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私立中山醫學院醫學研究所
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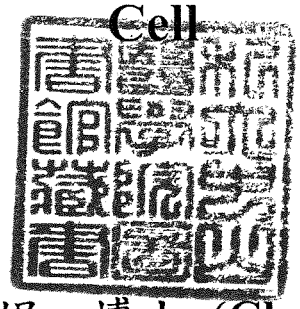
碩士論文
Master Thesis

淋巴母細胞中蛋白精胺酸甲基化之研究

Protein *N*-Arginine Methylation in Lymphoblastoid Cell



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中華民國八十八年六月

學生林佳慧論文題目為淋巴母細胞中蛋白質精胺酸甲基化之研究，其論文已經中山醫學院醫學研究所碩士論文考試委員會審合格及口試通過，並尤其指導教授核閱後無誤。

指導教授： 李　娟 博士　　簽名：

中華民國 87 年 6 月 10 日

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中山醫學院醫學研究所

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目錄

中文摘要

目前已經知道 *N*-arginine methylation 是細胞中最主要蛋白甲基化的形式，會發生在 RNA binding protein 的 RGG motifs 上。為了想了解發生在淋巴母細胞內的甲基化，我們藉由 SDS-PAGE 和 fluorography 分析以 AdOx 處理、淋巴母細胞中的甲基接受蛋白顯示：有許多多胜肽被內生性甲基轉移酶所催化，少數多胜肽會在加入 yeast GST-RMT1 後，訊號變得更強。而不論 yeast GST-RMT1 是否參與，這些甲基接受蛋白的甲基化會被 peptide GGRGRGGGF 特異性的競爭掉，這個結果顯示在淋巴母細胞中的甲基接受蛋白具有相似的 RGG motifs，且甲基化可能發生在 arginine residues。在接下來的研究中，我們將淋巴母細胞分為 nucleus、ribosome and cytosolic fractions 以進一步觀察甲基接受蛋白以及甲基轉移酶的分佈。實驗發現：在不同的 fraction 中都有外生性 RMT1 的甲基接受蛋白存在，而如果甲基化反應中不外加外生性 RMT1，也就是純粹觀察內生性甲基轉移酶的活性時發現：被甲基化的多胜肽存在 ribosomal fraction 中要比 nucleus and cytosolic fractions 來得多，而 ribosomal fraction 中多胜肽的甲基化可被 recombinant fibrillarin (核仁 RGG 蛋白) 競爭掉。當我們以 fibrillarin 作為受質加入反應中發現：酵素活性在 cytosolic fraction 中

最強，其次是 ribosomal fraction 、nucleus fraction。最後胺基酸分析的實驗則更直接的顯示發生在淋巴母細胞內的甲基化主要是精胺酸的甲基化。未來如果更進一步分析特定的甲基接受蛋白以及甲基轉移酶的分佈與調控，將能夠幫助我們瞭解甲基化為何普遍存在真核細胞的 RGG protein 中。

Abstract

N-arginine methylation in RNA binding proteins with arginine and glycine rich RGG motifs is known to be the major protein methylation in cells. Analysis of methyl-accepting polypeptides in AdOx-treated lymphoblastoid cells by SDS-PAGE and fluorography showed that many polypeptides between 29,000 and 90,000 Da were methylated by the endogenous methyltransferase. A few polypeptides could be methylated to a higher extent upon the addition of yeast GST-RMT1 fusion protein. A peptide (GGRGRGGGF) could compete for the majority of the methyl-accepting protein substrates in the AdOx-treated lymphoblastoid cell extracts, whether or not exogenous yeast RMT1 was included in the reaction. The results indicated that the protein methyl acceptors in lymphoblastoid cells share similar RGG motifs and that arginine residues should be the site of methylation. Then we fractionated the lymphoblastoid cell extracts to nucleus, ribosome and cytosolic fractions to further locate the substrates and the methyltransferase. In the presence of exogenous RMT1, hypomethylated methylaccepting polypeptides of a wide range of molecular weights were present in all three fractions. In the absence of exogenous methyltransferase, the methylaccepting polypeptides in the ribosomal fraction were heavily methylated while less polypeptides in the nucleus and cytosolic fractions could be methylated. Majority of the radioactivity on the methylaccepting polypeptides in the ribosomal fraction could be competed by recombinant fibrillarin (a nucleolar RGG protein) in a concentration-dependent manner, indicating the methylation should be specific to the RGG containing proteins. The

strength of activation of fibrillarin methylation by enzymes in the three fractions was cytosolic > ribosome > nucleus fraction. Finally, we demonstrated that the majority of the protein methylation in different subcellular fraction of lymphoblastoid cell appeared to be on the arginine residues by amino acid analysis. Further analysis of the specific methylaccepting substrates and the regulation of the distribution and the activity of arginine methyltransferase would be crucial for the understanding of the modification widely present in eukaryotic RGG protein.

緒論

在真核生物細胞中，許多蛋白質經核醣體轉譯出來後，會藉由在特定的胺基酸上進行轉譯後的修飾作用 (posttranslation modification) 來調控蛋白的活性，如甲基化 (methylation)、醣化 (glycosylation)、磷酸化 (phosphorylation)、雙硫鍵的形成 (disulfide bond formation) 等 (Beyer et al., 1997 ; Soulard et al., 1993 ; Wilk et al., 1985)。而其中蛋白甲基化的作用是由高度專一性的甲基轉移酶所催化。甲基轉移酶在細胞中廣泛存在，利用 S-adenosyl-L-methionine (AdOMet) 所提供的甲基以進行甲基化反應 (Kim and Paik, 1965；Paik et al., 1980)，甲基化反應可概略分為三類：1) *N*-methylation，主要在 histidine、lysine 以及 arginine 等胺基酸的側鏈進行甲基化。2) *O*-methylation 是在 glutamate 和 isoaspartate 的羧基或 cysteine 的 C 端進行甲基化。3) *S*-methylation 則在 cysteine 或 methionine 胺基酸上甲基化 (Paik and Kim, 1980, 1985 ; Clarke, 1985 ; Clarke et al., 1987 ; Kim et al., 1990)。發生在蛋白質精胺酸側鏈 guanidine 氨基上的 *N*-methylation 是由蛋白質甲基轉移酶 I (protein-arginine N-methyltransferase type I) 所催化，這種酵素廣泛存在於原生動物、植物和哺乳類動物組織中 (Paik and Kim, 1980；Kim et al., 1990)，它會

使蛋白形成 N^G-monomethylarginine 、 N^G,N^G-dimethylarginine 或 N^G,N'^G-dimethylarginine 三種形式 (圖一) (Paik and Kim, 1986；Gary et al., 1996)，蛋白像是 meylin basic protein 就會被甲基化形成 N^G-monomethylarginine 或 N^G,N'^G-dimethylarginine，其他蛋白像是 hnRNP A1、fibrillarin、nucleolin 等具有 RGG box 的 RNA 結合蛋白，會被甲基化形成 N^G-monomethylarginine 或 N^G,N^G-dimethylarginine。過去某些蛋白質 *O*-methylation 的甲基化便已知是與生物系統中的訊號傳遞有關，因為近來發現 arginine 甲基化可能也與與訊號傳遞 (signal transduction) 有關，甚至觀察到 arginine 甲基化對 nuclear RNA processing 的影響而倍受矚目 (Lin et al., 1996；Rajpurohit et al., 1994；Valentini et al., 1999；Shen et al., 1998)。因此我們便著手想探討 arginine 甲基化的作用機制。

如前所述：甲基化的修飾作用在很早以前就已經發現，但它在細胞內所扮演的角色及重要性到現在還不清楚，一直到最近第一個第一型甲基轉移酶才被選殖出來，相關的發現，也顯示 arginine 甲基化的重要性，我們根據近幾年文獻，歸納它可能扮演的角色：

1. 關於甲基轉移酶

第一型甲基轉移酶會將 histone、recombinant hnRNP A1 和其他

核蛋白甲基化形成 N^G,N^G-dimethylarginine (Ghosh et al., 1988；Rawal et al., 1994)。Lin et al.利用 yeast-two-hybrid 的實驗為了尋找會與 TIS21 interaction 的蛋白時，選殖出 rat 蛋白精胺酸甲基轉移酶的 cDNA，由於這個 clone 具第一型甲基轉移酶的活性，便把它定名為 PRMT1 (Protein *N*-Arginine Methyltransferase)，同時也發現 TIS21 可以調控 PRMT1 的活性，而 TIS21 是一種 immediate-early protein 與 signal transduction 有關，因此認為甲基化可能與訊號傳遞有關 (Lin et al., 1996)。值得一提的是：一九九七年 Abramovich et al.在鑑定與 interferon-α, β receptor 的 cytoplasmic domain interaction 的蛋白時發現 human PRMT1，而這個 cytoplasmic domain 往往是某些 signal protein 會來結合的位子，所以 Abramovich et al 也推測甲基轉移酶在訊息傳遞路徑中可能扮演某種角色 (Abramovich et al., 1997)。藉由這個哺乳類酵素的發現，yeast 的蛋白 arginine 甲基轉移酶 (protein-arginine methyltransferase ; RMT1) 也跟著被鑑定，它是由 OPD1 基因所表達，包含 348 個胺基酸的多胜肽鏈，分子量為 39.8 kDa，有趣的是：其 allelic HMT1 gene 也在一次尋找會與 yeast poly $(A)^+$-RNA-binding protein (Np13p) 相互作用的蛋白基因篩選 (genetic screen) 實驗中被發現 (Henry et al., 1996)，其受質甲基化後的形式主要為 N^G-monomethylarginine 和 N^G,N^G-dimethylarginine (Gary et al., 1996)。接

著 Katsanis 在 1997 年找到一與 human PRMT1 homologous 的 human gene；HRMT1L1，它所 encode 的蛋白具有部分 methyltransferase domain 是與 human PRMT1 相似的，因此將它定為 PRMT2，其基因位於第 21 對染色體上，蛋白 N 端則具 SH3 domain，但是不論 SH3 domain 存在與否，至今尚無結果顯示 recombinant PRMT2 可將其他蛋白甲基轉移酶的受質甲基化 (Scott et al ., 1998)，然而 dbEST 的搜尋顯示：在許多細胞株和不同的 growth stage 中有 HRMT1L1 的存在，因此 PRMT2 的酵素活性是會被高度調控或在 in vitro 的條件下無法偵測酵素活性則須要進一步的實驗證實。同樣利用 yeast-two-hybrid 的實驗，Tang et al.找到一會與 PRMT1 interaction 的蛋白，經胺基酸分析發現其與 PRMT1 具有高度相似性，因此定名為 PRMT3 (Tang et al., 1998)，PRMT3 並不如 PRMT1 般受 TIS21 調控，但經胺基酸序列的分析發現 ：這個蛋白的 N-terminal acidic amino acid rich (NAR) domain 包含 C2H2 zinc finger motif 和 tyrosine phosphorylation consensus sequence，PRMT3 的酵素活性可能就是受 NAR region 調控 (Tang et al., 1998)。免疫螢光染色分析則顯示 PRMT1 在 RAT1 細胞中主要位於 nucleus，PRMT3 則主要位於 cytoplasm (Tang et al., 1998)。在過去的文獻中指出：正常細胞中，arginine 甲基轉移酶是以大約 275-450 kDa 的分子量大小存在 (Rawal et al., 1994；Ghosh et al.,

1988；Najbauer et al., 1993；Liu et al., 1995；Tang et al., 1998)，然而計算 PRMT1 和 PRMT3 的胺基酸序列，得其分子量分別為 40.5 kDa 和 59.4 kDa，猜測在一般生理狀況下，甲基轉移酶是以較大分子量的複合體存在 (Tang et al., 1998)。Kim et al.由 rat liver cytosol 中純化出的蛋白 arginine 甲基轉移酶中進一步發現其多胜肽的序列與 10-formyltetrahydrofolate dehydrogenase 相同，並且在 assay 到精胺酸甲基轉移酶活性的同時也能 assay 到 10-formyltetrahydrofolate dehydrogenase 的活性 (Kim et al., 1998)，因此甲基轉移酶可會以不同的酵素形式存在不同的細胞或胞器中。

2. 關於甲基接受蛋白

Heterogeneous nuclear ribonucleoproteins (hnRNPs) 最先是由脊椎動物細胞的 nuclei 中分離出來，為一種 RNA 結合蛋白，它會出現在 nascent polymerase II transcripts 的 mRNA processing 和 nuclear transport 等過程中，其中 hnRNP A1 是由 320 個胺基酸組成，包含了兩個 N 端的 RNA Recognition Motifs (RRMs) 和一個 C 端的 arginine-glycine-rich domain 簡稱 RGG box (Dreyfuss et al., 1993)，除了 RRMs 會與 RNA 結合外，RGG box 則會調控蛋白、RNA 的相互作用 (Kiledjian & Dreyfuss, 1992；Henry et al., 1996)。hnRNPs 在 RNA metabolism 的功

能會藉由一些轉譯後的修飾作用來調控，目前已經發現許多 hnRNPs 會被磷酸化 (e. g., Cobianchi et al., 1993；Pinol-Roma & Dreyfuss, 1993)、醣化 (Soulard et al., 1993) 或甲基化 (e. g., Liu & Dreyfuss, 1995)，實際上在細胞 nucleus 中，hnRNPs 包含了 65%被甲基化的 arginine (Boffa et al., 1997)，且是發生在 hnRNPs RGG boxes 上的 arginine 並形成 asymmetric N^G,N^G-dimethylarginine (Rajpurohit et al., 1992；Kim et al., 1997)，我們已知 RGG box 會調控 RNA 結合蛋白與 RNA 結合的活性，那麼發生在 RGG box 的 arginine methylation 是否會影響這些蛋白與 RNA 結合的活性?一篇在一九九四年發表的文獻中指出甲基化可能會降低 RNA 結合蛋白與單股核酸的結合，蛋白的穩定性也會降低 (Rajpurohit et al., 1994)。然而 Valentini et al 在一九九九年發表的文獻中提出：Hrp1p 的甲基化與否並不會影響其與 RNA 結合的活性，Hrp1p 是 yeast 的一種 hnRNP，作用於 mRNA 3'端的 cleavage 和 polyadenylation，它會與參與 polyadenylation 的蛋白結合，Valentini et al.認為甲基化可能會調控 Hrp1p 與這些蛋白或其它與 nuclear export 有關蛋白的相互作用(Valentini et al., 1999)。而 yeast enzyme Hmt1p (hnRNP methyltransferase) (Gary et al., 1996；Henry & Silver, 1996)，在 in vitro 的條件下會將 Npl3p、Hrp1p 和 hnRNP A1 甲基化 (Henry & Silver, 1996；Shen et al., 1998)，Npl3p 和 Hrp1p 兩

