 Ci/mmol, Amersham) in the presence or absence of recombinant yeast GST-RMT1 arginine methyltransferase in methylation reaction buffer (50 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM EGTA). The final volume was 70 μl and the reaction was carried out for 2 h at 30°C. The reaction was stopped by incubation on ice, followed by dialysis using a mini dialysis unit (MW cut off 3,500; Pierce) for salt removal. Recombinant yeast GST-RMT methyltransferase was prepared as previously described (*31*).

Two-Dimensional Gel Electrophoresis—Samples (1 mg) were loaded onto immobilized pH gradient (IPG) strips (pH 3–10, 13 cm) through overnight rehydration at room temperature in a reswelling tray. The samples were prepared by lyophilization and then resuspension in 250 μl of 8 M urea, 2% (w/v) CHAPS, 2% IPG buffer, and 2.8 mg/ml dithiothreitol. Isoelectrophocusing (IEF) was carried out in a Multiphor II system as instructed by the manufacturer (Amersham Biosciences). Upon completion of IEF (17,000 Volt-hours), the IPG strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% (w/v) SDS, 15.4 mg/ml dithiothreitol, and 0.0125% bromophenol blue for 15 min. The strips were then stored frozen at –80°C or used immediately. Gradient SDS polyacrylamide gels (5–20%, 17 × 20 × 1 mm) were employed for the second dimensional separation in a Bio-Rad PROTEAN II xi system. Proteins were visualized by colloidal blue staining for 2 days and destaining was carried out in 25% methanol (*32*). Z3 (Compugen) and Melanie 3 (Genebio) software was used to analyze the gel images. Gels containing isotopically labeled proteins were treated with EN^{3}HANCE (Du Pont NEN), dried and then exposed to X-ray film (Kodak, MS) at –75°C. Since silver-stained gels gave poor fluorographic results and large gels tended to crack after drying, we used colloidal blue staining and 13 cm IPG strips for the analysis of radioactive samples.

In Gel Protein Digestion—Protein spots were excised from the polyacrylamide gel with pipette tips, and the gel plugs were washed extensively in 10% acetic acid and 50% methanol in water. They were then incubated in 200 mM Tris, pH 8.8, 50% acetonitrile for 30 min. The buffer was removed and the gel plugs were dehydrated by adding acetonitrile, followed by vacuum centrifugation. Lys-C protease solution (Wako, 0.033 mg/ml in 100 mM ammonium bicarbonate buffer, pH 9.2) or trypsin solution (Promega, 0.033 mg/ml in 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$) was then added to restore the gel plugs to their original volume. Digestion was carried out at 42 and 25°C overnight for Lys-C and trypsin proteases, respectively. The digests were then acidified by adding 2% TFA. Peptides were extracted from the gel plugs sequentially with 0.1% TFA, in water, 30% acetonitrile in water with 0.1% TFA, and finally 60% acetonitrile in water and 0.1% TFA. The solutions were combined, dried to less than 50 μl, and then desalted with a Ziptip (Millipore) pipette tip following the manufacturer's instructions.

MALDI-TOF MS Analysis—Peptide mass mapping was performed on a Bruker (Bruker-Daltonics, Bremen, Germany) REFLEX III time-of-flight mass spectrometer equipped with a SCOUT source and delayed extraction. Detection was performed in the positive ion reflector mode with each mass determination being the average of 100 spectra. Samples for mass measurement were prepared using the solution-phase nitrocellulose method (*33*) with α-cyano-4-hydroxycinnamic acid as the matrix. Alternatively, samples were deposited on a Bruker 600 micron AnchorChip according to the manufacturer's instructions. Angiotensin II (1,045.54 amu), adrenocorticotropic hormone fragment 18–39 (2,465.73 amu), and somatostatin (3,146.47) were used as external calibration standards. The peptide mass spectra obtained on MALDI-TOF analysis were ana-

lyzed using PeptidIdent (http://tw.expasy.org) and Profound (http://129.85.19.192/profound/).

RESULTS AND DISCUSSION

Stable Methylaccepting Proteins in Lymphoblastoid Total Extracts Analyzed by 2-D Electrophoresis—Arginine methylation is a stable posttranslational modification different from protein phosphorylation that is regulated reversibly by kinases and phosphatases. Even though six different protein arginine methyltransferases have been identified so far (*11, 34–39*), no demethylase activity has been reported. Therefore, once an arginine methylaccepting protein is methylated, the methyl group will stay on the protein. Proteomic analysis of protein phosphorylation benefits by the comparison of phophatase-treated or untreated samples (*40, 41*). In this study we used a methyltransferase inhibitor, AdOx, for the accumulation of proteins in the hypomethylation state. Extracts of AdOx-treated or -untreated lymphoblastoid cells were prepared and isolated on pH 3–10 IPG strips. The proteins were further separated in the second dimension by SDS-PAGE (8–15% gradient gel) and visualized by colloidal blue staining (Fig. 1). Typically, we were able to detect more than 1,000 spots on a 13-cm IPG strip loaded with 1 mg of protein. However, no consistent significant differences between the 2D patterns of the AdOx-treated and -untreated cells could be found using either the Z3 or Melanie 3 program. Even though AdOx treatment can cause the accumulation of methylaccepting sites in cellular proteins that can be methylated subsequently *in vitro* (*2, 4*), these sites available upon treatment (after confluency) are likely to comprise only a minor fraction of all the methylaccepting sites in proteins. It is also possible that arginine methylation does not significantly change the overall charge property or molecular masses of the proteins to be detected by 2-D electrophoresis.

We prepared total extracts of hypomethylated lymphoblastoid cells for *in vitro* methylation reactions in the presence of the radioactive methyl-group donor [*methyl*-^{3}H]-AdoMet in the presence of exogenous recombinant yeast GST-RMT1 enzyme. Since six protein arginine methyltransferases have been identified in mammalian systems and are likely to be regulated differently (*11, 34–39*), we used the recombinant yeast enzyme, which is stables and has a broader substrate specificity than the mammalian enzyme (*31*). The reaction products were then separated by two-dimensional gel electrophoresis and the methylated proteins were visualized by fluorography. As shown in Fig. 2, more than a hundred radioactive spots representing putative methylaccepting proteins were detected with our 2-D gel system. The addition of GST-RMT1 increased the intensity of some spots but few extra spots were detected (data not shown), indicating the exogenous enzyme recognized similar methylaccepting substrates to those recognized by the endogenous enzyme, as shown previously on SDS-PAGE analyses (*2*). Of the more than one hundred putative methylaccepting proteins revealed by two-dimensional gel electrophoresis of *in vitro* radio-labeled lymphoblastoid total extracts, less than one-fifth could be correlated with protein stains. The results indicate that most

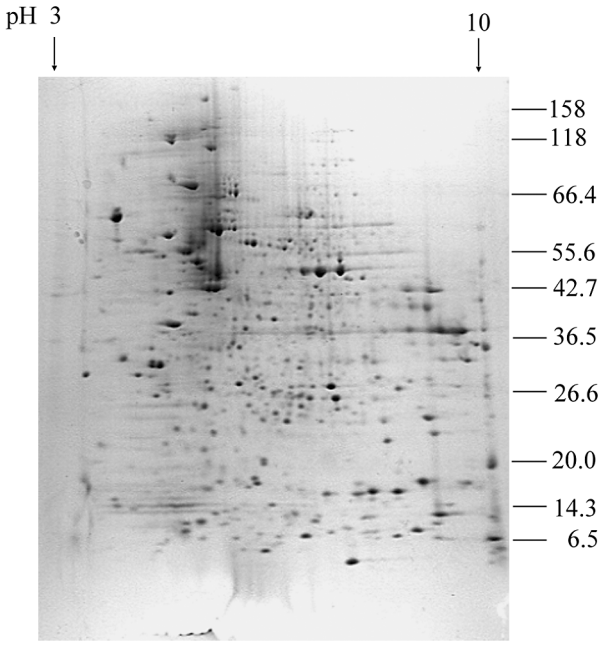


Fig. 1. **2-DE pattern of a lymphoblastoid total extract.** Lymphoblastoid total extracts (1 mg protein) prepared from AdOx-treated cells were analyzed by 2-DE and stained with colloidal blue as described.

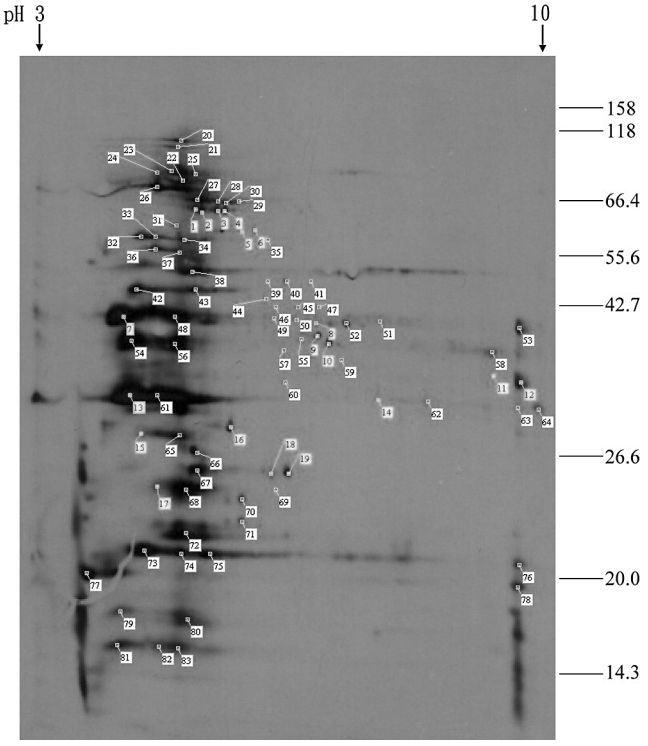


Fig. 2. **Protein methylacceptors revealed after *in vitro* methylation and 2-DE analyses.** Lymphoblastoid total extracts (200 μg) were incubated with exogenous recombinant yeast RMT1 methyltransferase and [*methyl*-^{3}H]-AdoMet. The reaction mixtures were then dialyzed with a mini dialysis unit, lyophilized and subjected to 2-DE analysis. The gel was stained with colloidal blue, soaked in ENHANCER and then dried for fluorography. The figure represents 2-week exposure. Spots 1–19 are the radioactive spots that can be matched with protein stains.

of the methylaccepting proteins are low abundant ones that are difficult to detect on colloidal staining. This can also help to explain why the AdOx treatment did not result in different protein patterns on 2-D gel electrophoresis.

Many of the reported arginine methyl-accepting proteins (*e.g.* fibrillarin, hnRNPs, histones and Sm proteins, see Table I) have high pI values that are beyond the resolution of our IEF gel (pH 3–10). We observed more than ten radioactive spots at the basic limit of the 2-D gel corresponding to molecular masses ranging from 40 to 14 kDa. These signals are likely to be due to proteins. Interestingly, the strongest signals on the 2-D gel came from the region between pH 4–6, differing from the pIs of most reported arginine methylaccepting proteins (Table I).

Identification of the Methylaccepting Proteins—To identify the methylaccepting proteins, we increased the loading of the methylated samples for 2-D gel electrophoresis. However, most of the heavily methylated signals could not be matched back to dye-stained spots. The results indicate that most of the methylaccepting proteins are low abundant ones that are difficult to detect on colloidal staining, and thus help to explain why the AdOx treatment did not result in different protein patterns on 2-D gel electrophoresis.

We were able to correlate about 20 radioactive signals with corresponding dye-stained protein spots. These spots were excised, protease-digested, and then subjected to MALDI-TOF analyses. We were able to identify six of the putative methylaccepting proteins using the peptide masses along with the pIs and molecular masses for the protein spots (Table II). Among these proteins, hnRNPA2/B1 and hnRNP K were known methylaccepting proteins containing RGG motifs (*20, 42*). It is likely that these proteins are more abundant ones than other methylaccepting proteins, and thus can be detected and analyzed by means of our approach.

For the other three putative novel methylacceptors, further experiments failed to show that protein disulfide isomerase (PDI) was arginine methylated. The recombinant yeast RMT enzyme failed to methylate bovine PDI (Calbiochem). This protein was not recognized by the mono- and di-methylarginine Ab (data not shown). To determine whether PDI is modified through another type of protein methylation or just has the same molecular weight and pI as another low abundant methylaccepting protein needs more experimental work. Whether or not prohibitin and proteasome subunit alpha type 5 are real methylacceptors is also under investigation.

Identification of the Methylaccepting Sites in the hnRNPA2/B1 Proteins—We detected the specific presence of hnRNPA2/B1 in the immunoprecipitants with a methylarginine-specific antibody (data not shown), further confirming the arginine methylation of the proteins. The difference between hnRNP A2 and B1 is the additional twelve amino acid residues at the N-terminus of B1. According to previous reports, we designated the more acidic one A2. We analyzed both the A2 and B1 spots using AdOx-treated or -untreated samples. Interestingly, as shown in Fig. 3, two extra peaks at 2,386.6 and 2,400.7 *m/z*, and a higher one at 2,509.6 *m/z* were observed for the trypsin digests of the B1 spots for extracts without AdOx treatment (theoretically in the normal methylation state), but not for those of the corresponding spots for AdOx-treated extracts (theoretically in the hypomethylation state). Although no peptide could be matched with these molecular masses on PeptIden analysis, mono- or di-methyl methylation of peptides containing RG-rich sequences was suggested by further FINDMOD analyses (http://tw.expasy.org/tools/findmod/; *47*). The peptide comprising residues 204 to 228 (GGNFGFGDS**R**GGGGNFGPGPGSNFR, mass 2,372.055) with one trypsin miss cleavage and mono or dimethylarginine at Arg-213 would well explain the extra two masses. It is possible that the higher signal, 2,509.6, could be due to monoarginine methylation at residue Arg-266 in the peptide covering residues 239–266 (GFGDGYNGYGGGPGGGNFGGSPGYGGG**R**, mass 2,495.039). We obtained similar results with the hnRNPA2 sample (data not shown).

