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蛋白質精胺酸甲基化之蛋白體分析（2/3）

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行政院國家科學委員會補助專題研究計畫

□成果報告　　☑期中進度報告

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共同主持人：

計畫參與人員：陳大晃 洪建仁 洪銓錨 吳冠賜 張宏豪　等

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摘要

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接受者在細胞中分佈的情形。（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，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 methylation 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, posttranslational modification

報告內容

Introduction

Protein arginine methylation is an irreversible posttranslational 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 et al., 1996).

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 PRMT6 (Frankel et al., 2002). Direct evidences of the catalysis by these methyltransferases to form the type I products 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 an hnRNPE1B-AP5 methylation in vivo (Kzhyshkowska et al., 2001) and its potential methyltransferase activity is important for its acting as a coactivator for 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). Its mouse orthologue was identified recently by a neural gene screen (Aubert et al., 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 seveviciae* histone synthetic lethal gene hsl7 (Ma et al., 1996). Another recently identified PRMT7 gene p82/p77 is a genetic suppressor element mediates 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, Miranda et al. suggested that PRMT7 can catalyze the formation of ω- N^G –monomethylarginine in peptides exhibiting the type III activity most recently (2004).

In comparison with eight members in mammalian PRMT family, there are only two related protein arginine methyltransferase genes in yeast *S. seveviciae.* 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 a long list such as 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) and is localized in the nucleus (Chen et al., 1999). Identified PRMT4 substrates include histone H3 (Xu et al., 2001), p300/CBP (Li et al., 2002), PABP1 (Lee and Bedford, 2002) and an mRNA –stabilizing protein HuR (Brahms et al., 2000). JBP1/PRMT5 is localized in the cytoplasm and the 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).

The specific aim of the project is to investigate one type of protein posttranslational

modification—protein arginine methylation using the proteomic tools, basically two dimensional gel electrophoresis (2-DE) followed by mass spectrometry to identify specific protein spots. Protein arginine methylation is an irreversible modification on the guanidino nitrogen of the arginyl residues that accounts for the majority of stable protein methylation events in cells. In this project, after one year getting working directions, we adjust our focus on three different areas, to help to better understand the roles of this posttranslational modification.

1. *We will perform global analysis of protein methylaccepting proteins by proteomic approach*
2. *We will characterize the novel protein methylacceptors identified through above approaches*.
3. *We will analyze the relationship of protein arginine methylation with specific human diseases by proteomic analyses of the arginine methylaccepting proteins in different biomedical samples*.

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 submitted to Archives of Biochemistry and Biophysics. 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 (Fig.1A, B for some representative results). This investigation also provides us with a good experimental system for future studying of *in vivo* methylated proteins proteomically.

Proteomic analyses of arginine methylaccepting proteins and basic proteins

Secondly, we are continuing the proteomic analyses of methylaccepting proteins. Since AdOx treatment is likely to affect a small fraction of total methylaccepting proteins, we have two approaches to directly analyze cellular proteins without AdOx treatment. One is to analyze HeLa extracts in pH 6-11, the pH range most arginine methylaccepting proteins presumably belong. We had spent some time in getting good IEF separation conditions at this pH range. We then compare the western blot probed by methylarginine specific antibodies to the protein staining pattern of 2-D electrograms (Fig. 2). We have just sent some of the putative methylarginine-containing proteins for MALDI analyses. Our preliminary analyses indicate a possible candidate hnRNP M. Another approach is to use chromatofocusing chromatography to pre-fractionate proteins prior to 2D analyses. We use a pH9-6 gradient to fractionate mouse brain extracts. Since many methylarginine containing proteins are RNA binding proteins, we treated the brain extract with nuclease or not before the chromatofocusing. The resolution of the column was satisfactory as we first examined the fractions by SDS-PAGE and silver stain (Fig. 3). We further picked up spots for MALDI analyses. For fractions with simple protein patterns, we can directly digest the proteins in the fractions for LC-MS/MS analyses. We can have a proteomic documentation of the mouse brain proteins and we will pay additional attention to the fractions with signals that can be detected by methylarginine specific antibodies for methylarginine containing signals by MS analyses. Preliminary result of this work is submitted to the poster presentation in International Proteomic Conference.