個蛋白都是 yeast hnRNP-like protein，當 yeast 缺乏 *HMT1* gene 時，Npl3p 和 Hrp1p 便不能移出 nucleus，顯示甲基化對這些 RNA 結合蛋白 nuclear export 功能的重要性 (Shen et al., 1998)。除了 hnRNPs 以外還有幾種其他的 RNA 結合蛋白，其 arginine 和 glycine rich motifs 的 arginine 胺基酸都會被甲基化形成 N^G-monomethylarginine 和 asymmetric N^G,N^G-dimethylarginine residues (Liu et al., 1985 ; Najbauer 1993 et al., ；Aris and Blobel, 1991 ; Lapeyre et al., 1985 ; Christensen et al., 1988)，包括 U3 small nuclear RNA-binding protein fibrillarin (Lischwe et al., 1985a)、pre-rRNA-binding protein nucleolin (Lischwe et al., 1985b) 等。早在一九八五年便定義 nucleolar scleroderma antigen 具 4.1 mol%的 N^G,N^G-dimethylarginine 以及 22.8 mol% glycine，之後發現這個蛋白應存在 nucleolus 中屬於 small nuclear RNP，因為藉由 scleroderma 病人血清中的抗體做免疫螢光染色，發現此蛋白存在 vertebrate nucleoli 的 dense fibrillar component 中，便定名為 fibrillarin，同時發現這個蛋白的分子量為 34 kDa 且在 N 端 31 個胺基酸中具有 6 個 dimethylarginine (DMA) 和 16 個 glycine。fibrillarin 會參與 ribosome synthesis：pre-rRNA processing、pre-rRNA modification 和 ribosome assembly (Tollervey et al., 1993)，關於這個蛋白在 cell cycle 不同階段的分佈等，自發現這個蛋白以來就有許多研究結果報告(e. g.,

Azum-Gelade et al., 1994)，但關於這個蛋白的甲基化至今並無進一步的探討，因此我們便著手分析 fibrillarin 的甲基化並進一步以 fibrillarin 為受質研究精胺酸的甲基化 (arginine mthylation)。

Adenosine dialdehyde (AdOx) 為一種 S-adenosylhomocysteine (AdoHcy) hydrolase 的抑制劑，當 S-adenosylhomocysteine (AdoHcy) hydrolase 被抑制，細胞內 AdoHcy 也就跟著增加 (Johnson et al., 1993 ; O'Dea et al., 1987)，進而抑制甲基轉移酶的活性 (圖二)，所以細胞經此藥物處理後可抑制細胞內蛋白質的甲基化作用而使細胞內大部份甲基接受蛋白呈低甲基化狀態，而這些低甲基化的甲基接受蛋白可藉由與甲基的提供者 S-adenosyl [*methyl*-^{3}H] L-methionine 的 incubation 被內生性或外生性甲基轉移酶標定，過去這個實驗在老鼠 pheochromocytoma (PC12) 細胞已有相當的研究，實驗發現：經過 AdOx 處理的 PC12 cells 大約有 90%蛋白甲基化的型式為 N^G-monomethylarginine 和不對稱型 (asymmetric) N^G,N^G-dimethylarginine (Najbauer and Aswad, 1990 ; Najbauer et al., 1993)。我們則選用了容易取得的淋巴母細胞，也用 AdOx 處理，以觀察淋巴母細胞內的甲基化。

材料與方法

材料:

GST-protein arginine *N*-Methyltransferase 表達的質體由美國加州大學 Dr. Steven Clarke 提供。

Lymphoblastoid 細胞株 003 由中山醫學院生命科學系主任李宣佑教授提供。

Fibrillarin 蛋白、表達的質體、單株抗體由 Dr. Michael Pollard at Scripps Institute (La Jolla, California, USA) 提供。

方法:

一、 專一性甲基轉移酶的表現與純化:

將 Dr. Clarke 提供的 *GST-PRMT1* (Glutathione S-Transferase-Protein Arginine Methyltransferase)或 *GST-RMT1* 質體送入大腸桿菌菌株 DH5α 內，將其塗抹在含有抗生素 (100 μg/ml ampicillin) 的 Luria-Bertani 培養基(LB agar)中，於 37℃培養箱中培養 12-16 小時，然後挑單一菌株到含抗生素 (100 μg/ml ampicillin) 的 2 ml Luria-Bertani

培養液 (LB medium) 中，同樣於 37℃培養 12-16 小時。最後以 1：100 的稀釋到新鮮的 LB 培養液 (100 μg/ml ampicillin) 中培養，直到菌液生長的濃度達到 OD_{600} 約 0.6-0.7 左右，加入 isopropyl-β-D-thiogalactopyranoside (IPTG) 使最後濃度為 1 mM 以誘導蛋白的表現，經過 37℃培養箱中培養五個小時後，取出菌液並將菌液以 10000g 離心 10 分鐘，離下的菌體以滅過菌的 phosphate-buffered saline (10 mM dibasic sodium phosphate ; 1.8 mM monobasic potassium phosphate ; 140 mM NaCl ; 2.7 mM KCl pH7.4) 洗掉殘留的培養液，再將 pellete 懸浮於 cell extract buffer (PBS ; 5% glycerol ; 1 mM sodium EDTA ; 1 mM sodium EGTA ; 1 mM dithiothreitol ; 40 μg/ml leupeptin ; 40 μg/ml aprotinin ; 20 μg/ml pepstatin ; 1 mM PMSF ; 0.5% Triton X-100)中，以超音波震盪器連續震盪 15 秒，再置於冰上 1 分鐘，重複 4 次將細胞震碎，直到菌液澄清為止。

取 1ml Glutathione-Sepharose 4B resin (Parmacia) 置入管柱中，並且加入 10 ml PBS 平衡管柱，然後將溶在 cell extract buffer 的菌體在 4℃下以 12000 g 離心 20 分鐘後，將其上清液過 0.45 μm 的過濾器，過濾液再通過已用 PBS 平衡過的 Glutathione-Sepharose 4B column，收取通過管柱後的液體 (Flow through)，接著以 10 ml 的 PBS 沖洗 column，最後加入 5 ml 的 Elution buffer (10 mM glutathione ; 50 mM

Tris-HCl pH8.0) 將結和在 Glutathione-Sepharose 的甲基轉移酶洗下來，每管收取 1 ml。

各取部分上清液以 12.5 % SDS-PAEG 分析，SDS-polyacrylamide gel 經由 Coomassie blue 染色以確定蛋白是否被 Glutathione-Sepharose 4B resin 純化出來，至於酵素活性則以甲基化實驗來偵測之。

二、 Fibrillarin 蛋白的表現與純化：

將 Dr. Michael Pollard 提供的 fibrillarin 蛋白表現質體送入大腸桿菌菌株 JM109 (DE3) (Promega) 內，將其塗抹在含有抗生素 (50 μg/ml Kanamycin) 的 Luria-Bertani 培養基(LB agar)中，於 37℃培養箱中培養 12-16 小時，然後挑單一菌株到含抗生素 (50 μg/ml Kanamycin) 的 2 ml Luria-Bertani 培養液 (LB medium) 中，同樣於 37℃培養 12-16 小時。最後以 1:100 的稀釋到新鮮的 LB 培養液 (50 μg/ml Kanamycin) 中以 25℃培養，直到菌液生長的濃度達到 OD_{600} 約 0.8 左右，加入 isopropyl-β-D-thiogalactopyranoside (IPTG) 使最後濃度為 1 mM 以誘導蛋白的表現，經過 25℃二十四個小時的誘導後，取出菌液並將菌液以 10000g 離心 10 分鐘，離下的菌體以滅過菌的 phosphate-buffered saline (10 mM dibasic sodium phosphate ; 1.8 mM monobasic potassium

phosphate ; 140 mM NaCl ; 2.7 mM KCl pH7.4) 洗掉殘留的培養液，再將 pellete 懸浮於 6 ml 的 buffer A (6 M Guanidine-HCl ; 0.1 M NaH_2PO_4 ; 0.01 M Tris-HCl pH8.0 ; 10 mM 2-mercaptoethanol ; 1 mM PMSF)，菌體將藉由在 25℃搖動一個小時而被溶解，再將經過 10, 000g 離心十分鐘後的上清液與 2 ml Ni-NTA agarose (Qiagen) 於 4℃轉動混合一個小時後 transfer 至管柱中。

待上清液與 2 ml Ni-NTA agarose 在管柱中靜置約 30-60 分鐘後，開始以每分鐘 0.5 ml 的流速收取通過管柱後的液體(flow through)，再利用含有不同 pH 值的 buffer (8 M Urea ; 0.1 M NaH_2PO_4 ; 0.01 M Tris-HCl ; 10 mM 2-mercaptoethanol ; 10 %(v/v) glycerol) 來清洗管柱，收取清洗管柱後的液體 (Wash 1 : pH8.0 ; Wash 2 : pH6.3 ; Wash 3 : pH5.9) 直到其 OD_{280} 低於 0.05。

最後以 pH4.5 的 buffer (8 M Urea ; 0.1 M NaH_2PO_4 ; 0.01 M Tris-HCl ; 10 mM 2-mercaptoethanol ; 10 %(v/v) glycerol)，將結合在 Ni-NTA resin 的 fibrillarin 蛋白洗下來，每 1 ml 收一管直到其 OD_{280} 低於 0.05。將收下來的 elute protein 分別取 10 μl，利用 12.5 % SDS PAGE 進行電泳分析，再以 Coomassie blue 染色方法來確定 fibrillarin 蛋白是否被 Ni-NTA resin 純化出來。整個純化過程在室溫下進行。

三、 SDS-聚丙烯醯板膠電泳法：

要分析的蛋白樣品加入 3× sample buffer 之後以 95℃煮沸 5 分鐘使蛋白變性，再 load 到使用 Laemmli (1970) 的緩衝溶液系統之膠體中 (0.75 mm x 7 cm resolving gel)，12.5% SDS-PAGE 之配方如下：

	Resolving gel	**Stacking gel**
Acrylamide-bisacrylamide (29:1)	3.125 ml	0.562 ml
1.5 M Tris-Hcl pH8.8	2.5 ml	
0.5 M Tris-Hcl pH6.8		1.25 ml
10% SDS	0.1 ml	0.05 ml
10% ammonium persulfate	75 μl	15 μl
TEMED	15 μl	4 μl
D_2H_2O	5 ml	3.188 ml

，running condition 為 100V、兩個小時 (BioRad；Mini-PROTEAN II)。最後將 SDS-PAGE 電泳分析之膠體浸泡於染色液 (0.1% Coomassie blue R 250 溶於 d2H2O : methanol : acetic acid = 6 : 3 : 1 之混合劑中) 一小時以上，再以去色液 (30% methanol : 10% acetic acid) 去色。

四、 西方點墨法：

將 SDS-PAGE 電泳分析之膠體、nitrocellulose paper (GelmanSciences)及濾紙，以三明治方法夾於 transfer cassette 中，置於 mini Trans-Blot Cell (Bio-Rad) 加入 transfer buffer (12 mM Tris pH 8.3 ; 96 mM Glycine ; 20 % Methanol) 中，以電流 100 伏特進行 1 小時或 30 伏特過夜。轉漬後的 membrane 以含 1% BSA 或含 5% 脫脂奶粉的 TTBS buffer (20 mM Tris-HCl pH 7.5 ; 150 mM NaCl ; 0.05% Tween 20)進行 blocking，即室溫下溫和搖晃 30 分鐘。倒出 blocking buffer，加入含有第一次抗體 (mouse monoclone anti-FMRP/1:5000 與 β-actin/1:200000 或 human polyclone anti-fibrillarin/1:1000) 的 TTBS buffer，於室溫下搖晃一個小時後倒出第一次抗體，以 TTBS buffer 洗 5 分鐘，重覆三次，然後加入含有第二次抗體 Anti-mouse IgG peroxidase conjugate /1:20000 或 anti-human IgG peroxidase conjugate /1:200000 的 TTBS Buffer，於室溫下搖晃 1 小時。倒出第二次抗體，以 TTBS buffer 洗 5 分鐘，重覆三次，然後加入 1:1 的 Working solution (Luminol/Enhancer solution: stable peroxide solution; Pierce)，於室溫下搖晃 5-10 分鐘，倒掉 Working solution，將 membrane 置於 X 光片下曝光，曝光時間依訊號強弱而定。

五、 淋巴母細胞株的培養：

淋巴母細胞株(Lymphoblastiod cell)培養於含培養液(10% FBS-RPMI1640-PSN-glutamine)的 25 cm^2 培養瓶中，將培養瓶置於 37℃，5 %二氧化碳培養箱。待細胞長滿時，更換新的培養液並加入 20μM 的甲基化抑制劑 AdOx (adenosine, periodate oxidized; Sigma)，同樣置於 37℃，5 %二氧化碳培養箱，待 72 小時後於 1500 rpm 離心 10 分鐘，將細胞收下來，用 PBS 洗二次後放置-80℃備用。

六、 淋巴母細胞質萃取液的製備：

待培養在 25 cm^2 flask 的細胞長滿後（約 3-5×10^6 個細胞），以 1500rpm 離心 10 分鐘收細胞，倒掉培養液後以滅過菌的 1×PBS 洗掉殘留的培養液，再將 pellete 懸浮於 200-500 μl 的 cell extract buffer (PBS ; 5% glycerol ; 1 mM sodium EDTA ; 1 mM sodium EGTA ; 1 mM dithiothreitol ; 40 μg/ml leupeptin ; 40 μg/ml aprotinin ; 20 μg/ml pepstatin ; 1 mM PMSF ; 0.5% Triton X-100)中，以超音波震盪器連續震盪 20 秒，再置於冰上 1 分鐘，重複 3 次將細胞震碎，直到菌液澄清為止。最後在 4℃離心機以 12000 g 離心 20 分鐘，取上清液，保

存於-20℃

七、 Subcellular Fractionation :