Nichols *et al.* (*20*) showed that the methylation state of RGG in hnRNP A2 might interfere with its subcellular localization. The addition of AdOx (low methylation) shifted the hnRNPA2 protein to the cytoplasm and then the nucleus. Removal of residues R203 to G265 reduced the methylation level of the recombinant protein to only 5%,

TABLE I. **List of known arginine methylaccepting proteins.**

Protein	SWISS PROT No.	MW	PI	References
Fibrillarin	P22087	33,784.22	10.18	*21*
Nucleolin	P19338	76,212.88	4.59	*22*
HnRNP				*41*
hnRNPA1	P09651	38,714.59	9.26	*23*
hnRNPA2/B1	P22626	37,429.70	8.97	*20*
(A2)		36,005.98	8.67	
E1B-AP5	O76022	95,809.60	6.49	*43*
Histone				
H2A	P28001	14,004.30	11.05	*36*
H3	P16106	15,272.89	11.13	*44, 45*
H4	P02304	11,236.15	11.36	*18, 46*
polyA binding protein II (PABP2)	O43484	32,749.07	5.04	*27*
ILF3 (NFAR2)	Q9BZH4	95,591.74	8.88	*13*
Sam68	Q07666	48,227.34	8.73	*17*
	Q99760	44,027.41	6.78	
EWS	Q01844	68,478.24	9.37	*26*
STAT1	P42224	87,334.76	5.74	*15*
MBP	P02686	33,117.10	9.79	*6*
SmD1	P13641	13,281.57	11.56	*7*
SmD3	P43331	13,916.25	10.33	*7*

TABLE II. **List of putative protein methylacceptors identified in this study.**

Spot ID	SWISS-PROT Accession No.	Protein name	Measured pI/MW	Theoretical pI/MW	Peptide matches/ detected peptides	PeptIden score	Profound score	Sequence coverage (%)
2	Q07244	hnRNPK	5.2/60	5.39/50.98	9/14	0.57	0.62	22
6	P30101	Protein disulfide isomerase	6.0/57	5.60/54.26	11/14	0.79	0.85	31
11	P22626	hnRNPA2	8.5/36.5	8.67/36.01	7/11	0.64	2.38	32
12	P22626	hnRNPB1	8.9/36.5	8.97/37.43	7/10	0.70	2.43	32
15	P28066	Proteasome subunit alpha type 5	4.5/27	4.69/26.47	6/10	0.40	2.43	27
16	P35232	Prohibitin	5.8/29	5.57/29.79	8/14	0.57	1.77	37

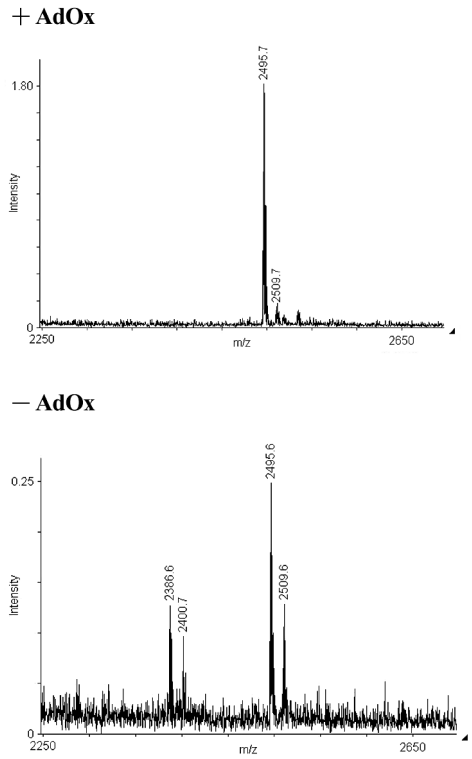


Fig. 3. **MALDI-TOF spectrum of tryptic peptide fragments derived from hnRNPB1.** (a) The hnRNPB1 spot was from 2-D gels used to separate extracts prepared from lymphoblastoid cells treated with AdOx (+AdOx). (b) The hnRNPB1 spot was from 2-D gels used to separate extracts prepared from lymphoblastoid cells not treated with AdOx (–AdOx).

indicating the major methylation site is within the deleted segment. However, the R203 residue in a peptide fragment (194–205) of hnRNPA2/B1 purified from a human tumor cell line was not methylated, as determined on peptide sequencing (*48*). Thus, our results are the first providing chemical evidence suggesting putative methylaccepting sites of hnRNPA2/B1 at Arg-213 and Arg-226.

To determine whether or not the other three putative methylaccepting proteins, protein disulfide isomerase, prohibitin and proteosome subunit alpha type 5, we identified are methylacceptors requires further investigation. Prohibitin had been reported to be post-translationally modified in younger but not older cells, but no positive results were obtained for prohibitin phosphorylation (*49*). Whether or not prohibitin can be modified by methylation is an interesting question. Since AdOx can inhibit the activity of all methyltransferases using S-adenosylmethionine as the methyl group donor, other types of protein methylation besides arginine methylation could also be revealed by this approach. Thus, for putative novel methylaccpetor proteins, more analyses are required. As for low abundant methylaccepting proteins, previous fractionation or concentration procedures should be included before the 2-D analyses for a better chance of detection.

REFERENCES

1. Pandey, A. and Mann, M. (2000) Proteomics to study genes and genomes. *Nature* **405**, 837–846
2. Li, C., Ai, L.S., Lin, C.H., Hsieh, M., Li, Y.C., and Li, S.Y. (1998) Protein N-arginine methylation in adenosine dialdehyde-treated lymphoblastoid cells. *Arch. Biochem. Biophys.* **351**, 53–59
3. Najbauer, J., Johnson, B.A., Young, A.L., and Aswad, D.W. (1993) Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. *J. Biol. Chem.* **268**, 10501–10509
4. Lin, C.H., Hsieh, M., Li, Y.C., Li, S.Y., Pearson, D.L., Pollard, K.M., and Li, C. (2000) Protein N-arginine methylation in subcellular fractions of lymphoblastoid cells. *J. Biochem.* **128**, 493–498
5. Gary, J.D. and Clarke, S. (1998) RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.* **61**, 65–131
6. Baldwin, G.S. and Carnegie, P.R. (1971) Specific enzymatic methylation of an arginine in the experimental allergic encephalomyelitis protein from human myelin. *Science* **171**, 579–581
7. Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L., and Luhrmann, R. (2000) The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. *J. Biol. Chem.* **275**, 17122–17129
8. Brahms, H., Meheus, L., de Brabandere, V., Fischer, U., and Luhrmann, R. (2001) Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B′ and the Sm-like protein LSm4, and their interaction with the SMN protein. *RNA* **7**, 1531–1542
9. Rajpurohit, R., Paik, W.K., and Kim, S. (1994) Effect of enzymatic methylation of heterogeneous ribonucleoprotein particle A1 on its nucleic-acid binding and controlled proteolysis. *Biochem. J.* **304**, 903–909
10. Valentini, S.R., Weiss, V.H., and Silver, P.A. (1999) Arginine methylation and binding of Hrp1p to the efficiency element for mRNA 3′-end formation. *RNA* **5**, 272–280
11. Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S., and Herschman, H.R. (1996) The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. *J. Biol. Chem.* **271**, 15034–15044
12. Abramovich, C., Yakobson, B., Chebath, J., and Revel, M.A. (1997) protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. *EMBO J.* **16**, 260–266
13. Tang, J., Kao, P.N., and Herschman, H.R. (2000) Protein-arginine methyltransferase I, the predominant protein-arginine methyltransferase in cells, interacts with and is regulated by interleukin enhancer-binding factor 3. *J. Biol. Chem.* **275**, 19866–19876
14. Aletta, J.M., Cimato, T.R., and Ettinger, M.J. (1998) Protein methylation: a signal event in post-translational modification. *Trends Biochem. Sci.* **23**, 89–91
15. Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R., and David, M. (2001) Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. *Cell* **104**, 731–741
16. Yun, C.Y. and Fu, X.D. (2000) Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in *Saccharomyces cerevisiae*. *J. Cell Biol.* **150**, 707–718
17. Bedford, M.T., Frankel, A., Yaffe, M.B., Clarke, S., Leder, P., and Richard, S. (2000) Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. *J. Biol. Chem.* **275**, 16030–16036
18. Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P., and Zhang, Y. (2001) Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* **293**, 853–857
19. Shen, E.C., Henry. M.F., Weiss, V.H., Valentini, S.R., Silver,

P.A., and Lee, M.S. (1998) Arginine methylation facilitates the nuclear export of hnRNP proteins. *Genes Dev.* **12**, 679–691
20. Nichols, R.C., Wang, X.W., Tang, J., Hamilton, B.J., High, F.A., Herschman, H.R., and Rigby, W.F. (2000) The RGG domain in hnRNP A2 affects subcellular localization. *Exp. Cell Res.* **256**, 522–532
21. Lischwe, M.A., Ochs, R.L., Reddy, R., Cook, R.G., Yeoman, L.C., Tan, E.M., Reichlin, M., and Busch, H. (1985) Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in NG,NG-dimethylarginine. *J. Biol. Chem.* **260**, 14304–14310
22. Lischwe, M.A., Cook, R.G., Ahn, Y.S., Yeoman, L.C., and Busch, H. (1985) Clustering of glycine and NG,NG-dimethylarginine in nucleolar protein C23. *Biochemistry.* **24**, 6025–6028
23. Rajpurohit, R., Lee, S.O., Park, J.O., Paik, W.K., and Kim, S. (1994) Enzymatic methylation of recombinant heterogeneous nuclear RNP protein A1. Dual substrate specificity for S-adenosylmethionine:histone-arginine N-methyltransferase. *J. Biol. Chem.* **269**, 1075–1082
24. Mears, W.E. and Rice, S.A. (1996) The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines *in vivo* methylation. *J. Virol.* **70**, 7445–7453
25. Klein, S., Carroll, J.A., Chen, Y., Henry, M.F., Henry, P.A., Ortonowski, I.E., Pintucci, G., Beavis, R.C., Burgess, W.H., and Rifkin, D.B. (2000) Biochemical analysis of the arginine methylation of high molecular weight fibroblast growth factor-2. *J. Biol. Chem.* **275**, 3150–3157
26. Belyanskaya, L.L., Gehrig, P.M., and Gehring, H. (2001) Exposure on cell surface and extensive arginine methylation of ewing sarcoma (EWS) protein. *J. Biol. Chem.* **276**, 18681–18687
27. Smith, J.J., Rucknagel, K.P., Schierhorn, A., Tang, J., Nemeth, A., Linder, M., Herschman, H.R., and Wahle, E. (1999) Unusual sites of arginine methylation in Poly(A)-binding protein II and *in vitro* methylation by protein arginine methyltransferases PRMT1 and PRMT3. *J. Biol. Chem.* **274**, 13229-13234
28. Pawlak, M.R., Scherer, C.A., Chen, J., Roshon, M.J., and Ruley, H.E. (2000) Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. *Mol. Cell Biol.* **20**, 4859–4869
29. Frankel, A. and Clarke, S. (1999) RNase treatment of yeast and mammalian cell extracts affects *in vitro* substrate methylation by type I protein arginine N-methyltransferases. *Biochem. Biophys. Res. Commun.* **259**, 391–400
30. Najbauer, J. and Aswad, D.W. (1990) Diversity of methyl acceptor proteins in rat pheochromocytoma (PC12) cells revealed after treatment with adenosine dialdehyde. *J. Biol. Chem.* **265**, 12717–12721
31. Gary, J.D., Lin, W.J., Yang, M.C., Herschman, H.R., and Clarke, S. (1996) The predominant protein-arginine methyltransferase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 12585–12594
32. Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) *Electrophoresis* **9**, 255–262.
33. Landry, F., Lombardo, C.R., and Smith, J.W. (2000) A method for application of samples to matrix-assisted laser desorption ionization time-of-flight targets that enhances peptide detection. *Anal. Biochem.* **279,** 1–8
34. Scott, H.S., Antonarakis, S.E., Lalioti, M.D., Rossier, C., Silver, P.A., and Henry, M.F. (1998) Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). *Genomics* **48**, 330–340
35. Tang, J., Gary, J.D., Clarke, S., and Herschman, H.R. (1998) PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. *J. Biol. Chem.* **273**, 16935–16945
36. Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174–2177
37. Rho, J., Choi, S., Seong, Y.R., Cho, W.K., Kim, S.H., and Im, D.S. (2001) Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. *J. Biol. Chem.* **276**, 11393–11401
38. Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S., and Clarke, S. (2001) PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. *J. Biol. Chem.* **276**, 32971–32976
39. Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S., and Bedford, M.T. (2002) The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* **277**, 3537–3543
40. Kaufmann, H., Bailey, J.E., and Fussenegger, M. (2001) Use of antibodies for detection of phosphorylated proteins separated by two-dimensional gel electrophoresis. *Proteomics* **1**, 194–199
41. Nollau, P. and Mayer, B.J. (2001) Profiling the global tyrosine phosphorylation state by Src homology 2 domain binding. *Proc. Natl. Acad. Sci. USA* **98**, 13531–13536
42. Liu, Q. and Dreyfuss, G. (1995) *In vivo* and *in vitro* arginine methylation of RNA-binding proteins. *Mol. Cell. Biol.* **15**, 2800–2808
43. Kzhyshkowska, J., Schutt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H., and Dobner, T. (2001) Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1. *Biochem. J.* **358**, 305–314
44. Schurter, B.T., Koh, S.S., Chen, D., Bunick, G.J., Harp, J.M., Hanson, B.L., Henschen-Edman, A., Mackay, D.R., Stallcup, M.R., and Aswad, D.W. (2001) Methylation of histone H3 by coactivator-associated arginine methyltransferase 1. *Biochemistry* **40**, 5747–5756
45. Ma, H., Baumann, C.T., Li, H., Strahl, B.D., Rice, R., Jelinek, M.A., Aswad, D.W., Allis, C.D., Hager, G.L., and Stallcup, M.R. (2001) Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. *Curr. Biol.* **11**, 1981–1985
46. Strahl, B.D., Briggs, S.D., Brame, C.J., Caldwell, J.A., Koh, S.S., Ma, H., Cook, R.G., Shabanowitz, J., Hunt, D.F., Stallcup, M.R., and Allis, C.D. (2001) Methylation of histone H4 at arginine 3 occurs *in vivo* and is mediated by the nuclear receptor coactivator PRMT1. *Curr. Biol.* **11**, 996–1000
47. Wilkins, M.R., Gasteiger, E., Gooley, A., Herbert, B., Molloy, M.P., Binz, P.A., Ou, K., Sanchez, J.-C., Bairoch, A., Williams, K.L., and Hochstrasser, D.F. (1999) High-throughput mass spectrometric discovery of protein post-translational modifications. *J. Mol. Biol.* **289**, 645–657
48. Zhou, J., Mulshine, J.L., Unsworth, E.J., Scott, F.M., Avis, I.M., Vos, M.D., and Treston, A.M. (1996) Purification and characterization of a protein that permits early detection of lung cancer. Identification of heterogeneous nuclear ribonucleoprotein-A2/B1 as the antigen for monoclonal antibody 703D4. *J. Biol. Chem.* **271**, 10760–10766
49. Liu, C., Stewart, C.A., King, R.L., Danner, D.A., Dell'Orco, R.T., and McClung, J.K. (1994) Prohibitin expression during cellular senescence of human diploid fibroblasts. *Biochem. Biophys. Res. Com.* **201**, 409–414