Modulation of stable protein methylation by KCl

Thirdly we were analyzing the methylaccepting proteins that can be differentially modified when the cell extracts were treated with 0.5M KCl (Fig. 4). The high salt condition enhanced the methylation of certain methylaccepting proteins but weakened the methylation of some other proteins. The high ionic strength most likely interrupted non-covalent associations of proteins. We proposed

that certain fraction of the type I methyltransferase activity might be associated with components that are associated with membrane or other large complexes. Release of the methyltransferase from the association affected the methyltransferase activity. We have performed the 2-D gel electrophoresis to separate the methylaccepting proteins and are going to identify the proteins by mass spectrometry soon.

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 manuscript "*Identification and phylogenetic analyses of the protein arginine methyltransferase gene family in fish and ascidians*", now under review of GENE. 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 (Fig. 5). Besides, We cloned the human PRMT6 as well as the zebrafish 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*, but are not able to detect methyltransferase activity in either recombinant protein yet.

Protein arginine methylation analyses of PAI-RBP1

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.

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. Thus in this project we will firstly obtain anti-Sm and anti-RNP autoantibodies from SLE patients and other antisera from the Project Core to search for the methylated autoantigens proteomically. 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. We are currently analyzing the proteins recognized by the affinity resin.

References

¥ Aubert, J., Stavridis, M.P., Tweedie, S., O'Reilly, M., Vierlinger, K., Li, M., Ghazal, P., Pratt, T., Mason, J.O., Roy, D. and Smith, A. 2003 Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice. Proc. Natl. Acad. Sci. U S A 100 Suppl. 1,11836-41.

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.

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 7,1531-42.

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, 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.

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., 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.

Goodstadt, L. and Ponting, C.P. 2001 CHROMA: consensus-based colouring of multiple alignments for publication. Bioinformatics 17,845-6.

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. 63,164-71.

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.

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.

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.

Li, H., Park, S., Kilburn, B., Jelinek, M.A., Henschen-Edman, A., Aswad, D.W., Stallcup, M.R. and Laird-Offringa, I.A. 2002 Lipopolysaccharide-induced methylation of HuR, an mRNA-stabilizing protein, by CARM1. Coactivator-associated arginine methyltransferase. J. Biol. Chem. 277,44623-30.

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, 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.

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.

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.

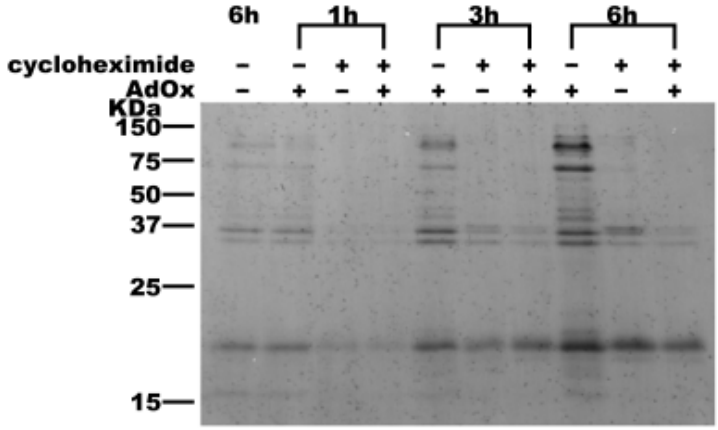
Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B.M., Montminy, M. and Evans, R.M. 2001 A transcriptional switch mediated by cofactor methylation. Science 294,2507-11.

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.

Zhang, X., Zhou, L. and Cheng, X. 2000 Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. Embo J. 19,3509-19.

Fig. 1 (A) Treatment of HeLa cells by AdOx and cycloheximide. HeLa cell extracts were prepared from cells incubated in the presence of both 20 μM of AdOx and 0.08 μg/ml of cycloheximide or only either one treatment for 1, 3 or 6 hrs. (A) *In vitro* methylation reaction of the above extracts without the addition of exogenous substrate was performed as described.



(B) *In vivo* methylation of HeLa cells treated with AdOx or not. *In vivo* methylation was performed as described in Materials and Methods. HeLa cells were *in vivo* labeled either with 24 hr AdOx-pretreatment, or with AdOx present in the labeling medium. The cells chased for 12 hr in non-radioactive medium after *in vivo* labeling were indicated. Cell lysates were collected, separated by SDS-PAGE and the *in vivo* methylated proteins were detected by fluorography.