將 3-5×10^6 個細胞以 PBS 洗三次後加入 1 ml 的 Extraction buffer (10 mM Tris-HCl, pH 7.4 ; 150 mM NaCl ; 1.5 mM $MgCl_2$; 100 mM NaF ; 10 ng/ml aprotinin ; 1 mM phenylmethylsulfonyl fluoride ; 1 mM dithiothreitol/DTT ; 40 μg/ml leupeptin; 20 μg/ml pepstatin ; 0.5% NP-40)，混合均勻，置於冰水中 10 分鐘後過最小號的針頭 (22G×1¼ ; 0.7×32mm) 5-6 次，藉以打破細胞，再以 3000 g 離心 5 分鐘，此時的 pellete 即為 nuclear fraction，上清液則再以超高速 100,000g 離心 60 分鐘，所得 pellete 即為 ribosome fraction，上清液為 cytosolic fraction。整個萃取過程維持在 4℃，蛋白萃取後保存在-20℃，最後蛋白濃度再以 BCA protein assay (Pierce) 定量。

八、 蛋白濃度的測定 (BCA Protein Assay；Pierce)：

先準備不同稀釋倍數的 BSA standards (2 mg/ml) 與 sample 各 5 μl，並在各管加入 1 ml working reagent (BCA reagent A：BCA reagent B

= 50：1) 混和均勻，於 60℃ incubate 30 分鐘後，待試管溫度回到室溫，以光度比色儀測波長 562 nm 的相對析光值，將得到的數值扣除背景值，再與基準蛋白 BSA 的斜率做比較，就可換算出欲測定蛋白的量。

七、 Protein Methylation Reaction (in vitro) :

為了觀察淋巴母細胞內的甲基化，基本上我們以等量的 Lymphoblastoid cell extract (30 μg) 加入 GST-RMT 酵素 (25 μg) 、0.75 μCi S-adenosyl-L- [*methyl*-^{3}H]methionine (Amersham 60 Ci/mmol) 及 reaction buffer (25 mM Tris pH7.5 ; 1 mM EDTA ; 1 mM EGTA) 的 reaction mixture 中以 30℃反應一個小時，Lymphoblastoid cell fractions 中可接受甲基的蛋白將被淋巴母細胞內生性甲基轉移酶或 GST-RMT 甲基化。整個反應在加入 3×SDS sample buffer 後終止，sample 以 95℃煮沸 5 分鐘並利用 12.5% SDS-PAGE 進行電泳分析，polyacrylamide gel 在電泳後以 Coomassie blue 染色一小時，接著去染液退染一小時，最後再將膠體浸泡於 EN^{3}HANCE (Du Pont NEN)中室溫下緩慢搖動一小時，乾膠後在-80℃下顯影在 X 光片 (Kodak, MS) 七天。另外在 fibrillarin competition 實驗中分別加入 0 μg 、5 μg、10 μg 及 20 μg

的 fibrillarin 於各反應中做競爭反應。或以等體積 25% TCA 將反應後的產物沉澱，在加 6 N HCl 水解後，以 HPLC 分析 (方法見 HPLC 胺基酸分析)。

九、 甲基化胺基酸的分析：

甲基化以後的產物以等體積 25% TCA 沉澱，再以-20℃的 Acetone wash、air dry，加 10 μl ddH_2O，轉移至胺基酸水解專用的玻璃管 (6×50 mm；KIMBLE) 中，真空抽乾，接著將 sample tube 置入加了 6 N HCl 的反應瓶中，於 105℃的烘箱抽真空 24 小時進行酸水解，水解後的產物加入 20 μl derivatization reagent (ethanol：ddH_2O：TEA：PITC = 7：1：1：1) 在室溫下靜置 20 分鐘後真空抽乾，保存於-20℃，待進行 HPLC 分析前再加入 200 μl sample diluent (Na_2HPO_4 pH 7.4；5% Acetonitrile)。sample 取 100 μl 以 C18 管柱 (PICO • TAG Amino Acid Analysis Column；3.9×150 mm；Waters) 分離，gradient 為：

Time (min)	Flow rate (ml/min)	%A	%B
Initial	1	100	0
5	1	87	13

10	1	75	25
15	1	67	33
20	1	63	37
25	1	61	39
30	1	60	40
31	1	0	100
40	1	100	0
45	1	100	0

註 1：Eluent A：8.225 mM sodium acetate；0.047% triethylamine pH 6.4；8.2266% acetonitrile

Eluent B：60% acetonitrile

註 2：PITC (Phenylisothiocyanate；Pierce)

以紫外光波長 254 來偵測，並由 0 分鐘開始每 15 秒收一管，共收 30 分鐘，所有收下來的 fraction 各取 100 μl 加入含 2 ml Scintillant solution (BCA；Amersham) 的 5ml Scintillation vial 中，以液體閃爍計畫數儀 (TRI-CARB 2100TR Liquid Scintillation Analyzer；PACKARD) 偵測放射線含量。

結果

第一部分　淋巴母細胞甲基化的觀察

一、 淋巴母細胞萃取液在有無AdOx處理的培養環境下，甲基接受蛋白的變化：

為了直接觀察甲基接受蛋白在淋巴母細胞萃取液中的存在，我們將細胞萃取液與甲基donor (S-adencsyl-L-[*methyl*-^{3}H]methionine) 一同在30℃反應，進一步藉由SDS-PAGE和fluorography分析。當細胞不以AdOx處理且反應中沒有外加外生性甲基轉移酶時，X光片上偵測不到任何的訊號 (圖三：lane 1)，相反的若在反應中加入yeast RMT1，我們發現至少有十個以上的甲基接受蛋白被偵測到(圖三：lane 2)，既然這些多胜肽在lane 1中並沒有出現，顯示這些受質雖不是內生性甲基轉移酶的受質卻能夠被yeast RMT1辨識，尤其是12.5% SDS-PAGE膠上，分子量小於14 kDa的位置有很強的訊號，表示yeast RMT1的受質主要是一些小分子量多胜肽。而lane 4同一位置也有一個很強的訊號，它是細胞以AdOx處理、甲基化反應加入外生性甲基轉移酶的結果，這個結果指出了此小分子量甲基接受分子為yeast

RMT而非內生性甲基轉移酶所辨識，且不論細胞有沒有以AdOx處理，它都是呈低甲基化的狀態。

當細胞生長在含AdOx的培養環境中所取得的細胞萃取液，在不含yeast RMT的甲基化反應中，有許多甲基接受蛋白在30,000-90,000 Da之間會被偵測到 (lane 3)，比較lane 1和lane 4可進一步證實當淋巴母細胞生長在含AdOx的培養環境中會造成許多低甲基化甲基接受蛋白的累積，且可因此in vitro的實驗中進一步被甲基化，然而我們並不能完全排除當淋巴母細胞生長在含AdOx的培養環境中會刺激活化內生性甲基轉移酶活性的可能性。當GST-RMT fusion protein參與甲基化反應 (lane 4)，則甲基化的pattern會與lane 3非常類似，只有分子量為32,000 Da的多胜肽在GST-RMT fusion protein參與甲基化反應時會產生八倍強的甲基化pattern，其它在50 kDa-100 kDa以及小於29 kDa位置的多胜肽則因為外加了yeast RMT1而變得更明顯易見，可見這些甲基接受蛋白應該是被yeast RMT1而非內生性甲基轉移酶甲基化。

二、 RGG peptide可成功競爭以AdOx處理的淋巴母細胞之內生性甲基轉移酶主要甲基接受蛋白的甲基化：

先前在PC12 cell的實驗中指出 ：RNA結合蛋白RGG box的*N*-methylation是主要base-stable甲基化的形式，這樣的甲基化可因細胞在含AdOx培養液中培養而被抑制 (Najbauer et al., 1993)。我們猜測在淋巴母細胞所觀察到大部分的甲基化應是發生在RGG box的arginine residues，因此我們合成一段出自fibrillarin和fragile X mental retardation (FMR-1) 基因產物 (FMRP ; Verkerk et al., 1991 ; Warren et al., 1995) 序列為GGRGRGGGF的peptide R9，這個序列屬於典型RGG box的序列，可被rat或mouse brain extract的*N*-arginine 甲基轉移酶或recombinant yeast RMT1甲基化行形成N^G-methylarginine (Ai et al., submitted)，如果經AdOx處理的淋巴母細胞其甲基接受蛋白也有相似的RGG domain則R9可能就會跟這些不論是內生性或外生性甲基轉移酶的`低甲基化受質競爭，因此我們在經AdOx處理、淋巴母細胞萃取液的甲基化反應中 (in vitro) 加入0、10、100和1000 μM的R9 peptide，結果再以SDS-PAGE和fluorography分析。如圖四：10 μM的R9 peptide即可競爭由經AdOx處理、淋巴母細胞萃取液的內生性甲基轉移酶催化的甲基化反應 (lane 2)，當加入1000 μM的R9 peptide時，多數多胜肽仍會被甲基化但intensity大大降低 (lane 4)，隨著R9濃度的增加，不同多胜肽甲基化降低的程度也不同，利用densitometry的分析結果顯示：32 kDa多胜肽的訊號隨著R9濃度的增加由lane 2的100降至33.7

最後降到13.3%，而60 kDa多胜肽的訊號則隨著R9濃度的增加同樣由lane 2的100降至64.2最後降到19.8%，另一個peptide K9是將R9所有的arginine residues替換成lysine residues，它競爭淋巴母細胞甲基化的能力，必須要在反應中加到1000 μM的濃度才看得到 (lanes 5-7)，R9和K9兩種peptide競爭多胜肽甲基化能力的不同，顯示出應該是R9具有與RGG box相似的序列所產生的競爭因而降低甲基化的程度，而RGG box的arginine residue應該就是甲基化主要的位置。

三、 RGG peptide競爭掉以AdOx處理的淋巴母細胞之外生性GST-RMT1甲基轉移酶主要甲基接受蛋白的甲基化：

接下來我們在以AdOx處理的淋巴母細胞萃取液、in vitro甲基化的反應中加入外生性GST-RMT1甲基轉移酶和R9 peptide。如圖五，甲基化多胜肽的訊號隨著R9 peptide濃度升高而減弱 (lanes 1-4)，分子量大約在32 kDa和50 kDa的多胜肽被甲基化的程度因為外加了yeast RMT1而增強，但60 kDa的多胜肽被甲基化的程度經densitometry的分析，並沒有因為外加了yeast RMT1而增強 (圖五)，而這個60 kDa多胜肽的甲基化較其它兩個多胜肽易被競爭，但大致上來說，這三個多胜肽都隨著R9 peptide濃度10 μM、100 μM和1000 μM增加，訊

號跟著降低，我們若以lane 2的訊號為100%，則訊號降至50-70、30-50甚至低於10%，另外K9即使在濃度加至1000 μM，也不見任何競爭反應 (圖五 lane 5-7)，有趣的是，我們發現有R9 peptide參與甲基化的幾個反應中 (lane2-4) ，非常接近底部的訊號似乎隨著R9 peptide濃度的增加變得越來越強，它們可能就是R9 peptide被甲基化的訊號，再與圖四比較：發現當反應中不含yeast RMT1時，這個訊號則減弱許多，所以這個peptide的甲基化可能主要就是經由yeast RMT1而非內生性甲基轉移酶所催化。甲基化的反應在R9 peptide濃度加到1000 μM時 (lane 4)，被甲基化的胜肽，其訊號並不比 lane 1沒有peptide參與競爭時的平均訊號來得強，因此，在高濃度peptide存在的反應中，甲基化的降低應該是因特異的競爭反應而非高濃度的甲基接受胜肽成功競爭了放射線標定的AdOxMet。

第二部分　淋巴母細胞不同subcellular fraction甲基化的觀察

一、淋巴母細胞不同subcellular fraction中，為yeast RMT1所辨識之甲基接受蛋白的分佈具特異性：

為了進一步鑑定甲基接受蛋白，我們以不同的離心力將淋巴母細胞的萃取液分為nucleus、ribosomal和cytosolic fraction (Corbin et al., 1997)。在把這些材料拿來做實驗前，由於FMRP主要會與核醣體結合而fibrillarin為nuclear protein，所以我們先利用anti-fibrillarin和anti-FMRP做西方點墨法，結果除了nuclear fraction有一點點訊號外，FMRP的確出現在ribosome fraction，至於fibrillarin則出現在nuclear fraction (圖六)，因此確立fraction分離的效果。分離好的subcellular fraction即可與甲基donor (S-adencsyl-L-[*methyl*-^{3}H]methionine) 一同在30℃反應，為了直接觀察甲基接受蛋白的分佈，我們進一步以SDS-PAGE分析然後壓片觀察。當細胞不以AdOx處理且反應中沒有外加外生性甲基轉移酶時，X光片上三個fraction幾乎都偵測不到任何的訊號 (圖七：lane 1, lane 3, lane 5)，這個結果與第一部分total extract的實驗結果符合，只除了ribosomal fraction和cytosolic fraction中大約60 kDa的

多胜肽被甲基化 (lane 3, lane 5)。相反的若在反應中加入yeast RMT1，我們發現在不同的fraction中都有顯著的甲基化pattern被偵測到 (圖七：lane 2, lane 4, lane 6)，這樣的結果再次證實了yeast RMT的受質似乎較廣範存在真核細胞中，值得注意的是：有些為yeast RMT1所辨識的甲基接受蛋白，只特異的出現在特定的fraction中，像是nuclear fraction (lane 2)，小於19.4 kDa的位置就有很強的訊號，其他尚有20個以上的多胜肽在30-100 kDa的位置被偵測到，ribosomal fraction (lane 4) 則有四個最強的訊號位於15、20、25和32 kDa的位置，其餘還有十幾個甲基接受蛋白被偵測到，而曾在lane 3出現、大約60 kDa的多胜肽被甲基化的訊號則因為這個外生性的yeast RMT1的作用變得更強，至於cytosolic fraction (lane 6) 雖然沒有像nuclear fraction、ribosomal fraction一樣具有特別強的訊號，但仍有超過20個甲基接受蛋白被偵測到。