J. Biochem. **136**, 00–00 (2004)
DOI: 10.1093/jb/mvh131

Effects of Adenosine Dialdehyde Treatment on *In Vitro* and *In Vivo* Stable Protein Methylation in HeLa Cells

Da-Huang Chen[1], Kuan-Tsu Wu[1], Chien-Jen Hung[1,2], Mingli Hsieh[1,3] and Chuan Li[1,*]

[1]Department of Life Sciences and [2]Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC; and [3]Department of Biology, Tunghai University, Taichung, Taiwan, ROC

Received June 4, 2004; accepted June 18, 2004

Adenosine dialdehyde (AdOx) is an indirect methyltransferase inhibitor broadly used in cell culture to accumulate methyl-accepting proteins in hypomethylated states for *in vitro* protein methylation analyses. In this study we included a translation inhibitor, cycloheximide, in the AdOx treatment of HeLa cells. The methyl-accepting proteins disappeared in the double treatment, indicating that they were most likely newly synthesized in the AdOx incubation period. AdOx treatment could also be used in combination with *in vivo* methylation, another technique frequently used to study protein methylation. AdOx treatment prior to *in vivo* methylation accumulated methyl-accepting proteins for the labeling reaction. The continued presence of AdOx in the *in vivo* labeling period decreased the methylation of the majority of *in vivo* methyl-accepting polypeptides. The level and pattern of the *in vivo* methylated polypeptides did not change after a 12-h chase, supporting the notion that the methylated polypeptide as well as the methyl groups on the modified polypeptides are stable. On the other hand, methylarginine-specific antibodies detected limited but consistent reduction of the methylarginine-containing proteins in AdOx-treated samples compared to the untreated ones. Thus, AdOx treatment probably only blocked a small fraction of stable protein methylation. Overall, it is likely that base-stable methylation are formed soon after the synthesis of the polypeptide and remain stable after the modification.

Key words: adenosine dialdehyde, cycloheximide, *in vivo* methylation, protein methylation.

Abbreviations: AdOx, adenosine dialdehyde; PRMT, protein arginine methyltransferase; AdoMet, S-adenosylmethionine; AdoHcy, *S*-adenosyl-L-homocystein; CHX, cycloheximide; ALLN, *N*-acetyl-leu-leu-norleucinal; sDMA, symmetric dimethylarginine; aDMA, asymmetric dimethylarginin; MMA, monomethylarginine.

Posttranslational modification of proteins can greatly enrich the diversity of the protein world beyond the combination of twenty amino acids encoded by the direction of the genetic information. Protein methylation can occur on various amino acid side chains such as glutamyl and isoaspartyl residues to form base labile methylesters or arginine or lysine residues to form chemically base-stable methylamines (*1*). It has been demonstrated that the majority of methyl-accepting sites are occupied in cells (*2*). To obtain methyl-accepting proteins with empty methyl-accepting sites for further studies, general inhibition of protein methylation can be accomplished by treating cells with methyltransferase inhibitors. For example, adenosine dialdehyde (AdOx), an indirect inhibitor that can be incorporated by cells, has been routinely used. AdOx inhibits *S*-adenosyl-L-homocystein hydrolase, resulting in the accumulation of *S*-adenosyl-L-homocystein (AdoHcy), a product inhibitor of methyltransferases that utilize *S*-adenosyl-L-methionine (AdoMet) as the methyl group donor (*3*, *4*). In this way, the methyl-accepting polypeptides can be retained in hypomethylated state upon the treatment and can later be probed by *in vitro* methylation with radioactive [methyl-^{3}H]-AdoMet.

Protein arginine methylation accounts for the majority of AdOx-accumulated stable protein methylation in rat pheochromocytoma PC12 cells (*5*) and human lymphoblastoid cells (*6*, *7*). Protein arginine methylation has been implicated in signal transduction, subcellular localization, transcription as well as protein–protein interactions (*8*, *9*). Unlike reversible modifications, such as phosphorylation of proteins by kinase and reversal of the modification by phosphatase, arginine methylation is chemically stable and irreversible. The reaction is catalyzed by a group of protein arginine methyltransferases (PRMT) that can catalyze the transfer of methyl groups from AdoMet to the side-chain ω-guanido nitrogens of arginine residues in protein substrates to form of monomethylarginines (MMA) and asymmetric N^G,N^G-dimethylarginines (aDMA; type I activity) or symmetric N^G,N'^G-dimethylarginines (sDMA; type II activity) (*10–16*). As no demethylase has been identified, the methyl group stays on the arginyl residues of the proteins once it is added.

In vivo methylation has frequently been employed approach to metabolically methylate proteins in cells. In this approach, the direct methyl group donor AdoMet cannot enter cells, so its precursor [methyl-^{3}H]-methio-

*To whom correspondence should be addressed. Tel: +886-4-24730022 ext 1807, Fax: +886-4-24757412, E-mail: cli@csmu.edu.tw

nine is generally used as the source of radioactive methyl groups. To prevent the incorporation of methyl groups into proteins by translation but not posttranslational modification, *in vivo* methylation has to be conducted in the presence of protein synthesis inhibitors (*17*). This technique has been widely used in different cell types to evaluate the protein methylation under different treatments or conditions (*18*). Specific *in vivo* methylation of certain proteins such as Sam 68 can also be demonstrated directly by their labeling during the *in vivo* methylation period (*19*, *20*).

HeLa cells have been used for hnRNP protein methylation studies (*17*) and other investigations related to protein methylation (*20*). In this study we evaluated the effect of methylation inhibitor AdOx on both the *in vitro* and *in vivo* protein methylation in HeLa cells.

MATERIALS AND METHODS

Cell Culture and Treatment—HeLa cells (ATCC CCL-2) were grown in MEM medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C in a 5% CO_2 incubator. Treatment of cells with AdOx for various time periods was performed as indicated. The cells were harvested and washed with phosphate-buffered saline, resuspended in buffer A (phosphate-buffered saline with 5% glycerol, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.5% Triton X-100 and Complete protease inhibitor cocktail [Roche]). Adenosine dialdehyde (AdOx, Sigma) was added to the medium at the indicated final concentration. Cycloheximide (CHX, Sigma) and *N*-acetyl-leu-leu-norleucinal (ALLN, Sigma) were added to the culture medium at the final concentration of 100 μg/ml and 15 μM respectively.

Extract Preparation and Methyltransferase Assay—HeLa cell extracts (20 μg of proteins) were incubated with 4 μCi of [methyl-^{3}H]-AdoMet (60 Ci/mmol, Amersham) in a total volume of 20 μl in buffer B (50 mM sodium phosphate, pH 7.5). After incubation at 37°C for 60 min, the reaction was terminated by the addition of one third of the volume of 3× SDS sample buffer, and the samples were subjected to SDS-PAGE (12.5% acrylamide) as described by Laemmli (*21*). The polyacrylamide gels were stained with Coomassie Brilliant blue, destained, and treated with EN^{3}HANCE (Du Pont NEN). Subsequently, the gels were dried and exposed to X-ray film (Kodak, MS) at –75°C for 3 d. Recombinant rat GST-PRMT1 and mouse fibrillarin protein were prepared as described (*7*, *22*).

Western Blotting—Equal amounts of protein (30 μg) were separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Gelman Science). The membranes were blocked in 5% skimmed dry milk in TTBS (10 mM Tris-HCl, pH = 7.5; 100 mM NaCl; 0.1% tween 20) for 30 min, incubated with primary antibodies (1:200 dilution for 7E6 antibody from Abcam; 1:550 dilution for SYM10, 1:900 dilution for SYM11 and 1:900 dilution for ASYM24 from Upstate) for 1 h, washed three times in TTBS, then incubated with secondary antibody (anti-mouse or rabbit IgG horse radish peroxidase conjugate from Sigma) for 1 h. Chemiluminescent detection was performed using the Supersignal kit according to the manufacturer's instructions (Pierce).

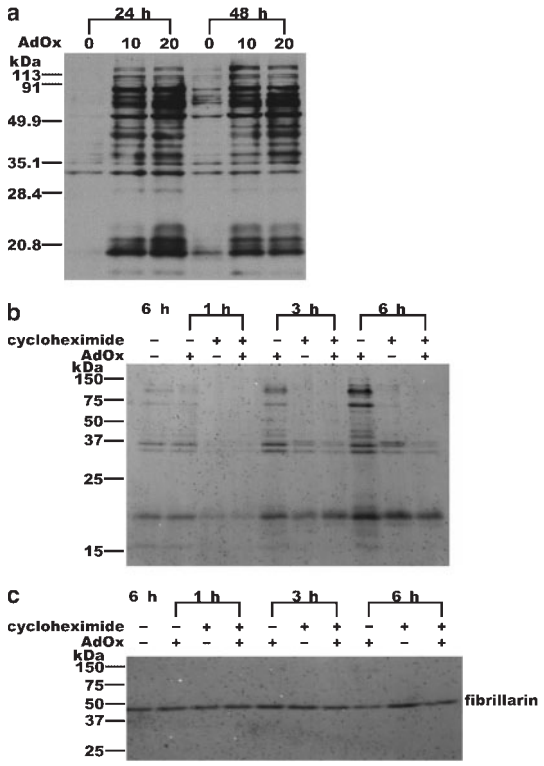


Fig. 1. **Treatment of HeLa cells with AdOx and cycloheximide.** (a) HeLa cell extracts were prepared from cells grown in the presence of 0, 10, 20 or 40 μM AdOx for 24 and 48 h. Accumulation of methyl-accepting proteins in HeLa cells was evaluated by *in vitro* methylation reaction as described in "MATERIALS AND METHODS." (b) HeLa cell extracts were prepared from cells incubated in the presence of both 20 μM AdOx and 0.08 μg/ml of cycloheximide or only either one for 1, 3 or 6 h. *In vitro* methylation of the extracts without the addition of exogenous substrate was performed as described. (c) Methylation of the extracts was performed with recombinant mouse fibrillarin (1 μg) as the exogenous methyl-accepting substrate. The molecular mass standards are indicated on the left in kilodaltons.

In Vivo Methylation—HeLa cells grown to near confluency were treated with AdOx for the indicated times. Cycloheximide (100 μg/ml) and chloramphenicol (40 μg/ml) were added 30 min prior to the labeling. For *in vivo* methylation, the medium was replaced with DMEM medium without methionine (GIBCO), 10% FBS (GIBCO, dialyzed), [methyl-^{3}H] methionine (10 μCi/ml) and translation inhibitors for the indicated labeling time. The cells were then collected, lysed by SDS-PAGE sample buffer and analyzed by SDS-PAGE and fluorography.