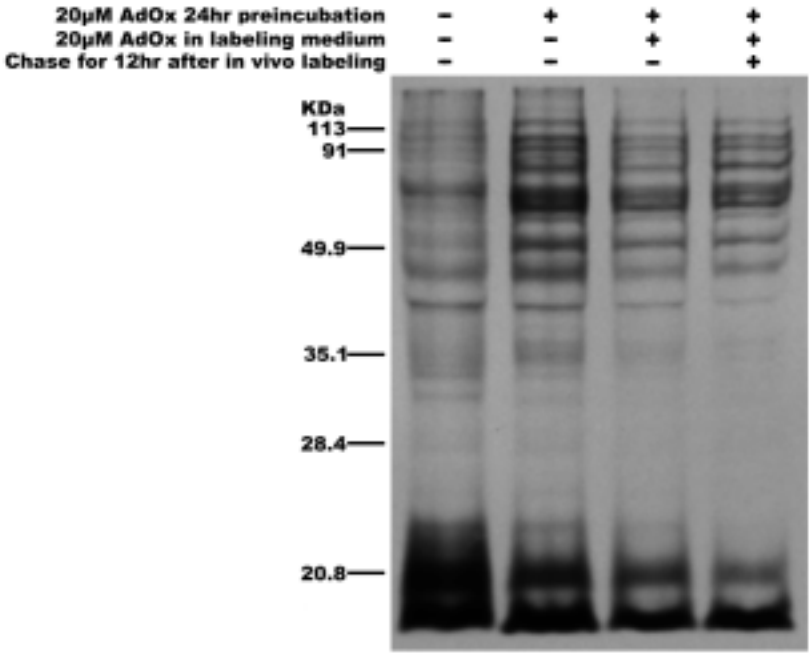


Fig. 2 Analyses of HeLa extracts in pH 6-11 2-D electrophoresis. HeLa cell extracts was prepared in IEF lysis buffer then separated on a 7-cm pH6-11 IPG strip and SDS-PAGE. The gel was then transferred and then detected by a mono- and di-methylarginine specific antibody 7E6 (upper panel). Parallel 2-D gel was stained with colloidal blue for protein pattern (lower panel). The positions where protein spots can be matched to the western signals were circled.