二、 淋巴母細胞不同subcellular fraction中甲基轉移酶的活性：

為了觀察甲基轉移酶在淋巴母細胞不同subcellular fraction中的活性，我們在細胞培養時以最終濃度為20 μM的AdOx處理72小時，AdOx

會間接抑制甲基轉移酶的活性，這樣一來淋巴母細胞內的甲基接受蛋白都會呈低甲基化的狀態，這麼一來我們就可以用來觀察淋巴母細胞中不同fraction內生性甲基轉移酶的活性。根據In vitro甲基化反應的結果顯示: 不同fraction中都具有甲基轉移酶的活性，在不加入外生性甲基轉移酶的條件下，X光片大約30 kDa-90 kDa的位置，不同subcellular fraction中都出現至少20個相似的訊號 (圖八：lane 1, lane 3, lane 5)，只是強弱的差異，這些訊號會因為外加yeast RMT而變得更強 (圖八：lane 2, lane 4, lane 6)，顯示內生性甲基轉移酶與yeast RMT所辨認的多胜肽是相似的。很明顯的，在不加入yeast RMT時，nucleus fraction中所偵測到的甲基接受蛋白，比起ribosomal和cytosolic fraction則少了許多，這可能表示內生性甲基轉移酶的活性在ribosomal和cytosolic fraction中較好，顯示核內甲基接受蛋白被內生性甲基轉移酶甲基化飽和程度最低。然而如果在甲基化的反應加入外生性甲基轉移酶 (圖八：lane 2, lane 4, lane 6)，則三個不同的fraction都出現許多原本不被內生性甲基轉移酶辨識的甲基接受蛋白的訊號。

三、 Recombinant fibrillarin競爭掉以AdOx處理的淋巴母細胞之內生性甲基轉移酶主要甲基接受蛋白的甲基化：

第一部分已經證實淋巴母細胞內的甲基接受蛋白多數應具有與RGG motif相似序列的區域，但當我們將淋巴母細胞萃取液分為不同的fraction後，為了再次確認，我們又做了競爭反應的實驗，由於早在一九八五年就已經發現fibrillarin這個含RGG box的核蛋白會被甲基化形成 N^G-monomethylarginine 和不對稱型 (asymmetric) N^G,N^G-dimethylarginine residues，所以這次我們改將送入*E. coli* 表達的recombinant mouse fibrillarin，加入in vitro甲基化反應中取代R9 peptide做競爭，由於recombinant mouse fibrillarin是送入沒有arginine修飾作用的*E. coli*中表達，所以可作為一個很好的甲基接受蛋白。結果隨著fibrillarin 0、5、10、20 μg濃度漸增，除了fibrillarin本身的訊號漸強外，其餘ribosomal fraction甲基接受蛋白的訊號則被競爭而越來越弱(圖九)，顯示淋巴母細胞ribosomal fraction甲基接受蛋白的甲基化主要應是與fibrillarin相似的arginine *N*-methylation。

四、 淋巴母細胞中三個不同的subcellular fraction甲基化多胜肽的胺基酸分析：

即使我們已知recombinant fibrillarin會競爭ribosomal fraction的甲基化，但為了想更直接的証實發生在淋巴母細胞、不同subcellular

fraction的蛋白甲基化確屬arginine methylation，因此我們將每一個以AdOx處理的fraction、in vitro methylation後的產物沉澱，進行胺基酸分析 (Table 1)，結果顯示：在cytosolic fraction中，有69.8%的甲基化是形成 N^G,N^G-dimethylarginine (DMA)，14.1%是形成 N^G-monomethylarginine (MMA)。在ribosomal fraction中，有66.3%的甲基化是形成DMA，25.5%是形成MMA。nuclear fraction則有33.8%的甲基化是形成DMA，20.6%是形成MMA，可見發生在淋巴母細胞、不同subcellular fraction的蛋白甲基化主要是arginine methylation。比較特別的是nuclear fraction中另有39.4%的放射線活性出現在第20.5分鐘的fraction，這個peak並不曾出現在ribosomal fraction和cytosolic fraction中。

值得注意的是：DMA比MMA的比值在nuclear、ribosomal和cytosolic fraction中依序為1.6、2.6和5，顯示DMA與MMA在三個不同subcellular fraction的比例不同，而當我們在甲基化的反應中加入外生性甲基轉移酶 (yeast RMT-1) 時，經胺基酸分析的結果：DMA比MMA的比值在nuclear、ribosomal和cytosolic fraction中依序增為2.1、3.1和7.3，符合yeast RMT-1甲基化蛋白質主要為形成 N^G,N^G-dimethylarginine。

早在一九八五年Lischwe et al.即鑑定fibrillarin為具4.1 mol% N^G,N^G-dimethylarginine的甲基接受蛋白，我們則利用純化的recombinant protein methyltransferase在in vitro的條件下將fibrillarin甲基化後的產物進行胺基酸分析，結果recombinant fibrillarin被甲基化的形式有87.7%為DMA，11.9%為MMA，表示recombinant PRMT對fibrillarin的作用與當年Lischwe et al.以in vivo的實驗觀察Novikoff hepatoma cell甲基化的情形相符：甲基化的形式為N^G-monomethylarginine和N^G,N^G-dimethylarginine，而主要是形成N^G,N^G-dimethylarginine。

五、觀察淋巴母細胞不同subcellular fraction中酵素的活性：

根據先前的的實驗結果顯示：淋巴母細胞三個不同的fraction，在不加入外生性甲基轉移酶時仍可觀察到許多多胜肽被甲基化，可見淋巴母細胞三個不同的fraction都具有甲基轉移酶的活性，且ribosomal fraction的活性較好。已知fibrillarin在in vivo的反應中會被甲基化，因此我們將送到*E. coli*中表達的recombinant mouse fibrillarin protein以作為本實驗的甲基接受蛋白，參與淋巴母細胞不同fraction甲基化的反應，來觀察淋巴母細胞不同fraction中內生性酵素的活性，

此時的淋巴母細胞因為沒有以AdOx處理，所以其甲基接受蛋白已在生長過程中被甲基化，應不會干擾內生性甲基轉移酶與fibrillarin in vitro的甲基化反應。實驗結果發現：淋巴母細胞三個不同的fraction都具甲基轉移酶的活性可將fibrillarin甲基化（圖十），cytosolic fraction的活性最強。接著依序是ribosomal fraction、nucleus fraction。

討論

第一部分　淋巴母細胞甲基化的觀察

我們發現當人類淋巴母細胞在培養時加入甲基化的抑制劑AdOx，會導致細胞內*N*-arginine甲基轉移酶的甲基接受蛋白以低甲基化的狀態累積，因此對我們研究甲基接受蛋白提供了更好的機會。雖然此種機制在PC12 cell已被研究，但在其它種類的細胞尚沒有研究報告，PC12 cell中蛋白甲基化主要是發生在arginine residue形成N^G-monomethylarginine和不對稱型 (asymmetric) N^G,N^G-dimethylarginine residues，由histone/hnRNP arginine甲基轉移酶催化 (Najbauer et al., 1993 ; Rajpurohit et al., 1994)。先前的研究是以內生性甲基轉移酶作為酵素來源，偵測在相同的萃取液中的甲基接受蛋白；而人類淋巴母細胞的甲基化以AdOx抑制後，內生性甲基轉移酶催化低甲基化的甲基接受蛋白的活性也很強，如果細胞是在不含AdOx的培養液中培養，則在由內生性甲基轉移酶所催化的in vitro甲基化反應結果中偵測不到訊號 (或是只有很弱的訊號會被偵測到)，顯示這些內生性甲基轉移酶的甲基接受蛋白在細胞內是幾乎完全被甲基化的，而如果

細胞在培養時，培養液中AdOx阻礙多胜肽合成時的修飾作用，此時in vitro甲基化反應方可偵測到這些多胜肽。

N-arginine甲基轉移酶的catalytic subunit，不論是在rat、human或yeast，都已陸續被clone到 (Lin et al., 1996；Abramovich et al., 1997；Gray et al., 1996；Henry et al., 1996)，因此我們選用純化的recombinant methyltransferase去偵測淋巴母細胞內的甲基接受蛋白，又因為哺乳類細胞的活性可能會受其相互作用蛋白 (如TIS21) 的調控，且細胞內可能有一個以上的甲基轉移酶存在 (Lin et al., 1996)，因此選用yeast enzyme，反應應該可以較為穩定，酵素的選擇性也較低 (Gray et al., 1996)。有趣的是：即使是沒有以AdOx處理的細胞萃取液，還是有一些低分子量的多胜肽會被yeast GST fusion enzyme甲基化，這可能是細胞在不含AdOx的培養基培養時，細胞內的多胜肽沒有完全被甲基化或這些多胜肽會由yeast GST fusion enzyme而非淋巴母細胞內生性甲基轉移酶所辨識，顯示淋巴母細胞內生性甲基轉移酶之受質蛋白特異性高於yeast GST fusion enzyme。另一方面，在以AdOx處理的細胞萃取液中，甲基化的反應若外加yeast RMT並不會增加大部分淋巴母細胞內生性甲基轉移酶之受質蛋白甲基incorporation的程度，也就是說淋巴母細胞內生性甲基轉移酶在in vitro反應中能使以AdOx處理的低甲基化蛋白的甲基化幾乎達飽合。分子量大約32 kDa的多胜肽

為一例外：甲基化的反應若外加yeast RMT，則其訊號會增加八倍，且這個多胜肽的甲基化會被R9 peptide競爭。值得注意的是：這個訊號的分子量與已知是arginine甲基接受蛋白的hnRNP、fibrillarin相當，當然這也有可能是未知的蛋白，而這個蛋白是yeast RMT1一個很好的受質。

當我們以RGG peptide的競爭反應來鑑定這些在鹼性環境下穩定的淋巴母細胞甲基接受蛋白時，發現每一個多胜肽的甲基化會被R9 peptide而非arginine被lysine取代的K9 peptide競爭，顯示這些蛋白甲基化主要是發生在含有RGG box蛋白的arginine residues上。

我們相信，進一步分析甲基接受蛋白的特異性以及arginine甲基轉移酶活性的分佈與調控，能幫助了解為何這樣的修飾作用廣泛存在真核細胞的RGG蛋白。

第二部分　淋巴母細胞不同subcellular fraction甲基化的觀察

為了更進一步鑑定蛋白arginine甲基轉移酶的活性與甲基接受蛋白，我們以不同的離心力將淋巴母細胞的萃取液分為nucleus、ribosomal和cytosolic fraction (Corbin et al., 1997)。我們發現這個實驗所觀察到的結果大致上與第一部分、淋巴母細胞total extract甲基化的反應吻合：當細胞不以AdOx處理且反應中沒有外加外生性甲基轉移酶時，X光片上幾乎偵測不到任何的訊號，相反的若在反應中加入yeast RMT1，我們發現在不同的fraction中都有超過十個以上的甲基接受蛋白被偵測到，而這個in vitro分fraction的實驗則讓我們進一步觀察到：淋巴母細胞不同的subcellular fraction中，出現在X光片上的訊號分佈不盡相同，具一定程度的特異性，換句話說，三個不同的fraction中都存在某些為yeast RMT1所辨識的甲基接受蛋白。

當我們細以AdOx抑制淋巴母細胞內的甲基化，並取其萃取液進行in vitro甲基化的反應發現:在不加入外生性甲基轉移酶的條件下，不同subcellular fraction中都可偵測到許多甲基接受蛋白，顯示淋巴母細胞不同的subcellular fraction中都具有甲基轉移酶的活性，可辨認三個fraction中某些低甲基化的甲基接受蛋白，尤以ribosomal fraction中可被內生性甲基轉移酶所辨識的低甲基化蛋白更多。而當我們另以

recombinant fibrillarin為甲基接受蛋白時發現內生性甲基轉移酶的活性是cytosolic fraction > ribosomal fraction > nucleus fraction。至於淋巴母細胞不同的subcellular fraction所偵測到不同分子量的訊號，這顯示可能有不同的甲基接受蛋白分別存在淋巴母細胞不同的subcellular fraction中。如果在甲基化的反應加入外生性甲基轉移酶，則三個不同的fraction都出現一些原本不被內生性甲基轉移酶辨識的甲基接受蛋白的訊號，而以nucleus及cytosolic fraction更明顯，顯示這兩個fraction中存在有不少可為arginine甲基轉移酶所辨識的低甲基化蛋白，但其被原fraction內內生性甲基轉移酶甲基化的程度較低。為了探討發生在淋巴母細胞內的甲基化是否主要是arginine methylation，我們進一步對淋巴母細胞不同subcellular fraction的甲基化後的產物進行胺基酸分析的結果顯示：存在這三個fraction的甲基化仍是以arginine methylation為主，有趣的是其DMA與MMA所佔的比例在三個不同的fraction中各有差異，配合fluorography的分析結果，我們猜測在淋巴母細胞不同fraction中的1) 甲基接受蛋白不同，2) 甲基轉移酶具選擇性，造成甲基化形式在不同fraction中所佔比例不同。目前已知第一型甲基轉移酶、含RGG box的受質主要是核蛋白，某些還會在細胞核和細胞質中游走。免疫螢光染色分析指出PRMT1主要是位於nucleus而PRMT3主要是位於cytoplasm (Tang et al., 1998)。另外，第一型蛋