RESULTS

The Translation Inhibitor Cycloheximide Block AdOx-Accumulated Methyl-Accepting Proteins—We first incubated HeLa cells with various concentrations of AdOx for different time periods to determine the best treatment time and dose of AdOx. Total cell extract was prepared, and *in vitro* methylation was performed to examine the level of methyl-accepting proteins accumulated upon the treatment. As shown in Fig. 1a, when no AdOx was present in the cell culture, a few methyl-accepting

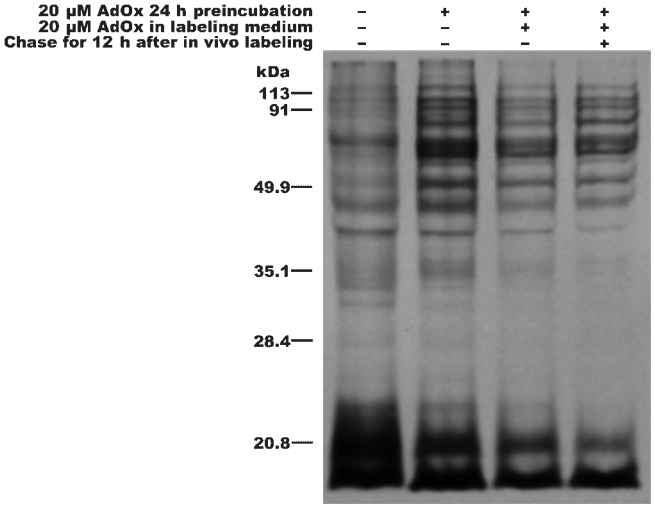


Fig. 2. ***In vivo* methylation of HeLa cells treated with AdOx.** *In vivo* methylation was performed as described in "MATERIALS AND METHODS." HeLa cells were *in vivo* labeled either by pretreatment with AdOx for 24 h, or by incubation in the labeling medium containing AdOx. Cells were also chased for 12 h in non-radioactive medium after *in vivo* labeling. Cell lysates were collected, separated by SDS-PAGE, and the *in vivo* methylated proteins were detected by fluorography.

polypeptides were modified only to a limited level, demonstrating that normally the methyl-accepting sites were mostly occupied. Addition of AdOx clearly enhanced the methylation signals of the methyl-accepting polypeptides that can be detected without AdOx treatment. AdOx concentration of 20 μM for 24 h was enough to reach the maximum accumulation of methyl-accepting proteins in cells.

The origin of the hypomethylated proteins accumulated upon AdOx treatment was further studied. HeLa cells were incubated with a translation inhibitor, cycloheximide (CHX), at the same time as the AdOx treatment. Since cells appeared to die upon double treatment for 12 h, we performed the experiments for periods of 1, 3 and 6 h. As shown in Fig. 1b, the AdOx treatment can accumulate methyl-accepting proteins in short intervals of 3 and 6 h. However, the presence of CHX completely blocked the accumulation of methyl-accepting proteins by AdOx treatment at 3 and 6 h. Cycloheximide treatment did not effect the type I arginine methyltransferase activity in HeLa cells as assayed by the addition of an exogenous methyl-accepting protein, fibrillarin (Fig. 1c). Thus the disappearance of methyl-accepting proteins by CHX treatment is probably due not to reduced protein methyltransferase activity in the extract but to the blockage of new protein synthesis. The results indicated that the methyl-accepting proteins accumulated for *in vitro* methylation by AdOx treatment are most likely to be newly synthesized during the incubation period.

Effects of AdOx Treatment on In Vivo Methylation—In vivo protein methylation in mammalian cells with [methyl-^{3}H] methionine has been used to evaluate the protein methylation under native conditions (*17*, *18*). To prevent the incorporation of radioactive methyl groups into proteins by translation but not posttranslational modification, protein synthesis inhibitors have to be included throughout the *in vivo* methylation period. Various methyl-accepting proteins were detected in earlier studies, which somehow seems not to agree with our results in Fig. 1b suggesting that methyl-accepting sites are on newly synthesized proteins. We performed *in vivo* methylation in HeLa cells following the protocol of Liu *et al*. (*17*) and as reported previously, detected many labeled polypeptides (Fig. 2). We then treated the cells with AdOx prior to the *in vivo* methylation. Except for few polypeptides, the pattern of the signals of AdOx-pretreated cells was basically the same as that of the untreated ones. However, addition of AdOx for 24 h before *in vivo* methylation clearly increased the labeling of polypeptides of molecular mass between 40 to 100 kDa. No new protein synthesis was detected under the *in vivo* methylation conditions as monitored by incubation with ^{35}S-Met (data not shown). The methyl groups were probably present on the unmodified proteins newly synthesized before the *in vivo* methylation period in the presence of CHX.

In addition, when AdOx was present in the labeling medium after the 24-h pretreatment, the elevated *in vivo* methylation pattern appeared to be suppressed back to a low level. When the *in vivo* labeling was followed by a 12-h chase in the non-radioactive medium, the majority of the *in vivo* labeled polypeptides were of similar level, indicating that the modified proteins were rather stable. Similar results were obtained from the 12-h chase experiments of no AdOx treatment or AdOx-pretreatment/labeling samples (data not shown).

The Level of Methylarginine-Containing Proteins in AdOx-Treated or Untreated Cell Extracts Detected by Methylarginine-Specific Antibodies—AdOx can accumulate proteins in hypomethylated states for *in vivo* and *in vitro* methylation. Nevertheless, the fraction of the AdOx-affected polypeptides in the whole methyl-modified protein is unknown. Protein arginine methylation accounts for the majority of AdOx-stable protein methylation in rat pheochromocytoma PC12 cells (*5*) and human lymphoblastoid cells (*6*, *7*). We also found that the major methyl-accepting substrates accumulated by AdOx treatment can be recognized by the predominant protein arginine methyltransferase PRMT1 and are likely to be modified to form methylarginines (data not shown). We thus determined the level of total methylarginine-containing proteins in the extracts by Western blot analysis. Methylarginine-specific antibodies SYM 10, SYM11 and ASYM24 are rabbit polyclonal antibodies against alternative RG sequences containing symmetric dimethylarginine (sDMA), SmD3 RG sequences containing sDMA, and alternative RG sequences containing asymmetric dimethylarginine (aDMA) in all the arginyl positions, respectively (*23*). Another antibody, 7E6, is specific for monomethylarginine (MMA) and aDMA (*24*). As shown in Fig. 3, reduced recognition of certain specific polypeptides by SYM10, SYM11, ASYM24 was observed for the AdOx-treated cell extracts. For SYM10 recognition, the intensity of the strongest 30-kDa signal was the same in both samples, while most of the signals above 36 kDa were reduced in the AdOx-treated sample (Fig. 3a). For SYM11, except for the highest signal above 113 kDa, most of the signals were slightly stronger in the untreated sample (Fig. 3b). The polypeptides with molec-

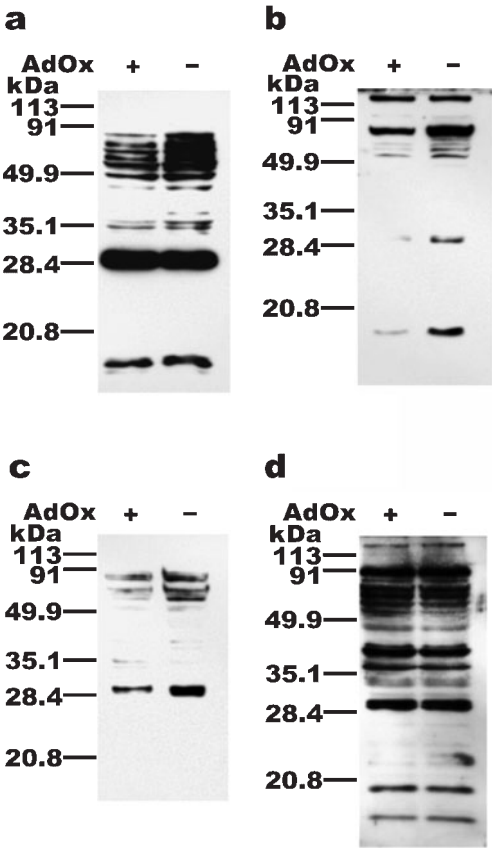


Fig. 3. **Detection of methylarginine-containing polypeptides by methylarginine-specific antibodies.** HeLa cell extracts of AdOx-treated or untreated cells were immunoblotted with commercially available methylarginine-specific antibodies SYM10 (a), SYM11 (b), AYM 24 (c) and 7E6 (d).

ular mass of 80, 28 and 16 kDa are probably the previously identified p80 coilin, SmB and SmD3, respectively (*25*). As for AYM24, generally the signals appeared to be slightly reduced in the AdOx-treated samples, probably including Sam68 at the molecular mass of 68 kDa (*20*). However, no difference was detected between the AdOx-treated and untreated cell extracts using the 7E6 methylarginine-specific antibodies (Fig. 3d). Whether this antibody might cross-react with certain other epitopes, thus masking minor differences in the level of methylarginines, needs more investigation.

Treatment of HeLa Cells with a Proteosome Inhibitor ALLN Does Not Affect AdOx-Mediated Accumulation of Hypomethylated Proteins—The *in vivo* labeled proteins appeared to be rather stable in our 12-h chase period. We then investigated whether the normally methylated proteins might be degraded rapidly when rendered unmethylated by AdOx-treatment. *N*-acetyl-leu-leu-norleucinal (ALLN) is an inhibitor that blocks proteolytic activity of both type I calpain and the 26 S proteosome. We treated the cells with ALLN to evaluate the effect of protein degradation on the accumulation of methyl-accepting sites by AdOx treatment. No significant changes to the AdOx induced hypomethylation pattern upon the addition of ALLN for 24 h were observed (Fig. 4). Thus, under our experimental conditions, ALLN-blocked proteosome degradation has no effect on the level of hypomethylated polypeptides.

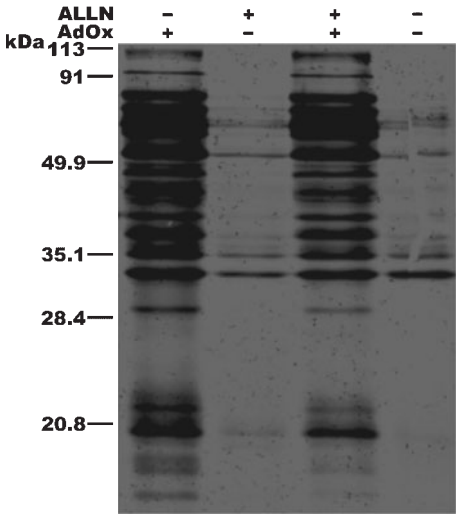


Fig. 4. **Treatment of HeLa cells with AdOx and ALLN.** HeLa cell extracts were prepared from cells incubated in the presence of 20 μM AdOx or 15 μM ALLN, or both for 24 h. *In vitro* methylation reaction of the cell extracts was performed as described in "MATERIALS AND METHODS."

DISCUSSION

In this study we demonstrated the best treatment time and dose of AdOx, a frequently utilized general protein methylation inhibitor, to accumulate methyl-accepting proteins in HeLa cells for further *in vitro* methylation. We showed that AdOx-accumulated hypomethylated polypeptides for *in vitro* methylation disappeared in the presence of a protein synthesis inhibitor, CHX, indicating that they were probably newly translated in the AdOx-incubation period. Since methyl-accepting sites on polypeptides are generally occupied in cells, it is likely that protein methylation occurs soon after the synthesis of the polypeptide and these sites are occupied thereafter.

In vivo protein methylation to label methyl-accepting proteins metabolically has been performed with protein translation inhibitors in various cell lines (*17–20*). The proteins that can be *in vivo* labeled thus are likely to be restricted to those that are newly synthesized but have not been modified before the labeling period. Interestingly, we demonstrated that *in vivo* methylation could also be affected by AdOx treatment. Firstly, pre-treatment (24 h) with AdOx increased the level of *in vivo* methylation. The pattern of the signals was similar to that of the untreated control, indicating that AdOx can accumulate methyl-accepting proteins in more hypomethylated states not only for *in vitro* methylation, but also for *in vivo* methylation. Secondly, when AdOx was present throughout the *in vivo* methylation period, the level of methylated proteins was reduced. Furthermore, when the *in vivo* labeled proteins were chased in the non-radioactive medium for 12 h, the radioactive proteins were of the same level as the non-chase samples. These results indicated that the methylated polypeptides were stable and no demethylation occurred in the chase period.

In comparison, the proteosome inhibitor ALLN did not change the appearance of methyl-accepting proteins upon AdOx treatment. This result indicated that the accumulated hypomethylated proteins probably are not subject to rapid proteosome degradation, otherwise inhibition of protein degradation by ALLN should accumulate more of these sites.

Our results represent the base-stable methylation of the AdOx-accumulated methyl-accepting proteins catalyzed by all endogenous methyltransferases in the extracts. Base-labile methylation that cannot survive the mild alkaline conditions of SDS-PAGE, such as carboxyl methylation, can be excluded, but C-terminal carboxyl methylation and N-methylation such as lysyl or arginyl methylation could not be distinguished (*1*). The irreversible modification on the guanidino nitrogen of the arginyl residues is known to account for the majority of the AdOx-accumulated stable protein methylation events in PC12 and lymphoblastoid cells (*5*, *6*). We detected limited but consistent reduction of the methylarginine-containing proteins in AdOx-treated samples compared to untreated ones by use of sDMA- and aDMA-specific antibodies. Specifically, decreased signals that probably correspond to methylation of coilin, SmB and Sm D3 were detected in AdOx-treated extracts. It is possible that since the cells treated with AdOx are of high confluency, most of the methyl-accepting proteins are already synthesized and methylated. If they are stable and turn over slowly, as indicated by the chase/*in vivo* methylation experiments, the newly synthesized proteins will be responsible for only a small fraction of the total pool of the methyl-accepting proteins in the cell, and the difference will be barely detectable. This is consistent with our inability to detect differences between the overall protein patterns from AdOx treated cells and untreated cells by two-dimensional gel electrophoresis (*26*). Thus, AdOx probably only affects a small portion of the total methyl-containing proteins, at least for the methylarginine-containing proteins.