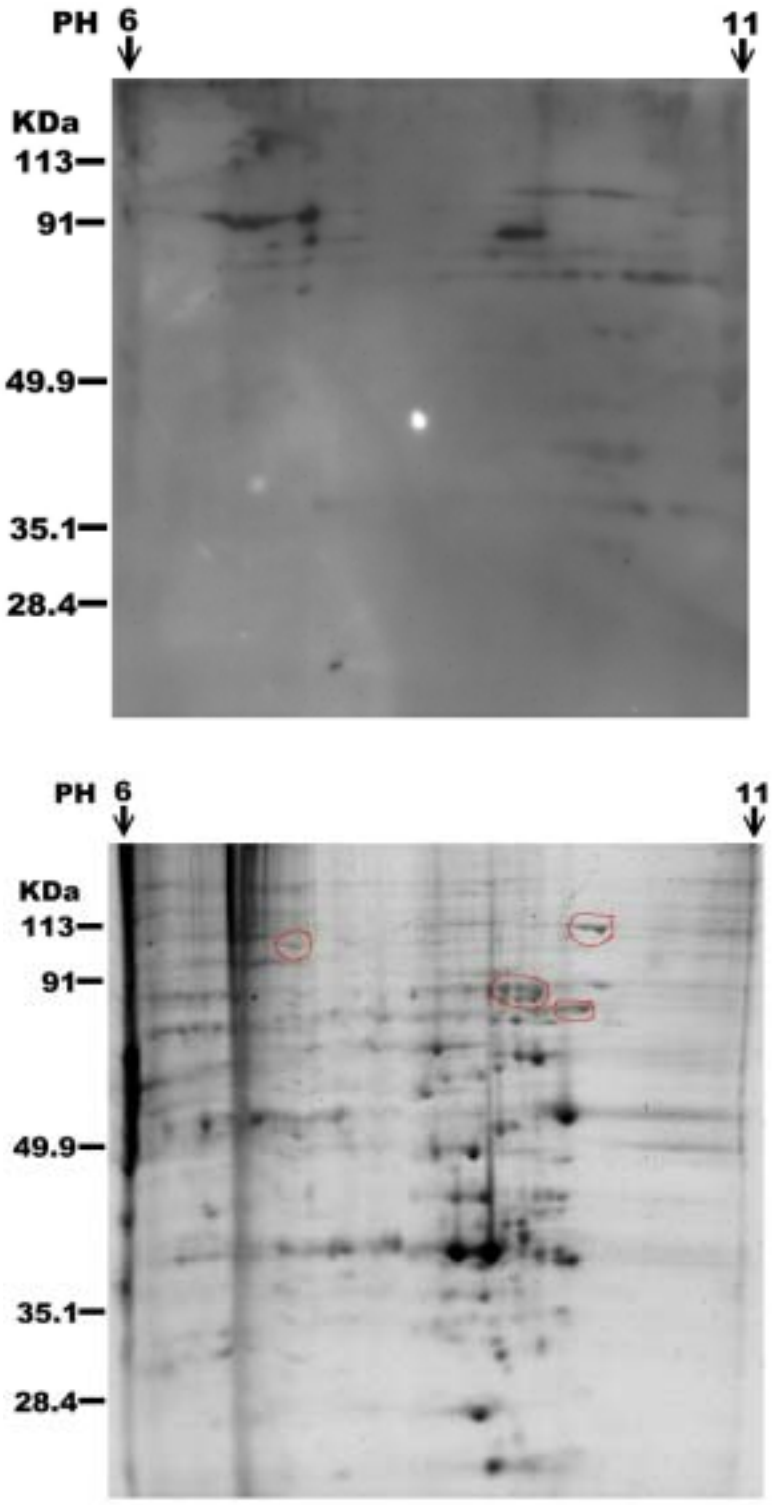


Fig. 3 Mouse brain extract treated with nuclease (upper panel) or not (lower panel) was separated by mono P chromatofocusing chromatography. The column was washed with 2 column volume (CV, 4 ml) of buffer A (0.075 M Tris-acetate, pH9.3), then eluted with the gradient of 100% buffer A to buffer 100% buffer 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.