白arginine甲基轉移酶的活性主要是催化histone、recombinant hnRNP A1和其它核蛋白的甲基化形成N^G,N^G-dimethylarginine (Ghosh et al., 1988；Rawal et al., 1994；Liu et al., 1995)，在過去的文獻中指出：正常細胞中，arginine甲基轉移酶是以大約275-450 kDa的分子量大小存在 (Rawal et al., 1994；Ghosh et al., 1988；Najbauer et al., 1993；Liu et al., 1995；Tang et al., 1998)，然而計算PRMT1和PRMT3的胺基酸序列，得其分子量分別為40.5 kDa和59.4 kDa，猜測在一般生理狀況下，甲基轉移酶是以較大分子量的複合體存在 (Tang et al., 1998)，Kim et al.由rat liver cytosol中純化出的蛋白arginine甲基轉移酶中進一步發現其多胜肽的序列與過去已被鑑定的10-formyltetrahydrofolate dehydrogenase相同，並且在assay到精胺酸甲基轉移酶活性的同時也能assay到10-formyltetrahydrofolate dehydrogenase的活性 (Kim et al., 1998)，而我們的實驗結果顯示：淋巴母細胞不同subcellular fraction都具有甲基化fibrillarin的活性。綜合以上觀察，是否因為不同type的甲基轉移酶存在不同的fraction中，因而造成甲基接受蛋白的特異性，甚至不同fraction中，甲基轉移酶的活性不同？關於這點我們並不清楚，但相信甲基接受蛋白與甲基轉移酶的分佈與活性，一定存在著某種重要的調控。如果我們進一步鑑定特定的甲基接受蛋白在淋巴母細胞內的分佈及被甲基化的情形，再加上淋巴母細胞不同

subcellular fraction內生性甲基轉移酶活性的鑑定，便能幫助瞭解arginine methylation在淋巴母細胞內的作用機制。

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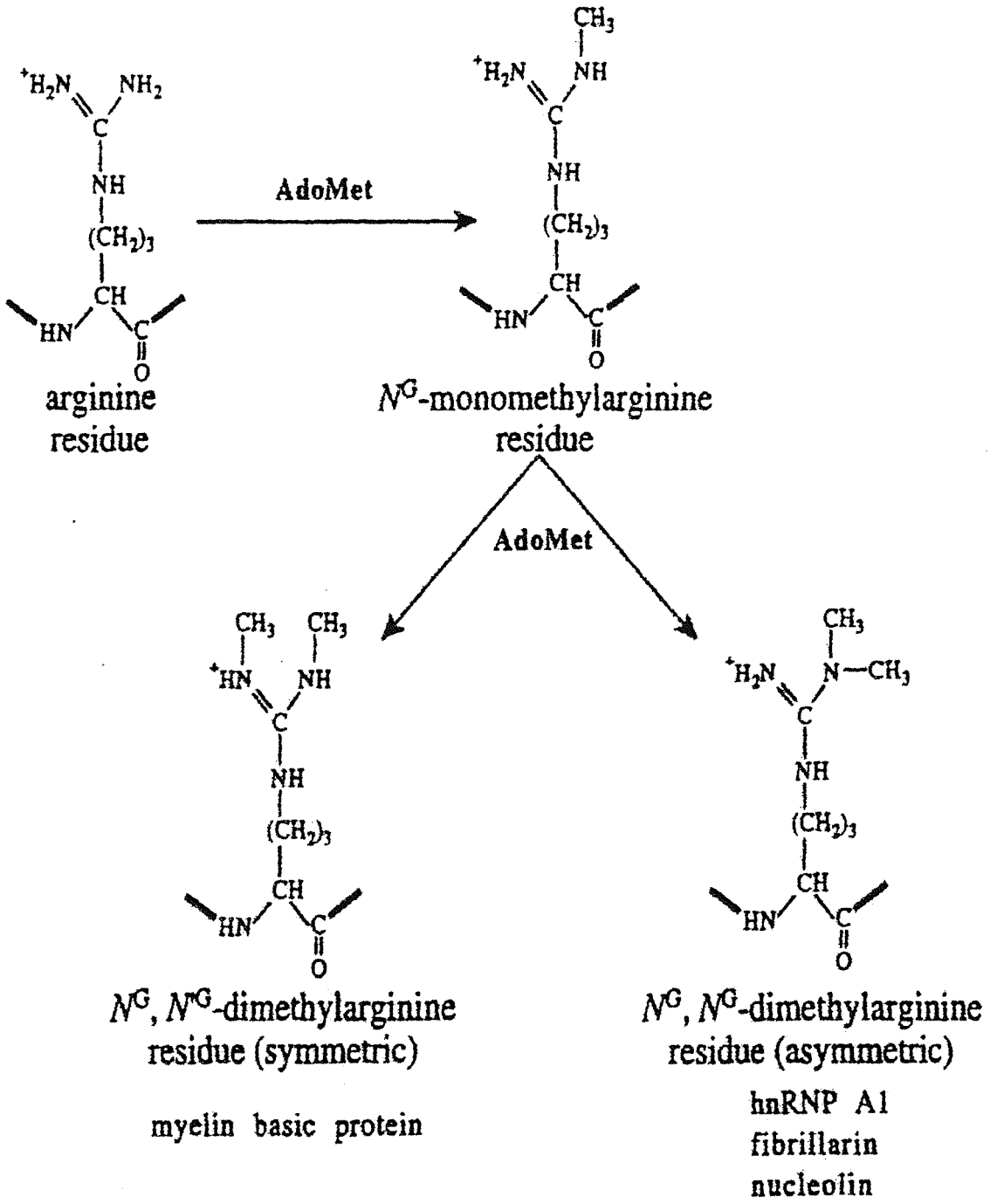
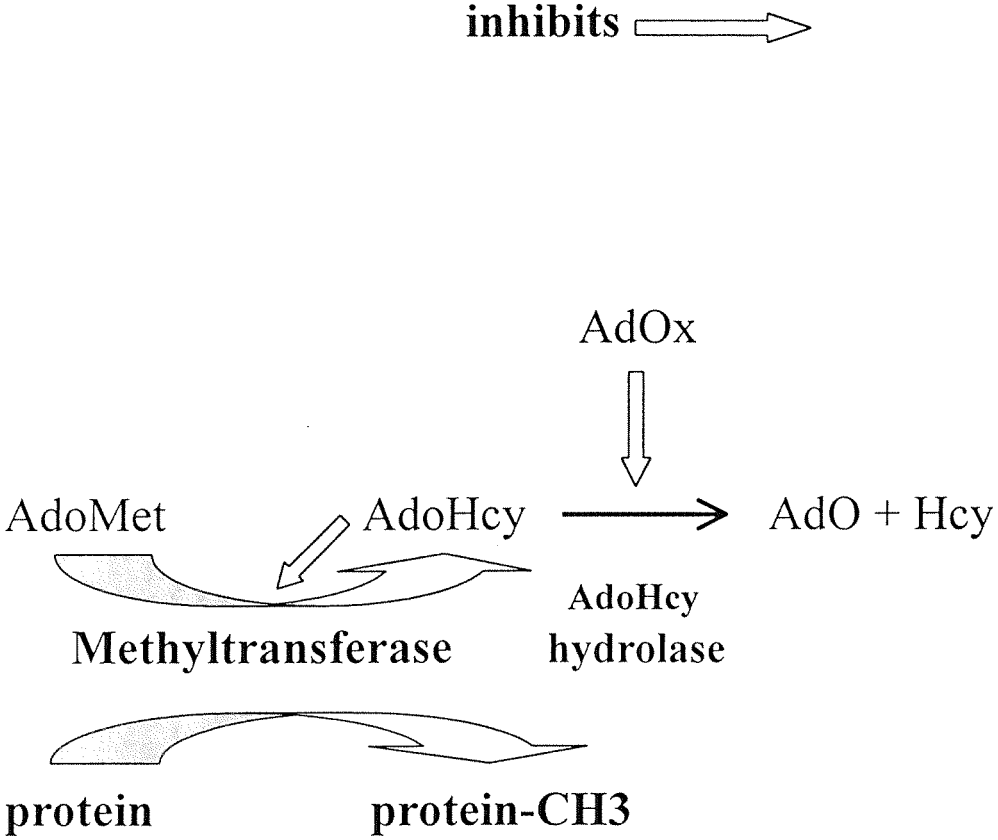


圖一　蛋白arginine甲基化之結構示意圖

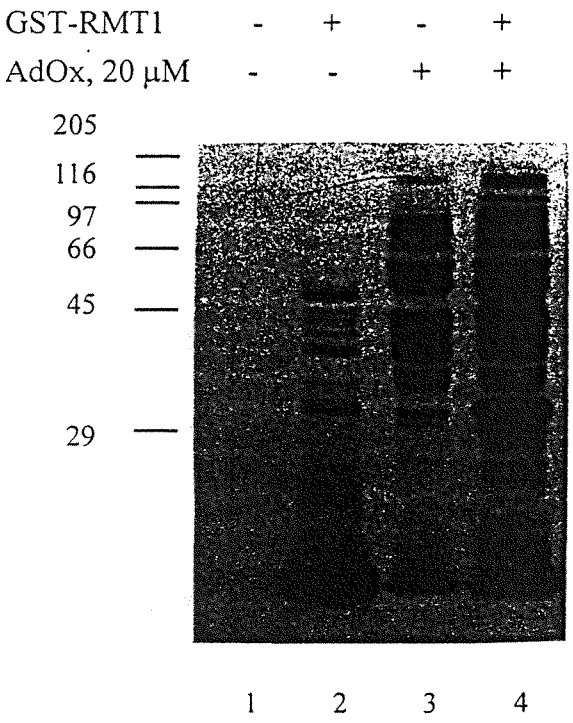


AdoMet : *S*-adenosyl-*L*-methionine

AdoHcy : *S*-adenosyl-*L*-homocysteine

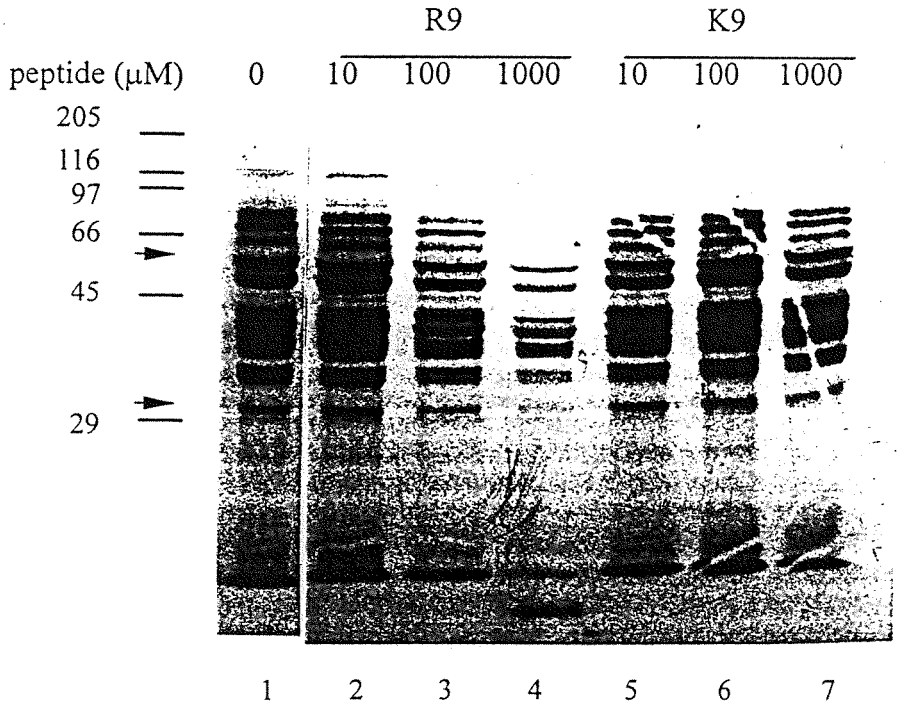
AdOx : adenosine dialdehyde

圖二、AdOx 抑制細胞內甲基化的作用機制



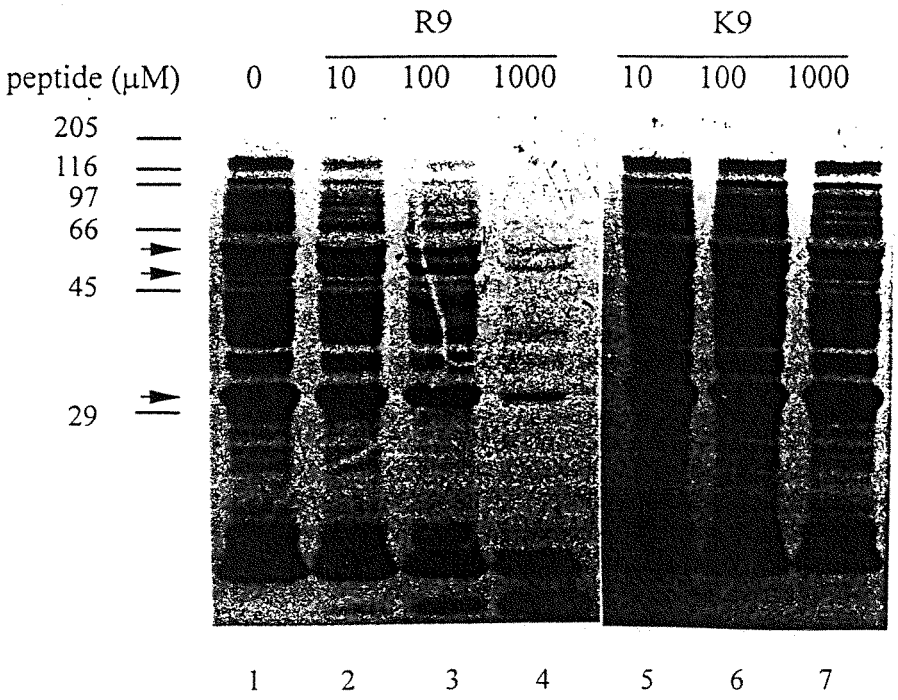
圖三、淋巴母細胞萃取液中甲基接受蛋白被甲基化的顯影分析

培養淋巴母細胞時，培養液中加入或不加入 AdOx 處理 72 小時後，製備細胞質萃取液並取濃度 25μg 的 total protein，加入 in vitro 甲基化反應中，藉由與 0.75μCi S-adenosyl-L-[*methyl*-^{3}H]methionine 於 30℃作用一小時，淋巴母細胞內的甲基接受蛋白即可被內生性甲基轉移酶或外加的外生性 GST-RMT1 標定 (參考材料與方法)，上圖是於-75℃顯影三天而得。



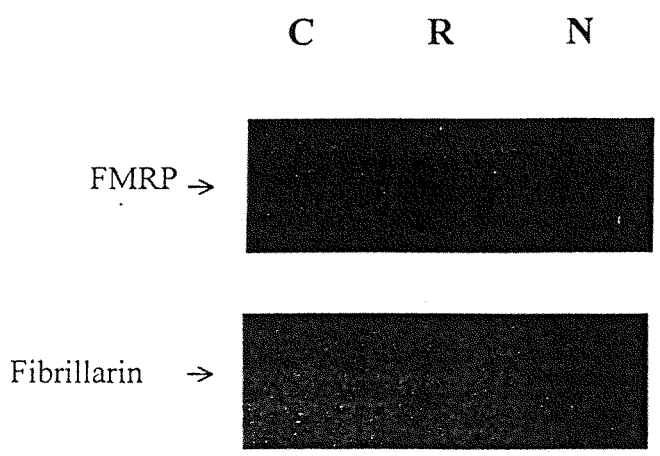
圖四、RGG peptide 競爭淋巴母細胞內生性甲基轉移酶催化的反應

除了外加 peptide R9 或 K9 (濃度如圖上標示)，內生性甲基轉移酶催化 in vitro 的甲基化反應方法同圖三，箭頭的標示則為文章中提出討論者。此結果是於-75℃顯影三天而得。