However, application of AdOx to inhibit specific protein methylation has already been shown in different cells. For example, we demonstrated that certain methylarginine-containing trypsin-digested peptides of hnRNPA2/B1 could be detected from AdOx-untreated lymphoblastoid cells but not from AdOx-treated cells (*26*). Nichols *et al*. also showed that of AdOx could affect the subcellular localization of hnRNPA2/B1 in HEK293 cells, presumably by affecting the methylation in the RGG domain (*27*). These investigations support the appropriateness of applying of AdOx treatment in specific protein methylation studies.

In summary, although AdOx treatment had been frequently used to accumulate methyl-accepting proteins for further *in vitro* methylation, the protein methylation inhibition is restricted to newly translated proteins during the treatment, which blocks only a small fraction of protein methylation in cells. In this study, we also show that AdOx can be applied to *in vivo* methylation as well as *in vitro* methylation. The timing of the AdOx treatment can be adjusted to enhance or to inhibit the *in vivo* methylation. In combination with chase experiments, *in vivo* methylation can be applied to follow the methylation of methyl-accepting proteins.

The study was supported by the National Science Council of the Republic of China (NSC 89-2320-B-040-072, 89-2745-P040-002, 91-3112-B-040-001 and 92-3112-B-040-001) and Chung Shan Medical University (CSMU 90-OM-018, 90-OM-090).

REFERENCES

1. Clarke, S. (1993) Protein methylation. *Curr. Opin. Cell Biol.* **5**, 977–983
2. Pawlak, M.R., Banik-Maiti, S., Pietenpol, J.A., and Ruley, H.E. (2002) Protein arginine methyltransferase I: substrate specificity and role in hnRNP assembly. *J. Cell. Biochem.* **87**, 394–407
3. Pilz, R.B., Van den Berghe, G., and Boss, G.R. (1987) Adenosine dialdehyde and nitrous oxide induce HL-60 differentiation. *Blood* **70**, 1161–1164
4. Johnson, B.A., Najbauer, J., and Aswad, D.W. (1993) Accumulation of substrates for protein L-isoaspartyl methyltransferase in adenosine dialdehyde-treated PC12 cells. *J. Biol. Chem.* **268**, 6174–6181
5. Najbauer, J. and Aswad, D.W. (1990) Diversity of methyl acceptor proteins in rat pheochromocytoma (PC12) cells revealed after treatment with adenosine dialdehyde. *J. Biol. Chem.* **265**, 12717–12721
6. Li, C., Ai, L.S., Lin, C.H., Hsieh, M., Li, Y.C., and Li, S.Y. (1998) Protein *N*-arginine methylation in adenosine dialdehyde-treated lymphoblastoid cells. *Arch. Biochem. Biophys.* **351**, 53–59
7. Lin, C.H., Hsieh, M., Li, Y.C., Li, S.Y., Pearson, D.L., Pollard, K.M., and Li, C. (2000) Protein *N*-arginine methylation in subcellular fractions of lymphoblastoid cells. *J. Biochem.* (*Tokyo*) **128**, 493–498
8. Gary, J.D. and Clarke, S. (1998) RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic. Acid. Res. Mol. Biol.* **61**, 65–131
9. McBride, A.E. and Silver, P.A. (2001) State of the arg: protein methylation at arginine comes of age. *Cell* **106**, 5–8
10. Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S., and Herschman, H.R. (1996) The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine *N*-methyltransferase. *J. Biol. Chem.* **271**, 15034–15044
11. Katsanis, N., Yaspo, M.L., and Fisher, E.M. (1997) Identification and mapping of a novel human gene, HRMT1L1, homologous to the rat protein arginine *N*-methyltransferase 1 (PRMT1) gene. *Mamm. Genome.* **8**, 526–529
12. Tang, J., Gary, J.D., Clarke, S., and Herschman, H.R. (1998) PRMT **3**, a type I protein arginine *N*-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. *J. Biol. Chem.* **273**, 16935–16945
13. Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., and Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174–2177
14. Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S., and Clarke, S. (2001) PRMT5 (Janus kinase–binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. *J. Biol. Chem.* **276**, 32971–32976
15. Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S., and Bedford, M.T. (2002) The novel human protein arginine *N*-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. *J. Biol. Chem.* **277**, 3537–3543
16. Gros, L., Delaporte, C., Frey, S., Decesse, J., de Saint-Vincent, B.R., Cavarec, L., Dubart, A., Gudkov, A.V., and Jacquemin-Sablon, A. (2003) Identification of new drug sensitivity genes using genetic suppressor elements: protein arginine N-methyltransferase mediates cell sensitivity to DNA-damaging agents. *Cancer Res.* **63**, 164–171

17. Liu, Q. and Dreyfuss, G. (1995) *In vivo* and *in vitro* arginine methylation of RNA-binding proteins. *Mol. Cell. Biol.* **15**, 2800–2808
18. Cimato, T.R., Ettinger, M.J., Zhou, X., and Aletta, J.M. (1997) Nerve growth factor-specific regulation of protein methylation during neuronal differentiation of PC12 cells. *J. Cell Biol.* **138**, 1089–1103
19. Hebert, M.D., Shpargel, K.B., Ospina, J.K., Tucker, K.E., and Matera, A.G. (2002) Coilin methylation regulates nuclear body formation. *Dev. Cell.* **3**, 329–337
20. Cote, J., Boisvert, F.M., Boulanger, M.C., Bedford, M.T., and Richard, S. (2003) Sam68 RNA binding protein is an *in vivo* substrate for protein arginine *N*-methyltransferase 1. *Mol. Biol. Cell.* **14**, 274–287
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
22. Pearson, D.L., Reimonenq, R.D., and Pollard, K.M. (1999) Expression and purification of recombinant mouse fibrillarin. *Protein Expr. Purif.* **17**, 49–56
23. Boisvert, F.M., Cote, J., Boulanger, M.C., and Richard, S. (2003) A proteomic analysis of arginine-methylated protein complexes. *Mol. Cell. Proteomics* **2**, 1319–1330
24. Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R., and David, M. (2001) Arginine methylation of STAT1 modulates IFNalpha/beta–induced transcription. *Cell* **104**, 731–741
25. Boisvert, F.M., Cote, J., Boulanger, M.C., Cleroux, P., Bachand, F., Autexier, C., and Richard, S. (2002) Symmetrical dimethylarginine methylation is required for the localization of SMN in Cajal bodies and pre-mRNA splicing. *J. Cell Biol.* **159**, 957–969
26. Huang, H.M., Tam, M.F., Tam, T.C., Chen, D.H., Hsieh, M., and Li, C. (2002) Proteomic analysis of stable protein methylation in lymphoblastoid cells. *J. Biochem. (Tokyo)* **132**, 813–818
27. Nichols, R.C., Wang, X.W., Tang, J., Hamilton, B.J., High, F.A., Herschman, H.R., and Rigby, W.F. (2000) The RGG domain in hnRNP A2 affects subcellular localization. *Exp. Cell Res.* **256**, 522–532

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Gene 340 (2004) 179–187

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

www.elsevier.com/locate/gene

Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians

Chuan-Mao Hung[a], Chuan Li[b,*]

[a]*Institute of Medicine, Chung Shan Medical University, 110 Sec. 1, Chein-Kuo North Road, Taichung, Taiwan, ROC*
[b]*Department of Life Sciences, Chung Shan Medical University, 110 Sec. 1, Chein-Kuo North Road, Taichung, Taiwan, ROC*

Received 5 April 2004; received in revised form 30 June 2004; accepted 23 July 2004
Available online 11 September 2004
Received by T. Gojobori

Abstract

Protein arginine methyltransferases (PRMT) involved in the regulations of signal transduction, protein subcellular localization, and transcription have been mostly studied in mammals and yeast. In this study orthologues of eight human PRMT genes (PRMT1-7 and HRMT1L3) were identified in both puffer fish *Fugu rubripes* and zebrafish *Danio rerio*. The fish PRMT genes appear to be conserved with their mammalian orthologues at the levels of amino acid sequences as well as genomic structures. All vertebrate PRMT genes contain 10–16 coding exons except PRMT6 that contains only one coding exon. Western blot analyses of zebrafish tissue extracts confirmed the expression of some PRMT proteins in zebra fish. We further identified six PRMT members (PRMT1, 3–7) in an invertebrate chordate *Ciona intestinalis*. Genomic structures of the PRMT orthologues are no more conserved in the ascidians, as PRMT3 and PRMT5 contain only one coding exon while PRMT6 contains six exons. PRMT2 and HRMT1L3 that are missing in *Ciona* appear to be vertebrate-specific. HRMT1L3 is a PRMT1 paralogue with highly conserved sequences and exact exon junctions, whereas the PRMT2 orthologues are very diverged. Different PRMT orthologues are likely to evolve at different rates and the PRMT1 orthologues appear to be most conserved through evolution. Furthermore, phylogenetic analyses using the core regions of various PRMT genes show that PRMT5 with the type II PRMT activity is separated in one branch. All other PRMT genes including PRMT1, 2, 3, 4, 6, 7 and HRMT1L3 clustered in the other branch, probably represent the genes for the type I activity.

Keywords: Protein arginine methyltransferase; PRMT; Gene family; Phylogenetic analyses; *Fugu rubripes*; *Ciona intestinalis*; Zebrafish

1. Introduction

Protein arginine methylation is an irreversible post-translational modification involved in various cellular functions such as signal transduction, protein subcellular localization, and transcriptional regulation and protein–protein interactions (McBride and Silver, 2001). Since the identification of the first protein *N*-arginine methyltransferase (PRMT) gene PRMT1 (Lin et al., 1996), by now at least eight PRMT genes have been identified in the mammalian system and seven of them has direct or indirect evidences to be able to catalyze the transfer of methyl groups from AdoMet to the side chain ω-guanido nitrogens of arginine residues in protein substrates. The PRMT activity can be further divided into type I and type II, depending on the catalyses of formation of asymmetric N^G,N^G–dimethylarginines (aDMA) or symmetric N^G,N'^G–dimethylarginine (sDMA) residues, respectively (Gary and Clarke, 1998).

The type I enzymes include PRMT1 (HRMT1L2) that appears to be the predominant methyltransferase in the mammalian system (Lin et al., 1996; Pawlak et al., 2000; Tang et al., 2000), the zinc-finger containing PRMT3 (Tang et al., 1998), the coactivator-associated arginine methyltransferase CARM1/PRMT4 (Chen et al., 1999) and

Abbreviations: PRMT, protein arginine methyltransferase; AdoMet, *S*-adenosylmethionine; sDMA, symmetric dimethylarginine; aDMA, asymmetric dimethylarginine; MMA, monomethylarginine; *Ci*, *Ciona intestinalis*.

* Corresponding author.
E-mail address: cli@csmu.edu.tw (C. Li).

0378-1119/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2004.07.039

PRMT6 (Frankel et al., 2002). Direct evidences of the formation of the type I products catalyzed by these methyltransferases have been reported. The N-terminal SH3 domain containing PRMT2/HRMT1L1,even though was the first identified PRMT1 homologue by sequence comparison (Katsanis et al., 1997; Scott et al., 1998), has no direct evidence of PRMT activity. However, it is most likely to be responsible for the methylation of hnRNPE1B-AP5 in vivo (Kzhyshkowska et al., 2001) and its potential methyltransferase activity is important for being a coactivator of estrogen receptor (Qi et al., 2002). Another PRMT-1-like PRMT gene (HRMT1L3) at chromosome 12p13 with 80% sequence identity with PRMT1 was identified in human but with no reported function yet (Zhang and Cheng, 2003).

The only type II PRMT identified is the Janus kinase-binding protein JBP1/PRMT5 (Pollack et al., 1999; Branscombe et al., 2001; Rho et al., 2001), which was first identified in *Schizosaccharomyces pombe* as an Shk1 kinase binding protein skb1 (Gilbreth et al., 1996) and a *Saccharomyces cerevisiae* histone synthetic lethal gene hsl7 (Ma et al., 1996). Another recently identified PRMT7 gene p82/p77 is a genetic suppressor element mediating cell sensitivity to DNA-damaging agents. Evidence of in vitro methylation of myelin basic protein, a typical type-II substrate, had been demonstrated for the p82/p77 immunoprecipitants (Gros et al., 2003). However, most recently Miranda et al. demonstrated that PRMT7 catalyzed the formation of ω-N^G monomethylarginine in an arginine/glycine containing peptide but not in known protein substrates (Miranda et al., 2004).

In comparison with eight members in mammalian PRMT family, there are only two related protein arginine methyltransferase genes in yeast *S. cerevisiae*. RMT1/HMT1 corresponds to the type I methyltransferase activity (Gary et al., 1996; Henry and Silver, 1996) while Hsl7p is responsible for the type II activity (Lee and Bedford, 2002). The survival of yeast HMT1 or Hsl7 mutants indicates that they are not essential genes (Gary et al., 1996; Gilbreth et al., 1996; Henry and Silver, 1996). On the contrary, mice null for PRMT1 die early during embryogenesis (Pawlak et al., 2000) and mice deficient in PRMT4/CARM1 show neonatal lethality (Yadav et al., 2003).