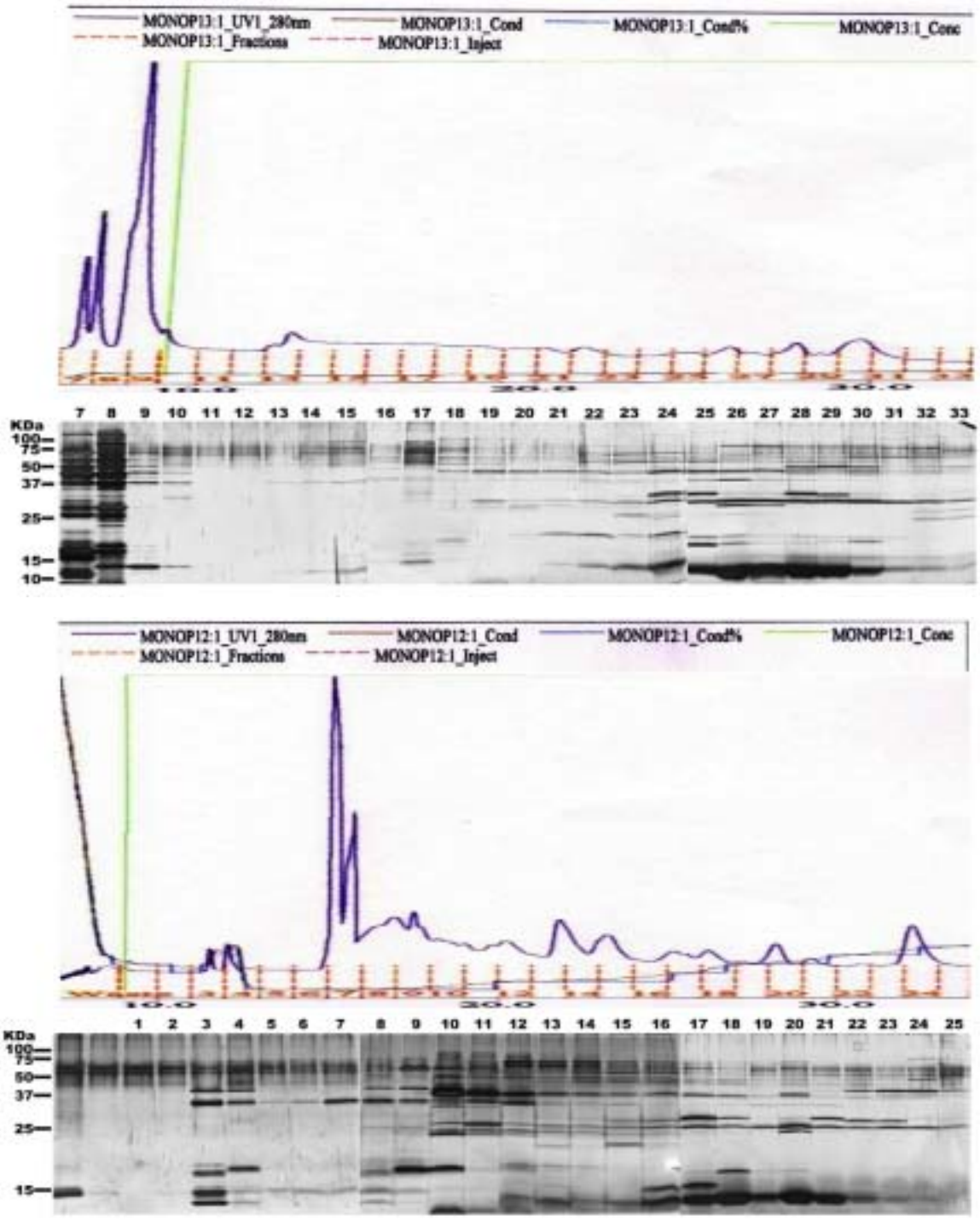
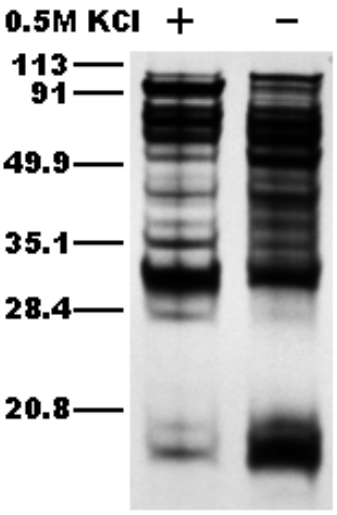
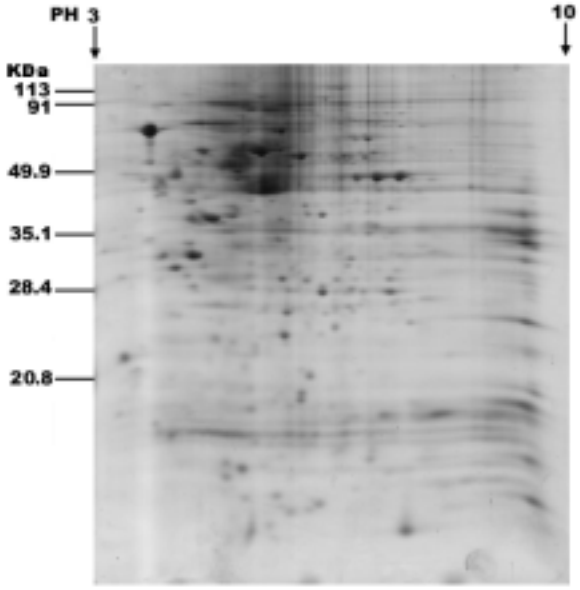


Fig. 4. High KCl concentration changed the pattern of in vitro protein methylation in HaLa cell extracts. The upper panel is the fluorography of in vitro protein methylation of HeLa cell extract with the addition of KCl or not. The reaction mixture had been dialyzed. The lower panel is the 2-D separation of the above dialyzed reaction mixture. The gels are under fluorography now.