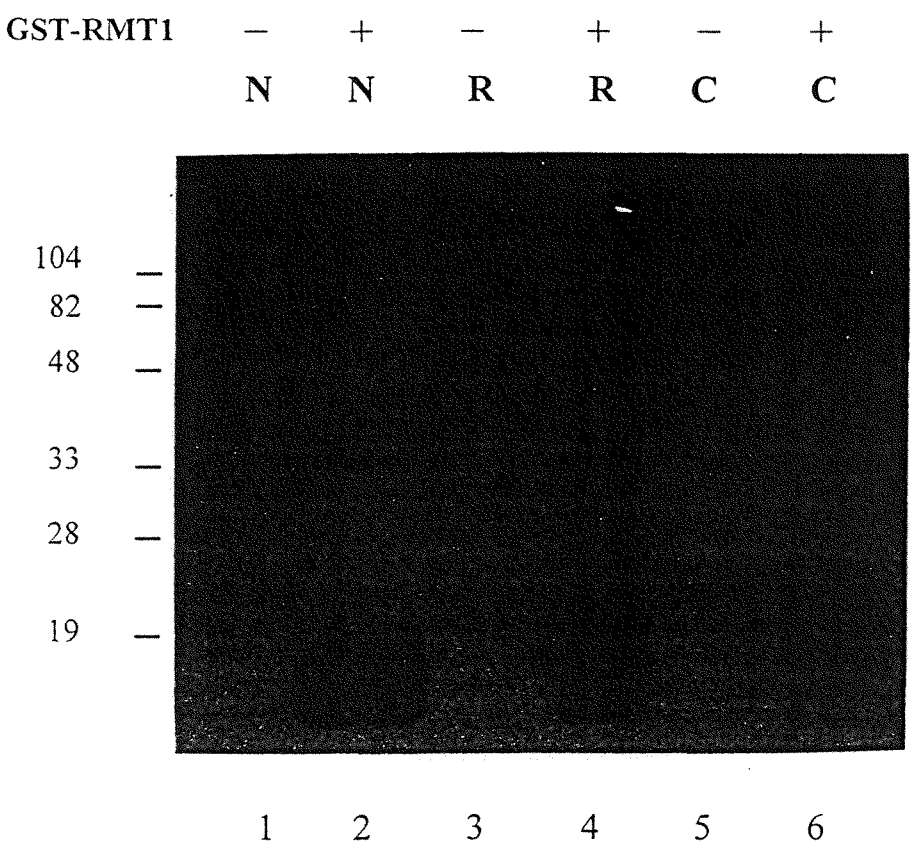
圖五、RGG peptide 競爭淋巴母細胞內除了內生性甲基轉移酶外，加上外生性 GST-RMT1 催化的反應

外生性甲基轉移酶催化 in vitro 的甲基化反應方法同圖三，外加的 peptide R9 或 K9 濃度一如圖上標示。此結果是於-75℃顯影三天而得。



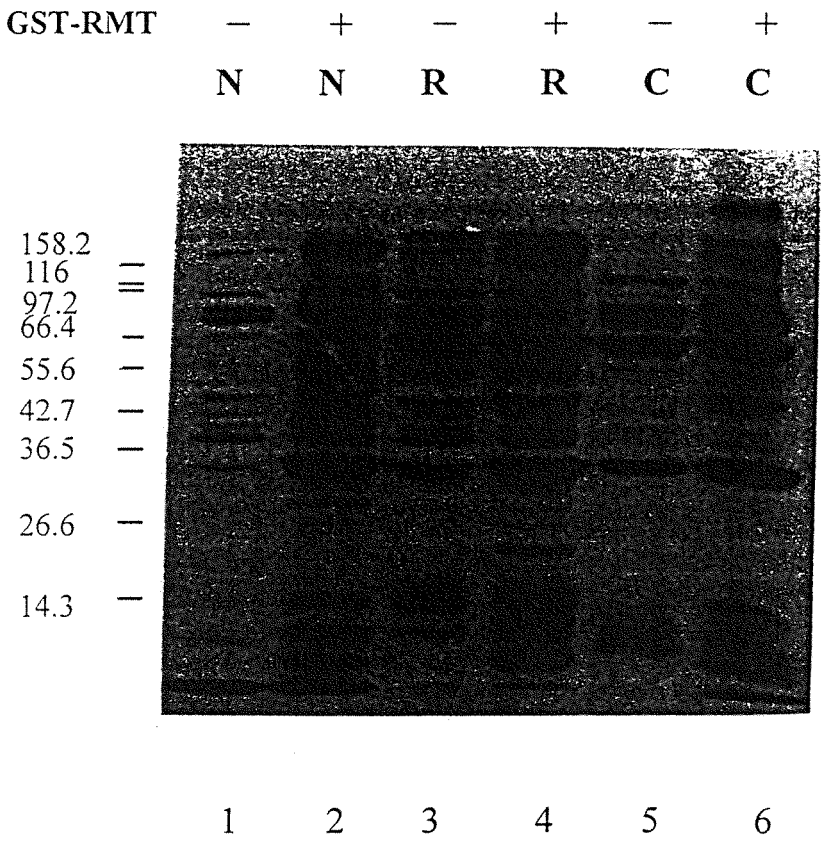
圖六、淋巴母細胞不同 **subcellular fraction** 分離效果的分析

Nuclear (N), ribosomal (R) and cytosolic (C) 等三的淋巴母細胞不同的 fraction 是藉由不同的離心力分離的（參照材料與方法），製備好後每一個 fraction 取等量 (30μg) 進行西方點墨法的分析，抗體為 anti-FMRP 和 anti-fibrillarin。



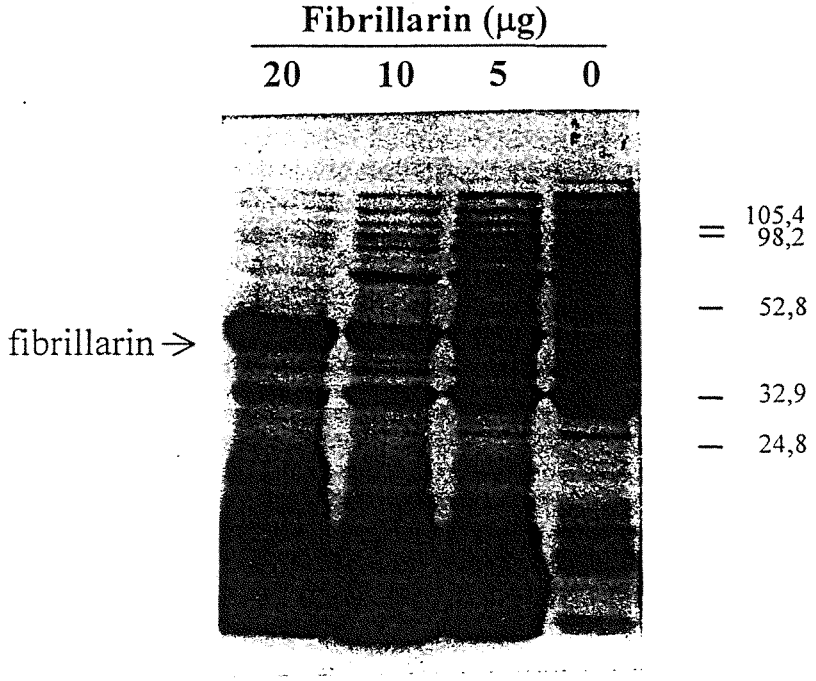
圖七、淋巴母細胞內不同 subcellular fraction 中內生性甲基轉移酶甲基化的分析

淋巴母細胞培養時不以 AdOx 處理，在製備成不同的 sucbcellular fraction 後各取等量的蛋白濃度進行 in vitro 甲基化的反應，藉由與 0.75μCi S-adenosyl-L-[*methyl*-^{3}H]methionine 於 30℃作用一小時，淋巴母細胞內的甲基接受蛋白即可被內生性甲基轉移酶或外加的外生性 GST-RMT1 標定 (參考材料與方法)，上圖是於-75℃顯影 7 天而得。



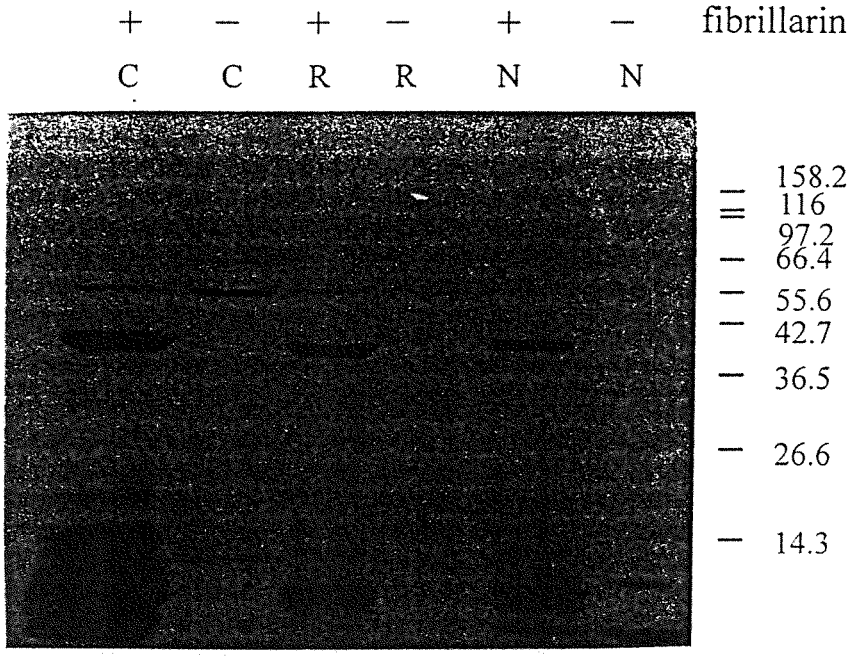
圖八、淋巴母細胞內不同 subcellular fraction 中內生性甲基轉移酶甲基化的分析

方法與圖七同，但淋巴母細胞培養時以 AdOx 處理，同樣於於-75℃顯影 7 天。



圖九、Recombinant fibrillarin 參與競爭內生性甲基轉移酶 in vitro 的甲基化反應

上圖為 ribosomal fraction 的甲基化結果，甲基化反應的方法與圖七同，多加了 recombinant fibrillarin 參與甲基化的反應，濃度如圖上所標示。



圖十、以 **recombinant fibrillarin** 作為甲基接受蛋白分析淋巴母細胞不同的 **subcellular fraction** 中內生性甲基轉移酶的活性

與圖七相同的甲基化反應，但外加 recombinant fibrillarin (5μg) 作為特異的甲基接受蛋白，分析淋巴母細胞不同的 subcellular fraction 中內生性甲基轉移酶甲基化 fibrillarin 的活性，此圖為-75℃顯影七天的結果。

Table 1、Percentage of radioactive methylated amino acids in different subcellular fraction of lymphoblastoid cell

	nuclear fraction	ribosomal fraction	cytosolic fraction	fibrillarin
N^G-monomethylarginine	20.6	25.5	14.1	11.9
N^G,N^G-dimethylarginine	33.8	66.3	69.8	87.7
The peak at 20.5 min	39.4	~ 0	~ 0	~ 0

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Protein *N*-Arginine Methylation in Adenosine Dialdehyde-Treated Lymphoblastoid Cells

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Protein arginine methyltransferase was recently identified to be associated with some proteins in signal transduction pathways. *N*-Arginine methylation in RNA binding proteins with arginine- and glycine-rich RGG motifs is known to be the major protein methylation in cells. Considering that arginine methylation might be involved in certain human disorders, we used human lymphoblastoid cells that can be easily prepared from lymphocytes as a model system to study the methylation. Lymphoblastoid cells grown in the presence of 20 μM indirect methyltransferase inhibitor adenosine dialdehyde (AdOx) for 72 h appeared to accumulate high levels of hypomethylated proteins for the endogenous protein methyltransferase or recombinant glutathion *S*-transferase-fused yeast arginine methyltransferase (RMT1). Analysis of methyl-accepting polypeptides in AdOx-treated lymphoblastoid cells by SDS–PAGE and fluorography showed that many polypeptides between 29,000 and 90,000 Da were methylated by the endogenous methyltransferase. A few polypeptides could be methylated to a higher extent upon the addition of yeast GST–RMT1 fusion protein. A peptide (GGRGRGGGF) could compete for the majority of the methyl-accepting protein substrates in the AdOx-treated lymphoblastoid cell extracts, whether or not exogenous yeast RMT1 was included in the reaction. When the arginine residues in the peptide were replaced by lysine, no competition was observed. The results indicated that the protein methyl acceptors in lymphoblastoid cells share similar RGG motifs and that arginine residues should be the site of methylation. © 1998 Academic Press

Key Words: **protein methylation; arginine methyltransferase; lymphoblastoid cell; adenosine dialdehyde.**

Protein *N*-methylation is generally regarded as an irreversible modification on newly synthesized polypeptides. Once the methyl-accepting sites on proteins have been occupied, no methyl groups can be incorporated *in vivo* or *in vitro* by the catalysis of protein methyltransferases. Adenosine dialdehyde (AdOx)[2] is an inhibitor of *S*-adenosylhomocysteine (AdoHcy) hydrolase and can elevate the cellular level of AdoHcy, the product inhibitor of methyltransferases, utilizing *S*-adenosylmethionine (AdoMet) as the methyl group donor (1, 2). It has been demonstrated that AdOx-treatment of rat pheochromocytoma (PC12) cells can accumulate protein methyl-accepting substrates in hypomethylated states presumably due to the inhibition of protein methyltransferases in cells (3–6). These hypomethylated polypeptides can be labeled *in vitro* after incubation with the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]-methionine by the endogenous enzyme in the extract (3–5). *N*-Arginine methylation has been shown to be responsible for the majority of the alkaline-stable protein methylation in cells that can be inhibited by the AdOx treatment (3, 5). About 90% of the total recovered protein methylation in AdOx-treated PC12 cells are N^G-monomethylarginine or N^G,N^G-dimethylarginine (asymmetric) (3, 5). Many RNA binding proteins such as fibrillarin (7, 8), nucleolin (9, 10), and heterogeneous nuclear ribonucleoproteins (hnRNPs; 11, 12) with arginine- and glycine-rich motifs (RGG box) are known to contain these modified arginine residues. Synthetic peptides GGRGGFGGRGGFGGRGGFG and GGFGG-RGGFG, based on the sequences with modified arginines in fibrillarin and nucleolin, inhibit the methylation of a large majority of the methyl-accepting proteins

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[2] Abbreviations used: RGG box, arginine- and glycine-rich motif; AdOx, adenosine dialdehyde; RMT, arginine methyltransferase; PRMT, protein arginine methyltransferase; GST, glutathione *S*-transferase; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; hnRNP, heterogeneous nuclear ribonucleoproteins; TCA, trichloroacetic acid; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride.