Different mammalian protein arginine methyltransferases are localized in different subcellular compartments and appear to have their own substrate sets and physiological roles. For example, the predominant PRMT1 appears to be located mainly in the nucleus (Tang et al., 1998). Its physiological substrates include histone H2A, many hnRNPs, fibrillarin and STAT1 (Lin et al., 1996; Tang et al., 1998; Mowen et al., 2001; Lee and Bedford, 2002; Wada et al., 2002). The other widely studied CARM1/PRMT4 were first identified as a coactivator-associated arginine methyltransferase (CARM) localized in the nucleus (Chen et al., 1999). Identified PRMT4 substrates include histone H3 (Chen et al., 1999), p300/CBP (Xu et al., 2001), PABP1 (Lee and Bedford, 2002) and an mRNA-stabilizing protein HuR (Brahms et al., 2000). The JBP1/PRMT5 substrates include myelin basic protein (Pollack et al., 1999; Branscombe et al., 2001; Rho et al., 2001), SmD1, D3 (Brahms et al., 2000), Sm B/B′ , Lsm4 (Brahms et al., 2001) and coilin (Hebert et al., 2002). Even though cytoplasmic localization of PRMT5 has been suggested (Rho et al., 2001; Frankel et al., 2002), the interactions of PRMT5 with complexes involved in transcriptional regulations (Fabbrizio et al., 2002; Kwak et al., 2003; Pal et al., 2003) indicate that certain portion of the protein should be present in the nucleus also.

Why so many different methyltransferases are required to modulate different biological functions in the mammalian system and how the methyltransferases are evolved are interesting questions. Nine PRMT genes (DART1–9) have been identified in *Drosophila* (Boulanger et al., 2004). However, only DART1, 4, 5, and 7 are the putative homologues of mammalian PRMT1, 4, 5, and 7. Other DART family members have closer resemblances to PRMT1 but do not have identifiable mammalian homologous. Knowledge of the distribution of the PRMT genes in other evolutionarily related biological systems to mammals should be helpful to answer the above questions. The genome of the pufferfish *Fugu rubripes* has been sequenced (Aparicio et al., 2002) and it is the second vertebrate genome for which the entire sequence is available. Numerous studies of gene families in *Fugu* have demonstrated that the genome contains approximately the same number of genes as the mammalian genomes (Aparicio et al., 2002). The structure of genes is conserved, as splice sites are located in positions identical to those found in human. Another important fish model system zebrafish (*Danio rerio*) has been used to analyze vertebrate development (Driever et al., 1996) and is an emerging model system for studying human diseases (Zon, 1999). Many EST as well as genomic DNA sequences, even though not complete yet, are available in this model organism. Invertebrate chordate *Ciona intestinalis*, an ascidians (sea squirt) with tadpole larva, is a model system at the critical evolutionary position as basal chordates. It is also an emerging model for whole genome analyses since its draft genome as well as transcriptome are available (Dehal et al., 2002). Data mining of the PRMT family members in these systems should provide interesting evolutionary clues of the PRMT genes.

2. Materials and methods

2.1. Database searches and analyses

Searches for the homologues of human PRMT genes (*PRMT1v3*:NP_938075; *PRMT2*: NP_001526; *PRMT3*: XP_058460; *PRMT4/CARM1*: XP_032719; *PRMT5/SKB1*: NP_006100; *PRMT6*: AAK85733; *PRMT7*: NP_061896;*HRMT1L3*: NP_062828) were performed by tblastn against *F. rubripes* or *C. intestinalis* genome data-

bases. Putative *Fugu* or *Ciona* PRMT homologues were identified by GeneScan (http://genes.mit.edu/GENSCAN.html) and GenomeScan (http://genes.mit.edu/GENSCAN.html) for gene prediction with human PRMT protein sequences as the templates. The predicted *Ciona* PRMT was further subjected to blastn against est_others database of NCBI. The matched *Ciona* EST sequences were then retrieved and assembled. Searches for the zebrafish PRMT genes were performed by tblastn against both zebrafish EST database and HTGS database using human PRMT protein sequences as the templates. The putative PRMT genes were predicted by similar approaches used above.

2.2. Alignment and phylogenetic analyses of the protein arginine methyltransferase

Multiple sequence alignments were performed with clustalX (1.83) (Thompson et al., 1997) and the output of the alignments was formatted by CHROMA (Goodstadt and Ponting, 2001). The phylogenetic tree was calculated using Neighbor-Joining method (Saitou and Nei, 1987) integrated in clustalX, then visualized with Treeview (v1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.3. Preparation of tissue extracts and Western blot analyses

Zebrafish was dissected into brain, inner organs, and muscle fractions. The extraction buffer (5% glycerol, 1 mM EGTA, 1 mM DTT, 1× Complete protease inhibitor cocktail [Roche], 0.5% Triton X-100, 1× PBS) was added in 1:6 weight/volume ratio. Fish brain and inner organs were homogenized by a Teflon pestle, and the muscle fraction was homogenized by a Polytron. Homogenized samples were then subjected to sonication followed by centrifugation at 17,530×*g* for 20 min at 4 °C. Equal amount of protein (30 μg) was separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Gelman Science). The membrane were then blocked in 5% skimmed dry milk in TTBS (10 mM Tris–HCl, pH=7.5; 100 mM NaCl; 0.1% Tween 20) for 30 min, incubated with primary Ab (1:500 dilution for anti-PRMT1 from Abcam or anti-PRMT6 from IMGENEX) for 1 h, washed two times in TTBS, and then incubated with secondary antibody (anti-mouse or rabbit IgG horse radish peroxidase conjugate from Sigma) for 1 h. Chemiluminescent detection was performed using the Supersignal kit according to the instructions of the manufacturer (Pierce).

3. Results

3.1. Identification of the fish orthologues of the mammalian PRMT genes

We retrieved the amino acid sequences and genomic structures of the eight human PRMT genes at first. Of all the human PRMT genes, the most well studied PRMT1 contains 10–12 exons according to different splicing forms. If minor alternative splicings were not considered, PRMT2 contains 10, PRMT3 contains 15, PRMT 4 contains 16, PRMT5 contains 15, PRMT7 contains 15 and HRMT1L3 contains 10 exons. Interestingly, in opposite to other PRMT genes that have 10–16 exons for the coding regions, the mRNA sequence of human PRMT 6 matches directly to the genomic sequence without any interruption, indicating that PRMT6 contains only one coding exon.

We then performed in silico data mining for the PRMT genes in other vertebrate systems. In pufferfish *F. rubripes* whose genomic sequence is complete, we identified PRMT orthologues of all eight PRMT family members. Protein sequences of putative *Fugu* PRMT genes based on the reported genomic sequences were manually examined for splice site selections to achieve highest similarity with the mammalian PRMT orthologues. For ambiguous sites, part of the *Fugu* genomic DNA was PCR amplified and sequenced. The original sequencing trace of the reported *Fugu* genome was also examined. In this way, we suggest the *Fugu* PRMT1 and HRMT1L3 with higher homology with their mammalian orthologues by changing one reported nucleotide at the splice site. The real first coding exon of *Fugu* PRMT1 might contain only few coding amino acid that is hard to be identified by searching the genomic DNA without the information of the transcriptome. We accordingly start the *Fugu* PRMT 1 sequence in Fig. 1 with VSQ with the most N-terminal sequence missing. The *Fugu* PRMT4 gene is at the end of the contig thus the C-terminus is missing. The predicted protein sequences of *Fugu* PRMT genes are aligned with their orthologues from other species (Fig. 1 and Figs. 1–5, Supplementary material).

Even though the zebrafish genomic sequences are not complete, all PRMT genes can be identified from either genomic or assembled EST sequences. The sequences of the putative zebrafish PRMT are also illustrated in Fig. 1. The genomic structures of the fish PRMT genes, if can be obtained, were compared with the mammalian ones. The genomic organizations of the PRMT genes examined were basically all conserved from fish to human (Fig. 1 and Figs. 1–5, Supplementary material). Interestingly, the HRMT1L3 gene highly homologous to PRMT1 has exact genomic structure as PRMT1 in both human and fish (Fig. 1). The fish PRMT6 gene also appears to have only one long coding exon (Fig. 1, Supplementary material).

Since the three-dimensional structures of rat PRMT1 and PRMT3 have been solved (Zhang et al., 2000; Zhang and Cheng, 2003), in Fig. 1 and Fig. 2 of the Supplementary material, we further indicate the secondary structural elements of the two PRMT genes with their amino acid sequences shown as discrete exons. Basically the mono-

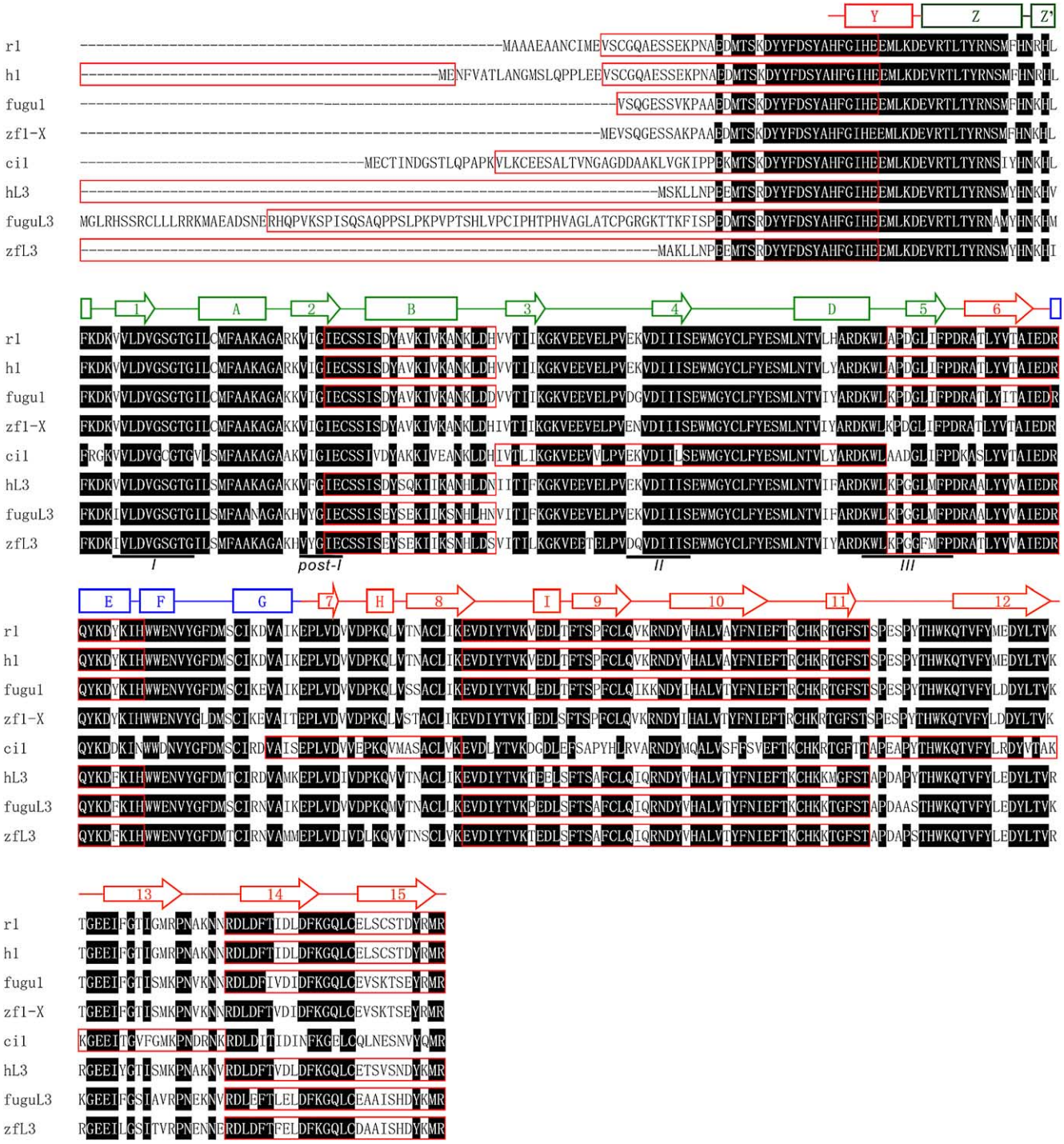


Fig. 1. Amino acid sequence alignments and genomic structures of PRMT orthologues. Orthologues of PRMT1 and HRMT1L3 are shown. The PRMTs used in this alignment are indicated by the species (r for rat, h for human, fugu for *F. rubripes*, zf for zebrafish, ci for *C. intestinalis*) and the number of the PRMT gene (L3 represents HRMT1L3). The dash cross (-X) behind the number indicates that the genomic organization is currently unavailable. The amino acids encoded by different exons are marked by red boxes and the black shadings show 80% identity among the sequence alignments. Characteristic methyltransferase I, post I, II and III motifs are indicated. The rectangles and arrowheads represent the helices and strands in rat PRMT1 as indicated in Zhang and Cheng (2003). The color coding is red for the N terminus including helix α Y, green for the AdoMet binding domain, orange for the β barrel structure, and blue for the dimerization arm. The accession numbers of the human, zebrafish, *Fugu* and *Ciona* PRMT genes are indicated in Materials and Methods or Tables 1 and 2. The accession number of rat PRMT1 is Q63009.

meric structure of PRMT1 or PRMT3 can be divided into four parts: N-terminal, AdoMet binding, β-barrel and dimerization domain. No strict exon-domain correlation can be found. However, the exon junctions of PRMT1 and PRMT3 are mostly located at the junctions of secondary structure elements or in the loop regions.