+KCl

-KCL



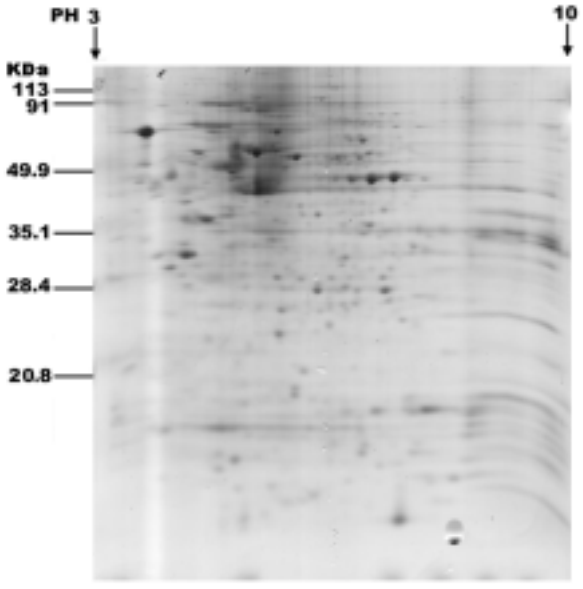


Fig. 5　Phylogram of the PRMT genes. The phylogeentic tree was constructed by Neighbor Joining methods. The exclude position with gap option and the correct for multiple substitutions option were disabled. Numbers at the nodes represent bootstrap proportions (BP) on 1000 replicates, and the scale bar represents 0.1 nucleotide substitutions per site. The core region of each PRMT was used in this analysis (see supplement materials). As for different species, h indicates human, r indicates rat, zf indicate zebra fish, ci indicate *Ciona intestinalis*, Ysp indicates *Schizosaccharomyces pombe* and Ysc indicates *Saccharomyces seveviciae*. 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.