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in extracts of AdOx-treated PC12 cells, suggesting that polypeptides containing the glycine- and arginine-rich sequence are the substrates of the predominant protein methyltransferase in these cells (6).

Many of the N^G,N^G-dimethylarginine-containing RGG box proteins such as fibrillarin, nucleolin, and some hnRNPs are known to be involved in RNA processing (13–15). Fibrillarin appeared to participate in major posttranscriptional activities in ribosome synthesis, pre-rRNA processing, pre-rRNA modification, and ribosome assembly (14) and was identified as the 34,000-Da nucleolar scleroderma antigen containing 4.1 mol% N^G,N^G-dimethylarginine (7). Nucleolin, which might play roles in pre-rRNA modification and ribosome assembly (14), is a 100-kDa nucleolar protein with multiple N^G,N^G-dimethylarginine surrounded by two or three glycine residues near the C-terminal of the protein (15). hnRNPs that are essential for pre-mRNA processing contain 65% of the asymmetric dimethylarginine in the cell nucleus (11, 12), among which hnRNPA1 is best characterized (16–18). The sites of arginine methylation in hnRNPA1 have been located (16, 18). The RGG box has been shown to be essential for the RNA-binding activity in some RGG proteins (19, 20). Why the arginine residues in the RGG box of RNA binding proteins are specifically methylated is still unclear. It is proposed that the methylation might regulate the RNA binding activity of these proteins (6). Rajpurohit *et al.* have shown that the RNA binding activity of methylated recombinant hnRNP A1 was reduced compared with that of the unmethylated form (17). One possible explanation for this result is that asymmetrically dimethylated arginine residues might make the protein to lose specific hydrogen bonds between the arginine residues and interacting nucleic acids and thus shift the mode of RNA binding from specific to nonspecific (21).

Two arginine methyltransferase activities were partially purified from calf brain and rat liver (22, 23). One activity can catalyze the methylation of myelin basic protein, which had been shown to contain N^G,N'^G-dimethylarginines (24, 25). The other histone protein, methylase I, was shown later to methylate recombinant hnRNP A1 and other nuclear proteins more efficiently to form N^G,N^G-dimethylarginines (22, 23, 16). A protein–arginine *N*-methyltransferase-specific for hnRNP and other RNA binding proteins containing the RGG motif has been partially purified and characterized from Hela cells (12). The enzyme preparation methylated the arginine residues in hnRNP A1 to mono- and asymmetric dimethylarginines but did not methylate myelin basic protein (12). The gene of the rat protein–arginine *N*-methyltransferase (PRMT1) has been cloned unexpectedly by two-hybrid analysis interacting with the TIS21 immediate-early gene product (2). Interestingly the human homologue of the rat PRMT1, which binds to the intracytoplasmic domain of interferon α and β receptors (27), was also identified by a two-hybrid screening. It is thus likely that the mammalian protein arginine methyltransferase might play roles in certain signal transduction pathways (26) and the arginine methylation of the RNA binding RGG box proteins might be tightly regulated to fine-tune the function of the proteins. The ODP1/RMT1 gene for the predominant protein–arginine *N*-methyltransferase in yeast has been identified by sequence comparison (28). Its allelic HMT1 gene was also found by a genetic screen for proteins that interact with an abundant yeast poly(A)$^+$-RNA-binding protein, Np13p (29). Glutathione *S*-transferase (GST) fusion protein of the rat PRMT1 or yeast RMT1 can modify appropriate protein substrates to form mono- and asymmetric dimethylarginines (26, 28). The yeast RMT appeared to have broader substrate specificity than the rat PRMT1 (28).

With the recent findings that the mammalian arginine protein methyltransferase might be regulated (26, 27) and connected to certain signal transduction pathway and the arginine methylation in the RGG box of RNA binding proteins might be critical for their RNA binding activity, it is important to analyze the methylation reaction in a system that can be easily sampled from different genetic backgrounds. The effects of AdOx treatment on cytotoxicity (30), cell growth (30), and cell differentiation (31) in various mammalian cell lines have been studied. The usage of the methylation inhibitor to probe the protein methylacceptors has been well established in PC12 cells (3–6) but not in other cell lines. Human lymphoblastoid cell lines are established from EB virus-transformed lymphocytes (32). Since lymphocytes can be easily sampled from individuals with specific genetic background, lymphoblastoid cell lines can preserve the precious genetic information in these persons. Considering that certain human diseases or syndromes might be related to abnormal N-arginine methylation, we are interested in the potential to use lymphoblastoid cells derived from such individuals to study *N*-methylation. We thus first analyzed the methylation status of the proteins in lymphoblastoid cells treated with the methylation inhibitor AdOx.

MATERIALS AND METHODS

Culture of lymphoblastoid cells. Lymphoblastoid cell lines were transformed by EB virus as described (32). One successfully established cell line (003) from a normal individual was cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C in a 5% CO_2 incubator. When the cells reached a density of about 5×10^5/ml, the cells were changed to fresh medium with 0, 2, 10, and 20 μM AdOx (adenosine, periodate oxidized; Sigma) and incubated for 0, 24, 48, and 72 h. The number of viable and dead cells in the culture was determined by trypan blue exclusion. Up to 20 μM and 72 h of AdOx treatment did not reduce the growth of lymphoblastoid cells. The dead cells in the culture did not increase upon AdOx treatment, indicating that the drug did not have a cytotoxic effect on the cells under these conditions. Thus, to prepare

AdOx-treated cell extracts for further methylation reaction, the cells were grown in the presence of 20 μM of AdOx for 72 h.

Preparation of lymphoblastoid cell extracts. Lymphoblastoid cells were harvested at 1000*g* for 10 min. The cell pellet harvested from one 75-cm^2 flask (grown in 50 ml of the culture medium) was resuspended in 0.5 ml of 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]. The cell suspension was homogenized with 4 strokes in a glass pestle, and then sonicated twice by an ultrasonicator for 30 s (1-min interval) on ice. The homogenate was centrifuged for 20 min at 20,000*g* and the supernatant was stored at −20°C as the cell extract. The protein content in the extract was determined by the Lowry method (33) with bovine serum albumin (BSA, Sigma) as the standard.

N-Protein methylation reactions. Recombinant yeast GST–RMT1 arginine methyltransferase was prepared from pGEX–RMT-1-transformed *Escherichia coli* strain DH5α as described (28). To determine the methyl-accepting substrates in the lymphoblastoid cell extract, the extract (about 120 μg of protein) was incubated in the presence or absence of the GST–RMT enzyme (about 25 μg), 40 μM S-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham; adjusted to 2000 cpm/pmol with unlabeled AdoMet), 25 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM EGTA buffer. After incubation at 30°C for indicated time, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid (TCA) and 20 μl of BSA (10 mg/ml) as carrier proteins. After centrifugation at 4°C at 2200*g* for 5 min, the precipitant protein was incubated with 0.1 ml of potassium borate (0.1 M, pH 11) at 60°C for 10 min to remove base-labile protein methyl esters (1, 16). One milliliter of 10% TCA was added for the second precipitation and the pellet was dissolved in 0.1 ml of 88% formic acid. Eighty microliters of the sample was added to 2 ml of scintillation solution (Biodegradable Counting Scintillant, Amersham) in a 5-ml scintillation vial to determine the radioactivity. For further SDS–PAGE and fluorography analysis, the methylation reaction was performed as described above, except 0.75 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (60 Ci/mmol) was included in a reaction of 15 μl and the reaction was terminated by the addition of one-third of the volume of 3 × SDS sample buffer.

SDS–PAGE analysis and fluorography. SDS–PAGE (12.5% acrylamide) was performed as described by Laemmli (34). After gel electrophoresis, the polyacrylamide gel was stained with Coomassie brilliant blue, destained, and soaked in EN^{3}HANCE (Du Pont NEN) for 1 h 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. The densities of the signals were analyzed by an AlphaImager 2000 Digital Imaging & Analysis System (Alpha Innotech Corporation).

RESULTS

AdOx treatment increased the level of base-stable methyl-accepting substrates in lymphoblastoid cells. Lymphoblastoid cell extracts prepared from cells grown in the presence or absence of 20 μM AdOx for 72 h were incubated *in vitro* with *S*-adenosyl-L-[*methyl*-^{3}H]-methionine to evaluate the effects of AdOx treatment on the hypomethylation state of the *N*-methyl-accepting substrates in lymphoblastoid cells. As shown in Fig. 1, the level of methylation increased with progressively decreasing rate in the 120-min reaction interval whenever only the endogenous methyltransferase of the lymphoblastoid cell extract was present or the exogenous GST–RMT1 enzyme was included. When exogenous recombinant GST–RMT1 fusion protein was included in the reaction, the maximum level of methyl-acceptors was about 200 pmol/mg of extract protein at the reaction time of 120 min. When no exogenous enzyme was included in the reaction, the AdOx-treated extracts can be methylated by the endogenous methyltransferase to the maximum level of approximately 60 pmol/mg of extract protein in 120 min. Since the reaction products had been incubated with 0.1 M of borate buffer (pH 11), base-labile methylation products such as methylesters of the L-isoaspartyl residues could not survive the conditions and would not contribute to the radioactivity detected (1, 16). If the cells were grown in the absence of AdOx, the level of methyl-acceptors in the extract for the GST–RMT1 or endogenous methyltransferase was much reduced (maximum level about 66 and 5 pmol/mg, respectively, in 120 min). Thus, the AdOx treatment apparently increased the level of base-stable methyl-incorporation into the lymphoblastoid cell extract.

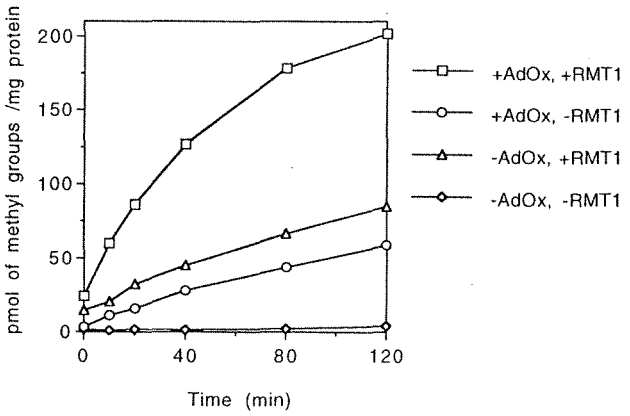

FIG. 1. Time course of *in vitro* methylation in lymphoblastoid cell extracts. Lymphoblastoid cell extracts were methylated in the presence of 40 μM of *S*-adenosyl-L-[*methyl*-^{3}H]methionine for various time intervals as described under Materials and Methods. Squares and open circles represent the methylation of lymphoblastoid cell extracts prepared from cells grown in the presence of 20 μM of AdOx for 72 h by exogenous GST–RMT1 or by endogenous methyltransferase (without the addition of exogenous GST–RMT1), respectively. Triangles and diamonds represent the methylation of lymphoblastoid cell extracts from cells grown in the absence of AdOx by exogenous GST–RMT1 or endogenous methyltransferase. Points represent the average of duplicate measurements.

Methyl-accepting polypeptides in the lymphoblastoid cell extracts grown in the presence and absence of AdOx. To directly look at the methyl-accepting polypeptides present in the lymphoblastoid cell extracts, we incubated the extracts with *S*-adenosyl-L-[*methyl*-^{3}H]-methionine and analyzed the methylation reaction products by SDS–PAGE and fluorography. There were no detectable signals if the lymphoblastoid cells were grown without AdOx treatment and the reaction was performed without exogenous methyltransferase (Fig. 2, lane 1). When yeast GST–RMT1 fusion protein was

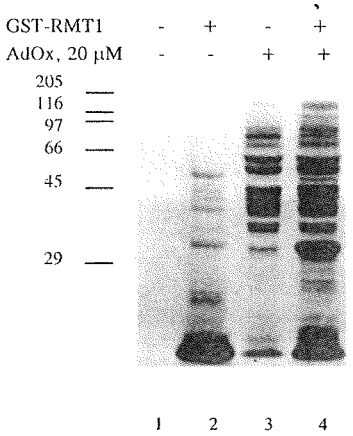

FIG. 2. Fluorography of stable methyl incorporation in polypeptide acceptors in lymphoblastoid cell extract. Lymphoblastoid cell extracts (containing 25 μg of total protein) prepared from cells grown in the presence or absence of 20 μM of AdOx for 72 h were methylated by endogenous methyltransferase or exogenous GST–RMT1 (6 μg) with 0.75 μCi of S-adenosyl-L-[*methyl*-^{3}H]methionine for 1 h as described under Materials and Methods. The figure represents the results of a 3-day exposure at −75°C.

included in the reaction, we observed at least 10 methyl-accepting polypeptides of various molecular weights (Fig. 2, lane 2). Since these polypeptides did not show up in lane 1, they probably were not substrates for endogenous lymphoblastoid methyltransferases but can be recognized by the yeast RMT1. A very strong low-molecular-weight signal ran with the dye front (MW < 14,000) of the 12.5% polyacrylamide gel, indicating the presence of low-molecular-weight polypeptides as major methyl-accepting substrates for the yeast GST fusion enzyme. An intense signal at the same position was present in lane 4, where the yeast RMT1 was included in the methylation reaction to AdOx-treated lymphoblastoid cell extract. The results indicated that the low-molecular-weight methyl-acceptors well recognized by yeast RMT1 probably were not substrates for the endogenous lymphoblastoid methyltransferase and remain hypomethylated whether the cells were grown in the presence or absence of AdOx.