3.2. Protein expression analyses of PRMT gene family in zebrafish

Zebrafish EST data indicate that all PRMT genes are likely to be transcribed in fish. To further illustrate PRMT protein expression in fish, Western blot analyses with commercially available antibodies against mammalian PRMT family members were performed. As shown in Fig. 2, PRMT1 was expressed in all brain, inner organ, and muscle fractions of zebrafish. PRMT6 was expressed in brain but was rare in other fractions. Signals of the similar molecular weight can be detected in mouse and porcine brain. However, the fish (as well as the mouse or porcine) PRMT6 protein appeared to be 5 kDa larger than that in human. Since recombinant fish PRMT6 protein can be recognized by the same antibody (data not shown), the detected signals are likely to be the fish PRMT6. Expression of PRMT2 and 3 was also detected in zebrafish but with high backgrounds. We were unable to detect signals of PRMT4 and 5 in the fish samples with commercially available antibodies (data not shown).

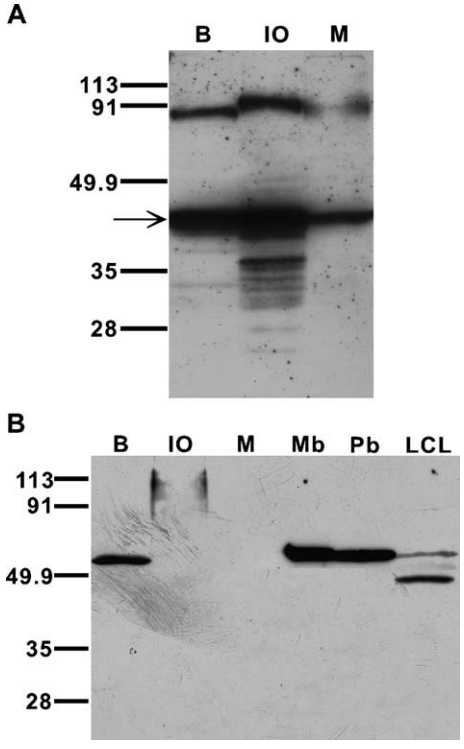


Fig. 2. Analyses of PRMT1 and PRMT6 protein expression in zebrafish. Extracts of zebrafish tissues are prepared, separated by SDS-PAGE and immunoblotted as described in Materials and Methods. Anti-PRMT1 and anti-PRMT-6 antibodies were used in (A) and (B), respectively. The arrow in (A) indicates the position of PRMT1. Other signals are likely to be nonspecific. (B) IO and M indicate fish brain, inner organ, and muscle fractions, respectively. Mb, Pb and LCL indicate mouse brain, porcine brain and human lymphoblastoid cell extract, respectively. The molecular mass standards were indicated on the left in kilodaltons.

Table 1
Protein sequence identity/similarity between full-length human (h), zebrafish (zf), and *Takifugu rubripes* (fugu) PRMT genes

	h1	h2	h3	h4	h5	h6	h7	hL3
zf1 AAH57480	88/96	26/52	31/45	21/36	12/29	31/58	14/29	80/93
fugu1 BK004168	88/95	26/51	30/45	19/37	12/28	31/58	13/27	80/94
zf2 BK004174	29/53	52/74	24/46	20/39	13/35	32/54	14/32	28/55
fugu2 BK004169	26/51	46/67	23/45	22/42	12/32	29/50	13/32	25/52
zf3 BK004175	30/47	23/48	57/78	19/37	15/41	23/44	16/38	29/46
fugu3 BK004170	30/46	25/47	57/77	19/41	16/41	24/42	15/36	28/44
zf4 BK004176	20/39	24/42	20/38	82/90	15/41	23/41	14/39	21/38
fugu4 BK004171	22/41	25/46	21/41	76/83	15/41	24/43	12/38	21/41
zf5 CAD60861	12/31	15/35	17/43	15/39	78/90	13/32	15/39	11/29
fugu5 BK004172	13/32	17/34	18/42	14/40	77/92	12/33	15/39	12/30
zf6 BK004177	40/66	29/51	22/41	19/38	12/32	50/73	13/30	40/66
fugu6 BK004173	38/61	30/50	23/40	18/35	11/31	51/73	14/29	38/65
zf7 BK004179	13/29	16/35	15/41	17/41	14/40	14/31	62/80	13/27
fugu7 BK004167	11/26	11/33	15/40	15/42	15/40	13/28	59/76	12/26
zfL3 BK004178	75/90	24/51	28/44	20/35	13/30	31/56	13/29	88/97
fuguL3 BK004166	67/80	28/59	32/52	21/42	14/35	29/56	15/32	72/81

3.3. Identification of the PRMT orthologues in C. intestinalis

From previous analyses, members of the PRMT gene family are conserved from fish to human in the vertebrates. A marine invertebrate chordate *C. intestinalis* (*Ci*) was

Table 2
Protein sequence identity/similarity between full-length human (h) and *C. intestinalis* (ci) PRMT genes

	h1	h3	h4	h5	h6	h7
ci1 BK004868	70/84	30/48	20/37	13/31	32/58	12/29
ci3 BK004869	26/43	43/67	22/40	16/42	22/40	17/39
ci4 BK004870	22/39	19/37	50/69	14/38	22/42	15/40
ci5 BK004871	11/30	16/44	16/39	51/73	13/36	14/38
ci6 BK004872	30/50	16/38	21/42	12/34	33/52	16/37
ci7 BK004873	14/30	15/40	11/38	15/39	13/30	41/66

proposed to represent a plausible approximation to the ancestral chordates for the origin of vertebrates and chordate. Its draft genome and cDNA database are both available. We thus searched for the PRMT genes further in this system. We identified orthologues of PRMT1, 3, 4, 5, 6 and 7 in *C. intestinalis* (predicted amino acid sequences and genomic structures in Fig. 1 and Figs. 1–5, Supplementary material). Interestingly, PRMT1 in *C. intestinalis* appeared to be the orthologue of both PRMT1 and HRMT1L3 (Fig. 1). No PRMT2 orthologue can be identified in the ascidians.

In general, the genomic structures of the ascidians PRMT genes are fairly different from their vertebrate counterparts. However, genomic structure of *Ci* PRMT1 is similar with that of its vertebrate orthologues. Only exons 3, 5, 6 of *Ci* PRMT1 are longer than those in the vertebrate PRMT1

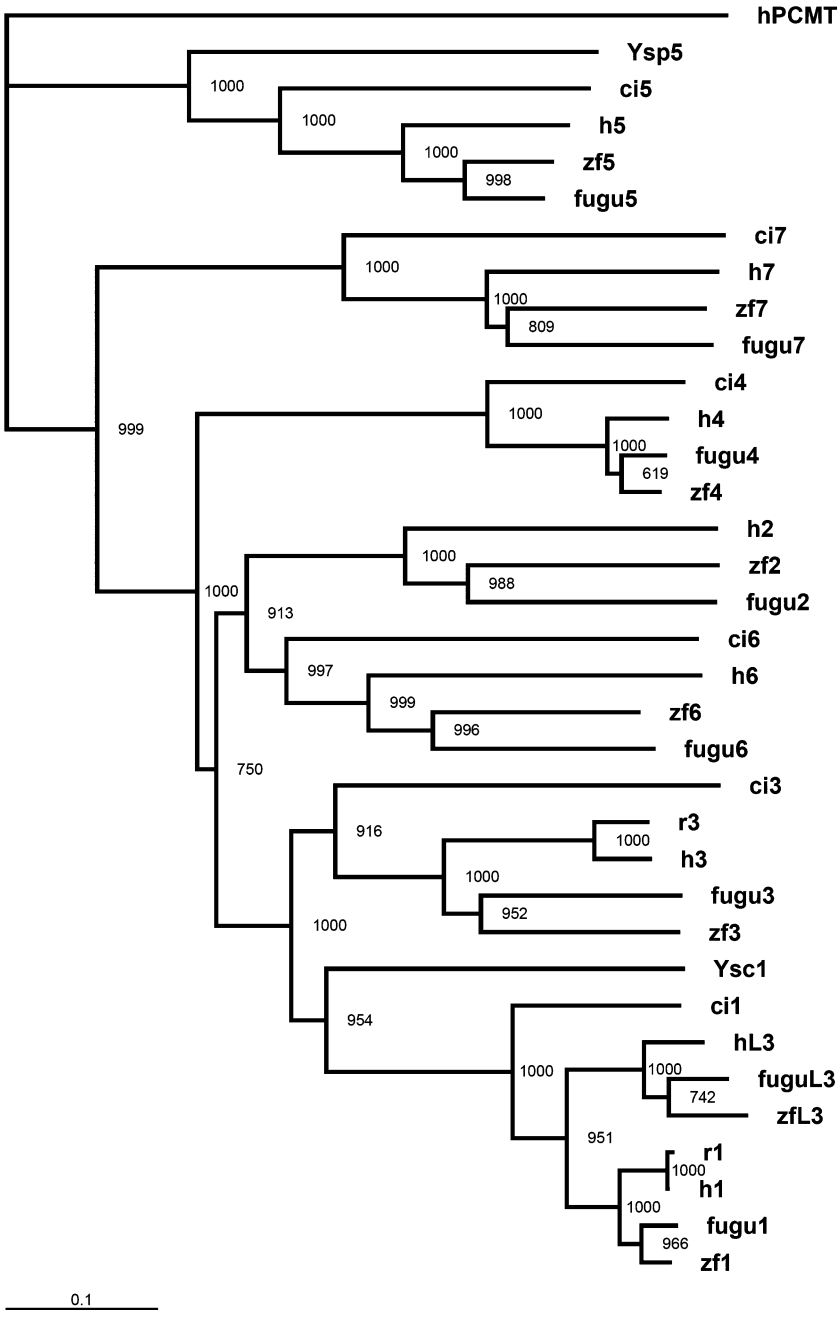


Fig. 3. Phylogram of the PRMT genes. The phylogenetic tree was constructed by neighbor–joining methods. The exclude position with gap option and the correct for multiple substitutions option were disabled. Numbers at the nodes represent bootstrap proportions (BP) on 1000 replicates, and the scale bar represents 0.1 nucleotide substitutions per site. The core region of each PRMT was used in this analysis (see Table 3 in Supplementary material). As for different species, h indicates human, r indicates rat, zf indicates zebra fish, ci indicates *C. intestinalis*, Ysp indicates *S. pombe* and Ysc indicates *S. cerevisiae*. The PRMT was indicated by the number of the PRMT gene at right. hPCMT is the human protein-L-isoaspartate(D-aspartate) *O*-methyltransferase (PCMT) used as the outgroup.

while other exons are with exact junctions as those in the vertebrate ones (Fig. 1). The *Ci* PRMT4 contains 14 compared with 16 exons of human PRMT4. Among these exon junctions, five sites are conserved (Fig. 3, Supplementary material). The exon number of PRMT7 is greatly reduced from 14 to 4 from human to *Ciona*. Among the few exon junctions of *Ci* PRMT7, one site is conserved as that in human (Fig. 5, Supplementary material). Most importantly, PRMT6 with characteristic one coding exon in vertebrates now contains six separate exons in the ascidians (Fig. 2, Supplementary material), while both *Ci* PRMT3 and PRMT5 have only one coding exon (Figs. 2 and 4, Supplementary material).

3.4. Comparison of the conservation of PRMT genes

In both *Fugu* and zebrafish, eight putative PRMT genes have been identified. As shown in Tables 1 and 2, PRMT1 is the most conserved one among all PRMT genes. Zebrafish HRMT1L3 also share 88% sequence identity with its human orthologue. The zebrafish and human PRMT4/CARM1 or PRMT5 are highly conserved with 82% or 78% identity, respectively. For other zebrafish PRMT proteins, PRMT7, 3, 2 and 6 share 62%, 57%, 52% and 50% identity with their human orthologues. The identities of the *Fugu* PRMT proteins with their human orthologues were of similar level as those between zebrafish and human. (Comparison of full-length sequence identity/similarity between zebrafish/*Fugu* as well as *Ciona/Fugu*, see Tables 1 and 2 in Supplementary material). The identity between human and *Ciona* PRMT orthologues were of highest 71% to lowest 33% corresponding to PRMT1 and PRMT6, respectively (Table 2).

3.5. Phylogenetic analyses of the PRMT genes

We then performed phylogenetic analyses of the PRMT genes of yeast, *C. intestinalis*, fish and mammals (Fig. 3). Since all PRMTs share a conserved methyltransferase core with varied C-terminal and N-terminal sequences, core region of each PRMT gene was utilized for the analyses. In the type II division, it is apparent that PRMT5 is conserved through yeast to human. Except the type II PRMT5, all PRMT located in one division in the phylogenetic tree. In this division, it is apparent that PRMT1 is most closely related to HRMT1L3. The vertebrate PRMT1 and L3 gene is of similar distance to the *Ci* PRMT1 gene. Yeast RMT1 appears to belong to this lineage. PRMT3 then clustered with the PRMT1/HRMT1L3 lineage. Another cluster next to PRMT1/3 includes both PRMT2 and PRMT6. PRMT4 is further outside the PRMT2/6 and PRMT1/3 subgroup. It is interesting that the newly identified PRMT7 is located most outside in this branch. Even though PRMT7 show more sequence homology with PRMT5 than with PRMT1 when full-length sequences are compared (Tables 1 and 2), it is probably just due to the long protein sequences of both PRMT7 and 5. PRMT5 is divided from all other PRMT genes probably indicating the separation of the type II protein arginine methyltransferase with other PRMTs.