Fig.3

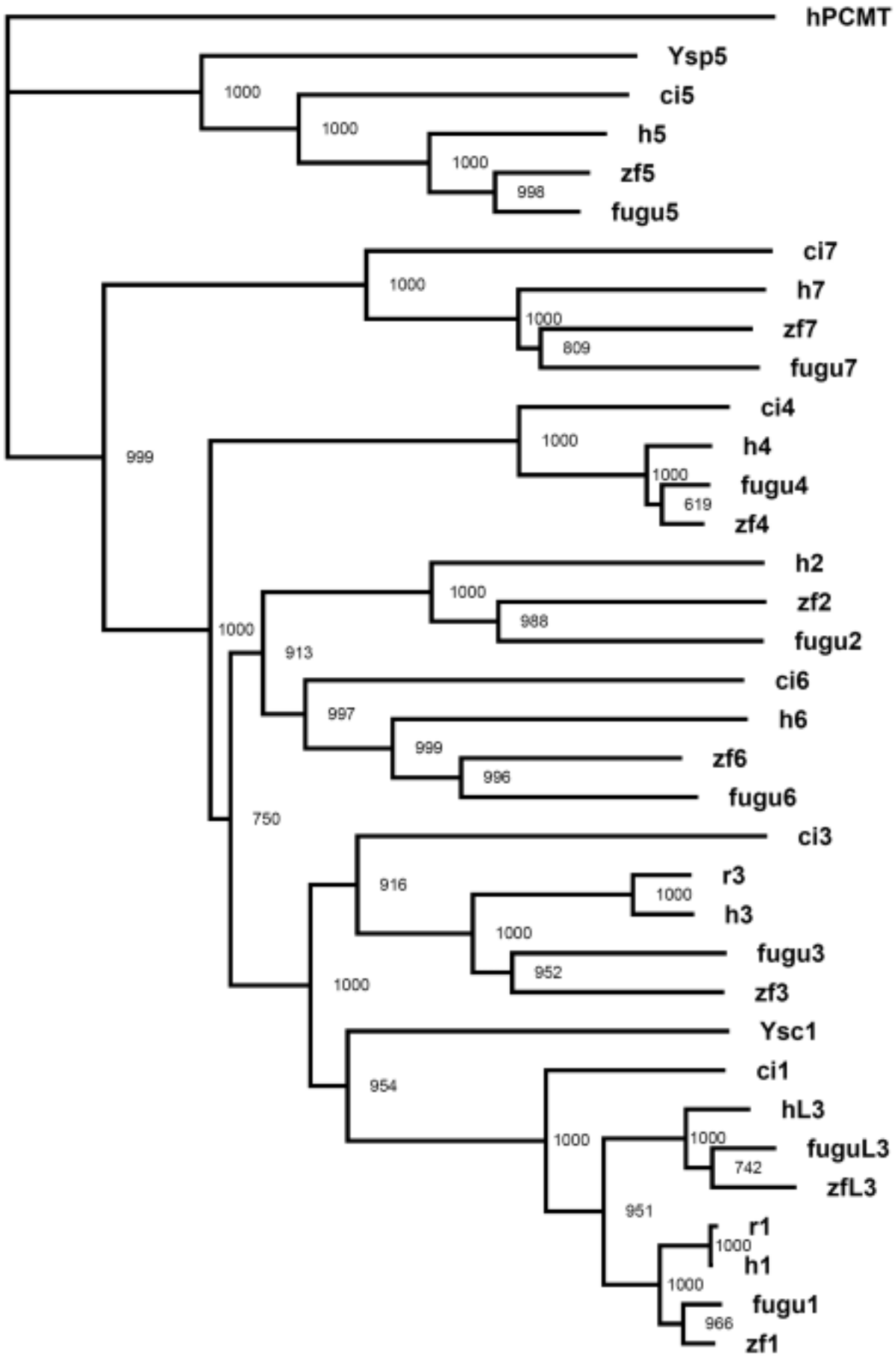

hPCMT
Ysp5
ci5
h5
zf5
fugu5
ci7
h7
zf7
fugu7
ci4
h4
fugu4
zf4
h2
zf2
fugu2
ci6
h6
zf6
fugu6
ci3
r3
h3
fugu3
zf3
Ysc1
ci1
hL3
fuguL3
zfL3
r1
h1
fugu1
zf1
1000
1000
1000
998
1000
1000
809
999
1000
1000
619
1000
988
913
997
999
996
750
916
1000
1000
952
1000
954
1000
1000
742
951
1000
1000
966
0.1

Fig. 6. ***In vitro*** **methylation of PAI-RBP1 by porcine brain extract.**

Recombinant PAI-RBP1 (RGG) was used as the methylacceptor and porcine brain extract (S3) was used the methyltransferase source. Even though the PAI-RBP1 protein (mw. About 50 kDa) appeared to be specifically methylated by certain methyltransferase in S3, we were not able to modify the protein with recombinant arginine methyltransferase such as rat PRMT1 or yeast RMT1. Mass spectrometric analyses also could not provide support for the PAI-RBP1 protein.

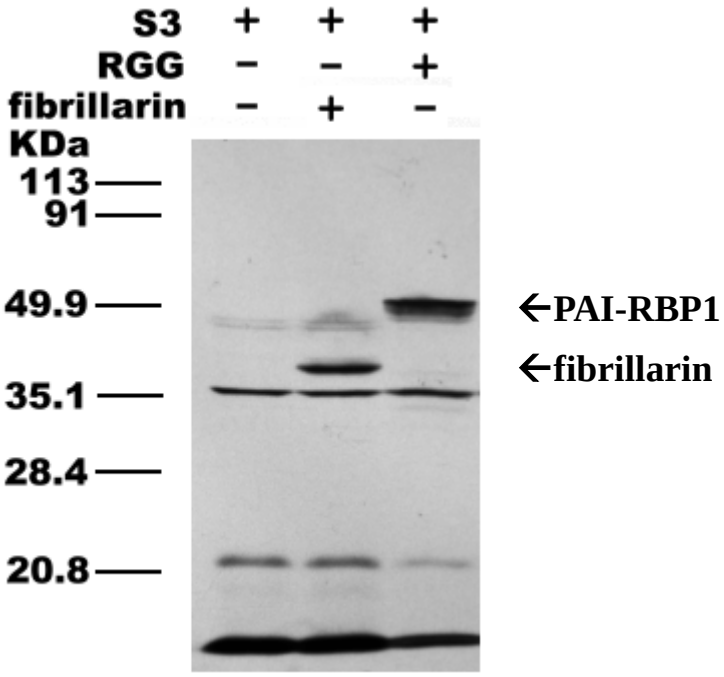


Fig. 7. Immunoprecipitation of in vitro methylated proteins by anti-Sm. AdOx-treated lymphoblastoid cell extracts were in vitro methylated with radioactive [*methyl*-^{3}H]-S-adenosylmethionine (AdoMet) and then used for the immunoprecipitation with protein-A agarose. The immunoprecipitant in the presence of anti-Sm or not were analyzed by gel electrophoresis and fluorography.