When cells were grown in the presence of AdOx, many methyl-accepting species, mostly between 30,000 and 90,000 Da, could be detected in the extracts even without the addition of recombinant enzyme in the methylation reaction (lane 3). The results (compare lane 3 with lane 1) further confirmed that the methyl-accepting polypeptides could be accumulated at hypomethylated states when the lymphoblastoid cells were grown in the presence of AdOx and could thus be further methylated *in vitro*. However, we could not exclude the possibility that AdOx treatment activates the endogenous methyltransferase. If GST–RMT1 fusion protein was included in the reaction (lane 4), the methylation pattern was very similar to that of lane 3. Additionally a polypeptide of molecular weight 32,000 was heavily methylated in the presence of the yeast enzyme. Analysis of the densities of the 32,000-Da signals in lane 4 and lane 3 revealed at least an eightfold increase. Other polypeptides at 50,000 and 100,000 Da, and at least eight polypeptides below 29,000 Da, also became visible upon the addition of the yeast enzyme. These bands were most likely to be methylated by the yeast GST fusion enzyme but not the endogenous lymphoblastoid methyltransferase.

RGG peptide competes the majority of the methyl-accepting proteins for endogenous methyltransferase in AdOx-treated lymphoblastoid cells. Previous investigations of PC12 cells indicated that *N*-arginine methylation in the RGG box RNA binding proteins is responsible for the majority of the base-stable methylation that could be inhibited when cells were grown in the presence of AdOx (3, 6). We suspected that most of the base-stable methylations in lymphoblastoid cell extracts that we observed were on the arginine residues in RGG box. We synthesized a peptide R9 with the sequence GGRGRGGGF derived from fibrillarin (7, 8) and fragile X mental retardation (*FMR-1*) gene product (FMRP; 35, 36). The sequence is a typical RGG box sequence and the peptide could be methylated by the *N*-arginine methyltransferase present in rat or mouse brain extract or the recombinant yeast RMT1 enzyme to form N^G-methylarginine (Li *et al.*, manuscript in preparation). If the methyl-acceptors in the AdOx-treated lymphoblastoid cells share similar RGG domains, the R9 peptide might be able to compete with these hypomethylated substrates for either endogenous or exogenous *N*-arginine methyltransferases. We thus performed *in vitro* methylation reactions of the AdOx-treated cell extract in the presence of 0, 10, 100, and 1000 μM R9 peptide. The methylation reaction was analyzed by SDS–PAGE and fluorography. As illustrated in Fig. 3, the methylation reaction catalyzed by the endogenous enzyme in the lymphoblastoid cell extract treated with AdOx could barely be competed in the presence of 10 μM of the R9 peptide (lane 2). At 1000 μM, most of the polypeptides were still methylated, but with greatly reduced intensity (lane 4). As the concentration of R9 increased, the methylation in different polypeptides appeared to decrease to different extents. For example, from the analysis by densitometry, the signals of 32,000-Da polypeptide were reduced from 100 to 33.7 and to 13.3% when 10, 100, and 1000 μM R9 were included. The signals of the 60,000-Da polypeptide were reduced from 100 to 64.2 and 19.8% when 10, 100, and 1000 μM R9 were included. Another peptide K9 with all the arginine residues in the R9 peptide substituted by lysine residues could barely compete the methylation in the polypeptides of the lymphoblastoid extract only at 1000 μM (lanes 5–7). The differences in the ability of the two peptides to

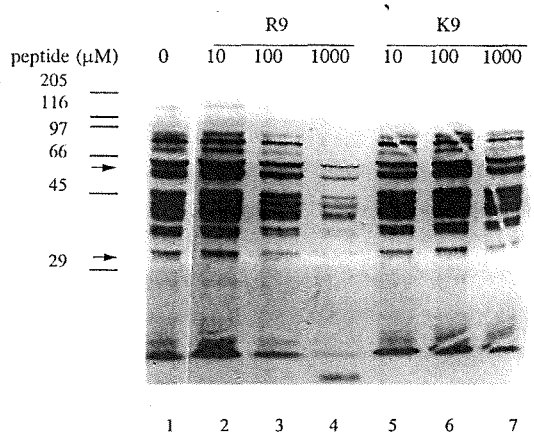

FIG. 3. Fluorography of RGG peptide competition of methyl incorporation into lymphoblastoid polypeptides catalyzed by endogenous methyltransferase. Methylation reaction was performed by the endogenous lymphoblastoid methyltransferase as described in the legend to Fig. 2 except R9 or K9 peptide was included for the indicated concentrations. The polypeptides discussed in the text are indicated by arrows. The figure shows the results of a 3-day exposure at −75°C.

compete the polypeptide methylation indicated that the reduced methylation was most likely due to the competition of the RGG sequence of the R9 peptide, and the arginine residues in the RGG box of the RGG box proteins should be the major methylation sites.

RGG peptide competes the majority of the methyl-accepting proteins for endogenous and exogenous GST–RMT1 methyltransferase in AdOx-treated lymphoblastoid cells. We performed *in vitro* methylation reactions of the AdOx-treated cell extracts including exogenous GST–RMT1 enzyme in the presence of R9 peptide. As illustrated in Fig. 4a, the intensities of the methylated polypeptides appeared to decrease to different extent with increasing concentration of the R9 peptide (lanes 1–4). The changes of the intensities of the 32,000- and 50,000-Da polypeptides that can be methylated to higher extents upon RMT-1 addition as well as a 60,000-Da polypeptide that was not further methylated by RMT-1 were analyzed by densitometric analysis of the fluorography (Fig. 4b). It appeared the radioactive label on the 60-kDa polypeptides can be more easily competed than the label on the other two polypeptides. In general, the intensities of the signals of the three polypeptides decreased to 50–70, 30–50, and about 10% upon the addition of 10, 100, and 1000 μM R9 peptide. On the other hand, the K9 peptide could not compete with the methylation of polypeptides in the cell extract even at the concentration of 1000 μM (Fig. 4a, lanes 5–7). Interestingly, we observed signals at the very bottom of the lanes for the reactions containing the R9 peptide. The signals were likely to be methylated R9 peptides since the strength of the signals increased with increasing concentration of the peptide in the reaction. The majority of the peptide methylation probably was catalyzed by the yeast GST fusion protein but not by the endogenous methyltransferase since the intensity of the signals was much lower when no RMT1 fusion was included in the reaction (compare Figs. 3 and 4a). The intensity of the methylated peptide when 1000 μM R9 was included in the reaction (lane 4) was not stronger than the average intensity of signals in lane 1 where no peptide was included for competition. Therefore, the decreased methylation in polypeptides in the presence of high concentration of R9 probably was not due to the depletion of radioactive AdoMet by large amounts of methyl-accepting peptides, but rather to the specific competition of the RGG peptide.

DISCUSSION

We demonstrated that when human lymphoblastoid cells were grown in the presence of AdOx, the methylation inhibitor, methyl-accepting polypeptides for *N*-arginine methyltransferase appeared to be accumulated in a hypomethylated state, providing better opportunities to study these methyl-accepting polypeptides. Even though accumulation of methyl-accepting proteins by the treatment with AdOx in cultured cells such as PC12 cells (3–6) has been studied, the effects of the treatment on protein methylation in other cell types has not been well characterized. The extracts of PC 12 cells grown in the presence of AdOx appeared to contain highly reactive methyltransferase and abundant methyl-accepting substrates (3). The majority of the protein methylation in PC 12 cells appeared to be on the arginine residues to form N^G-monomethylarginine or N^G,N^G-dimethylarginine (3) that probably was catalyzed by the histone/hnRNP arginine methyltransferase (6, 16). Previous investigations used endogenous methyltransferase as the enzyme source to probe the methylacceptors present in the same extract. The endogenous methyltransferase of human lymphoblastoid cells could also effectively methylate the hypomethylated polypeptides formed by the AdOx treatment. If the cells were grown without AdOx, no (or very low levels of) protein could be detected to be *in vitro* methylated by the endogenous methyltransferase. It is thus likely that these methyl-accepting polypeptides of endogenous methyltransferase revealed by AdOx treatment are fully methylated in intact cells. These polypeptides could be detected by *in vitro* methylation reaction only by growing the cells in the presence of AdOx to block the modification during or shortly after the synthesis of polypeptides.

The catalytic subunit for the *N*-arginine methyltransferase has been cloned in rat, human, and yeast (26–29). We thus used purified recombinant methyltransferase to probe the methyl-acceptors in the lymphoblastoid cell extracts. Since the activity of the mammalian enzyme might be regulated by the presence of

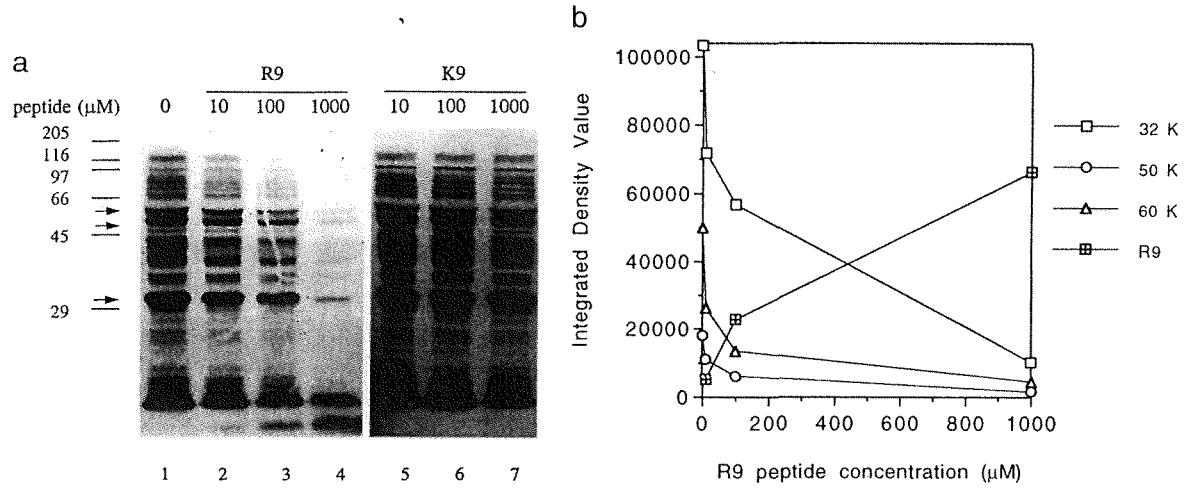

FIG. 4. (a) Fluorography of RGG peptide competition of the methyl incorporation into lymphoblastoid polypeptides catalyzed by exogenous GST–RMT1 in addition to endogenous methyltransferase. Methylation reaction was performed with the addition of exogenous GST–RMT1 enzyme as described in the legend to Fig. 2 except R9 or K9 peptide was included for the indicated concentrations. The positions of the 32-, 50-, and 60-kDa polypeptide further analyzed in (b) are indicated by arrows. The figure shows the results of a 3-day exposure at −75°C. (b) Analysis of the changes of the intensities of the signals of the 32-, 50-, and 60-kDa and a low-molecular-weight peptide upon the addition of the R9 peptide. Signals in the fluorogram in (a) were analyzed by an Image analyzer as described under Materials and Methods.

interacting proteins such as the immediate early gene TI21 product (26) and may contain more than one methyltransferase in the cells (26), we thus use yeast enzymes that are more stable and have broader substrate specificity (28). Interestingly, even in the control extracts without the AdOx treatment, some polypeptides can be methylated by the yeast GST fusion enzyme. The majority of the methylation was concentrated on low-molecular-weight polypeptides. The polypeptides methylated *in vitro* by the yeast arginine methyltransferase probably were not fully methylated in cells even if no AdOx was present in the culture. It is possible that these polypeptides might contain domains that can be recognized by the yeast arginine methyltransferase but not (or barely) recognized by the endogenous lymphoblastoid enzyme that has restricted substrate specificity as other mammalian enzymes (26). On the other hand, for AdOx-treated lymphoblastoid cell extracts, the addition of yeast RMT1 enzyme could not increase the level of methyl incorporation of most of the hypomethylated polypeptides catalyzed by the endogenous lymphoblastoid enzyme. Therefore, the arginine methyltransferase in the lymphoblastoid extracts was able to saturate the available hypomethylated sites in these polypeptides formed by AdOx treatment. One major exception was a polypeptide of about 32,000 Da. The intensity of the signal increased at least eightfold upon the addition of the exogenous yeast RMT1 enzyme and the methylation could be competed by the R9 peptide. It is thus likely that in AdOx-treated lymphoblastoid cell extracts the hypomethylated polypeptide can be partly methylated by the endogenous enzyme and even further methylated by the yeast enzyme. Interestingly, the molecular weight of this signal corresponds to that of the well-studied arginine methyl-accepting substrate hnRNP A1 and fibrillarin. The yeast GST fusion enzyme had been shown to catalyze the methylation of recombinant human hnRNP A1 (28). Endogenous hnRNP A1 present in hypomethylated AdOx-treated RAT1 cell extracts had been shown to be a substrate for the rat recombinant GST–PRMT1 fusion proteins (26). It is still possible that the 32,000-Da lymphoblastoid polypeptide is an unidentified polypeptide that is excellent for yeast arginine methyltransferase but a poor substrate for human lymphoblastoid enzyme.

The identities of the base-stable methyl-accepting substrates in the lymphoblastoid cells were further suggested by the RGG peptide competition experiments. The methylation on individual polypeptides could be specifically competed by the addition of the GGRGRGGGF R9 peptide but not the K9 peptide with the arginines substituted by lysines. The results indicated that the majority of the stable protein methylation was indeed on the arginine residues of the RGG box-containing proteins.

We have performed the methylation reaction in other lymphoblastoid cell lines. The methylation patterns in these cell extracts were basically the same as the pattern of lymphoblastoid cell line 003 (data not shown). Therefore, the methylation pattern we observed should be typical for human lymphoblastoid cell extracts. Although no human diseases or syndromes have been proposed to be related to abnormality in arginine methylation, there are diseases or syndromes related to dysfunction of some of the RNA binding RGG box proteins.

For example, fibrillarin was characterized as a major scleroderma antigen (7) and the absence of the expression of functional *FMR-1* protein is directly related to the fragile X syndrome (36). It is not known if *N*-arginine methylation in the RGG box might be involved in fine-tuning the function of the proteins and might be related to the development of the diseases. Lymphoblastoid cell lines established from blood samples of certain human subjects might be useful to these studies. Basic investigation of the effects of AdOx treatment on the *N*-arginine protein methylation in lymphoblastoid cells should be beneficial for further investigation in this system.

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