4. Discussion

In this study we performed data mining and bioinformatic analyses of the PRMT genes. Orthologues of the eight human PRMT genes in two fish model systems pufferfish *F. rubripes* and zebrafish *D. rerio* were identified based on their amino acid sequence homology. Basically, the genomic structures of PRMT orthologues are conserved from fish to human. The conservation indicates that these genes are conserved in vertebrates and the divergence of these PRMT genes probably occurred before the origin of vertebrates. We demonstrated the expression of a few PRMT proteins in zebra fish, the model organism that is amenable to genetic manipulations and has been widely used in developmental studies. Since the PRMT genes are highly conserved from fish to human, we suggest that zebrafish would be a valuable model system to study protein arginine methylation.

C. intestinalis was proposed to represent a plausible approximation to the ancestral chordates for the origin of vertebrates and chordate. Paralogous vertebrate genes such as gene families for cell signaling (such as the fibroblast growth factor) and transcription factors (such as SMAD) often match to a single gene in *C. intestinalis* (Dehal et al., 2002). Similarly, the PRMT genes are found in a simplified form with two family members (PRMT2 and HRMT1L3) missing in *Ciona*. Interestingly, the PRMT1 (HRMT1L2) homologue HRMT1L3 has identical exon junctions and share high sequence identity with PRMT1 in human and fish. The information strongly suggests that HRMT1L3 occurred by gene duplication of PRMT1 after the divergence of the vertebrate ancestors from other chordates and can be viewed as a paralogue of PRMT1 in vertebrates. Furthermore, we found that *Ci* PRMT3 as well as PRMT5 contains only one exon, compared with the 15 exons of both human PRMT3 and 5. Since PRMT3 and PRMT5 in yeast *S. pombe* also have multiple coding exons (Figs. 2 and 4, Supplementary material), it is possible that these one-exon genes are derived from lineage-specific gene rearrangements in the ascidians.

It is likely that some PRMT genes accumulate mutations faster than other PRMT family members. Basically, putative PRMT2, 3, 6 and PRMT1-like (HRMT1L3) genes identified in chordates, fish and mammals share more than or about 50% similarity to human PRMT1. It is thus likely that PRMT1 is the most critical type I PRMT with least variations through evolution. Other type I PRMT genes in

different biological divisions are most likely to be derived from lineage-specific duplication of an ancestral PRMT1 and evolved with different rates. Interestingly, the degree of sequence conservation seems to be related to the functional importance of the PRMT genes known for now. It is apparent that the three frequently reported PRMT1, 4 and 5 with direct evidences to play roles in various transcriptional controls and signaling pathways are the most conserved ones. PRMT3 though identified early, has limited reports on its physiological functions (Tang et al., 1998; Frankel and Clarke, 2000). Recently, fission yeast PRMT3 was identified as a ribosomal protein arginine methyltransferase (Bachand and Silver, 2004). Interestingly, even PRMT3 can be identified in *Drosophila* and fission yeast with typical N-terminal C_2H_2 zinc finger, three residues of the four critical cysteins and histidines of the finger in the *Ciona* PRMT3 have been changed (Fig. 2, Supplementary material). Compared with PRMT3 with 57% sequence conservation between the fish and human orthologues and can be traced back to the fission yeast *S. pombe*, PRMT7 is more conserved (62% sequence identity between fish and human) but is present through *Caenorhabditis elegans* to human. It should have certain important roles to be conserved through evolution. PRMT 7 was identified as a mediator of cell sensitivity to DNA-damaging agents (Gros et al., 2003) in a rodent cell line. Although its catalyses of monomethylarginine (MMA) formation on peptide substrates has been demonstrated, no formation of dimethylarginines on known protein substrates can be detected by in vitro methylation (Miranda et al., 2004). From our phylogenetic analyses, PRMT7 appears to be the most outside lineage in the type I PRMT branch that is separated with the only type II PRMT5. The identity of the PRMT7-catalyzed activity requires more biochemical evidences to illustrate.

In this study, we found that PRMT6 and PRMT2, converged in one lineage parallel to the PRMT1/3 lineage, are the least conserved PRMT from fish to human, with about 50% sequence identity. Whether the two PRMT genes are evolved at a higher rate is an interesting issue. However, the N-terminal SH3 domain before the catalytic core of PRMT2 and the characteristic one coding exon in PRMT6 is conserved from fish to human. Surprisingly, we found that *Ci* PRMT6 contains six exons, indicating that the one coding exon character of PRMT 6 is specific only in the vertebrates. It is interesting that we could not identify PRMT2 in *Ciona*. It is attractive to propose that PRMT2 is evolved from a duplication of a common ancestor of both PRMT6 and PRMT2 in the early vertebrates but are diverged with faster rates than other PRMT genes. By now no direct methyltransferase activity has been demonstrated for PRMT2 and no physiological importance of PRMT6 is known. Whether they are of less physiologically importance thus accumulate mutations much faster than other PRMT genes is an interesting question.

Acknowledgement

We thank Dr. Hui-Chin Pan for providing the zebrafish, Dr. Venkatesh for *Fugu* genomic DNA, Dr. Wen-Chang Lin for critical suggestions of the study and comments of the manuscript and Da-Huang Chen for preparation of the manuscript. The studies were supported by National Science Council, ROC (NSC 91-3112-B040-001; 92-3112-B040-001; 93-3112-B040-001) and Chung Shan Medical University (CSMU 91-OM-A-013).

Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2004.07.039.

References

Aparicio, S., et al., 2002. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science 297, 1301–1310.

Bachand, F., Silver, P.A., 2004. PRMT3 is a ribosomal protein methyltransferase that affects the cellular levels of ribosomal subunits. EMBO J. 23, 2641–2650.

Boulanger, M.C., Branscombe Miranda, T., Clarke, S., DiFruscio, M., Suter, B., Lasko, P., Richard, S., 2004. Characterization of Drosophila protein arginine methyltransferases DART1 and DART4. Biochem. J. 379, 283–289 (Pt.).

Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L., Luhrmann, R., 2000. The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. J. Biol. Chem. 275, 17122–17129.

Brahms, H., Meheus, L., de Brabandere, V., Fischer, U., Luhrmann, R., 2001. Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein. RNA 7, 1531–1542.

Branscombe, T.L., Frankel, A., Lee, J.H., Cook, J.R., Yang, Z., Pestka, S., Clarke, S., 2001. PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins. J. Biol. Chem. 276, 32971–32976.

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W., Stallcup, M.R., 1999. Regulation of transcription by a protein methyltransferase. Science 284, 2174–2177.

Dehal, P., et al., 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298, 2157–2167.

Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., Boggs, C., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. Development 123, 37–46.

Fabbrizio, E., El Messaoudi, S., Polanowska, J., Paul, C., Cook, J.R., Lee, J.H., Negre, V., Rousset, M., Pestka, S., Le Cam, A., Sardet, C., 2002. Negative regulation of transcription by the type II arginine methyltransferase PRMT5. EMBO Rep. 3, 641–645.

Frankel, A., Clarke, S., 2000. PRMT3 is a distinct member of the protein arginine *N*-methyltransferase family. Conferral of substrate specificity by a zinc-finger domain. J. Biol. Chem. 275, 32974–32982.

Frankel, A., Yadav, N., Lee, J., Branscombe, T.L., Clarke, S., Bedford, M.T., 2002. The novel human protein arginine *N*-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. J. Biol. Chem. 277, 3537–3543.

Gary, J.D., Clarke, S., 1998. RNA and protein interactions modulated by protein arginine methylation. Prog. Nucleic Acid Res. Mol. Biol. 61, 65–131.

Gary, J.D., Lin, W.J., Yang, M.C., Herschman, H.R., Clarke, S., 1996. The predominant protein-arginine methyltransferase from *Saccharomyces cerevisiae*. J. Biol. Chem. 271, 12585–12594.

Gilbreth, M., Yang, P., Wang, D., Frost, J., Polverino, A., Cobb, M.H., Marcus, S., 1996. The highly conserved skb1 gene encodes a protein that interacts with Shk1, a fission yeast Ste20/PAK homolog. Proc. Natl. Acad. Sci. U. S. A. 93, 13802–13807.

Goodstadt, L., Ponting, C.P., 2001. CHROMA: consensus-based colouring of multiple alignments for publication. Bioinformatics 17, 845–846.

Gros, L., Delaporte, C., Frey, S., Decesse, J., de Saint-Vincent, B.R., Cavarec, L., Dubart, A., Gudkov, A.V., Jacquemin-Sablon, A., 2003. Identification of new drug sensitivity genes using genetic suppressor elements: protein arginine *N*-methyltransferase mediates cell sensitivity to DNA-damaging agents. Cancer Res. 63, 164–171.

Hebert, M.D., Shpargel, K.B., Ospina, J.K., Tucker, K.E., Matera, A.G., 2002. Coilin methylation regulates nuclear body formation. Dev. Cell 3, 329–337.

Henry, M.F., Silver, P.A., 1996. A novel methyltransferase (Hmt1p) modifies poly(A)+-RNA-binding proteins. Mol. Cell. Biol. 16, 3668–3678.

Katsanis, N., Yaspo, M.L., Fisher, E.M., 1997. Identification and mapping of a novel human gene, HRMT1L1, homologous to the rat protein arginine *N*-methyltransferase 1 (PRMT1) gene. Mamm. Genome 8, 526–529.

Kwak, Y.T., Guo, J., Prajapati, S., Park, K.J., Surabhi, R.M., Miller, B., Gehrig, P., Gaynor, R.B., 2003. Methylation of SPT5 regulates its interaction with RNA polymerase II and transcriptional elongation properties. Mol. Cell 11, 1055–1066.

Kzhyshkowska, J., Schutt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H., Dobner, T., 2001. Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg–Gly–Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1. Biochem. J. 358, 305–314.

Lee, J., Bedford, M.T., 2002. PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. EMBO Rep. 3, 268–273.

Lin, W.J., Gary, J.D., Yang, M.C., Clarke, S., Herschman, H.R., 1996. The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine *N*-methyltransferase. J. Biol. Chem. 271, 15034–15044.

Ma, X.J., Lu, Q., Grunstein, M., 1996. A search for proteins that interact genetically with histone H3 and H4 amino termini uncovers novel regulators of the Swe1 kinase in *Saccharomyces cerevisiae*. Genes Dev. 10, 1327–1340.

McBride, A.E., Silver, P.A., 2001. State of the arg: protein methylation at arginine comes of age. Cell 106, 5–8.

Miranda, T.B., Miranda, M., Frankel, A., Clarke, S., 2004. PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. J. Biol. Chem. 279, 22902–22907.

Mowen, K.A., Tang, J., Zhu, W., Schurter, B.T., Shuai, K., Herschman, H.R., David, M., 2001. Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. Cell 104, 731–741.

Pal, S., Yun, R., Datta, A., Lacomis, L., Erdjument-Bromage, H., Kumar, J., Tempst, P., Sif, S., 2003. mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in transcriptional repression of the Myc target gene cad. Mol. Cell. Biol. 23, 7475–7487.

Pawlak, M.R., Scherer, C.A., Chen, J., Roshon, M.J., Ruley, H.E., 2000. Arginine *N*-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable. Mol. Cell. Biol. 20, 4859–4869.

Pollack, B.P., Kotenko, S.V., He, W., Izotova, L.S., Barnoski, B.L., Pestka, S., 1999. The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. J. Biol. Chem. 274, 31531–31542.

Qi, C., Chang, J., Zhu, Y., Yeldandi, A.V., Rao, S.M., Zhu, Y.J., 2002. Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. J. Biol. Chem. 277, 28624–28630.

Rho, J., Choi, S., Seong, Y.R., Cho, W.K., Kim, S.H., Im, D.S., 2001. Prmt5, which forms distinct homo-oligomers, is a member of the protein-arginine methyltransferase family. J. Biol. Chem. 276, 11393–11401.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Scott, H.S., Antonarakis, S.E., Lalioti, M.D., Rossier, C., Silver, P.A., Henry, M.F., 1998. Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). Genomics 48, 330–340.

Tang, J., Gary, J.D., Clarke, S., Herschman, H.R., 1998. PRMT 3, a type I protein arginine *N*-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation. J. Biol. Chem. 273, 16935–16945.

Tang, J., Frankel, A., Cook, R.J., Kim, S., Paik, W.K., Williams, K.R., Clarke, S., Herschman, H.R., 2000. PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. J. Biol. Chem. 275, 7723–7730.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Wada, K., Inoue, K., Hagiwara, M., 2002. Identification of methylated proteins by protein arginine *N*-methyltransferase 1, PRMT1, with a new expression cloning strategy. Biochim. Biophys. Acta 1591, 1–10.

Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B.M., Montminy, M., Evans, R.M., 2001. A transcriptional switch mediated by cofactor methylation. Science 294, 2507–2511.

Yadav, N., Lee, J., Kim, J., Shen, J., Hu, M.C., Aldaz, C.M., Bedford, M.T., 2003. Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 100, 6464–6468.

Zhang, X., Cheng, X., 2003. Structure of the predominant protein arginine methyltransferase PRMT1 and analysis of its binding to substrate peptides. Structure (Camb) 11, 509–520.

Zhang, X., Zhou, L., Cheng, X., 2000. Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. EMBO J. 19, 3509–3519.

Zon, L.I., 1999. Zebrafish: a new model for human disease. Genome Res. 9, 99–100.