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子計畫一:自體免疫疾病中蛋白質精胺酸甲基化之蛋白質體研究(2/3)

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一、 摘要

在此研究計劃中，我們想要探討蛋白質精胺酸甲基化（分為對稱型或不對稱型双甲基精胺酸）和自體免疫疾病的關係。許多蛋白質包含了甲基精胺酸，如：fibrillarin、部分的 hnRNP 蛋白、myelin basic protein 以及 SmD1、D3 蛋白，而這些蛋白也已被發現是許多自體免疫疾病的自體免疫抗原。在這個計劃中，我們首先從紅斑性狼瘡病人中取得 anti-Sm 和 anti-RNP 自體免疫抗體血清，尋找抗血清中是否有因精胺酸甲基化差異而對蛋白質辨識差異之抗體，再利用蛋白質體學方法找出有甲基化的自體免疫抗原。我們用一位病人血清作西方點墨法時，發現低甲基化之細胞萃取蛋白和正常甲基化組比較，有數調訊號減低情況。將相關蛋白點挖出，以蛋白脢水解後作質譜分析，有二蛋白質 ZNF9 以及 CARG binding protein 含有典型精胺酸甲基化蛋白質中出現之 RGG 序列，我們建構此二蛋白之重組蛋白，並證明此二蛋白在試管中可被精胺酸甲基轉移脢甲基化。此蛋白甲基化與否和自體免疫抗體辨識是否有關正在研究中。

Abstract

In this project we are going to find out the relationships between protein methylation, mostly protein *N*-arginine methylation to form symmetric or asymmetric N^G, N^G –dimethylarginines (sDMA or aDMA), and 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. Sm protein D1 and D3 were reported to contain symmetric di-methylarginines and a few different anti-Sm autoantisera recognized only the sDMA peptide of SmD1 and D3 but not unmethylated or asymmetric dimethylarginine peptides. We thus examined if the anti-Sm sera from local SLE patients also preferentially recognize the methyl-modified Sm D proteins and if there are other proteins that can be differentially recognized by the anti-Sm sera due to their methylation states. We treated HeLa cells with adenosine dialdehyde (AdOx), an inhibitor of protein methylation. Anti-Sm autosera from three different SLE patients were used in western blot analyses of AdOx-treated (proteins presumably at hypomethylation state) and untreated (proteins at normal methylation states) HeLa cell extracts. Reduced signals between molecular mass of 18 to 21 kDa and about 31 kDa were consistently detected from cell extracts treated with AdOx compared to the ones without AdOx treatment by one-dimensional SDS-PAGE analyses. However, there were no significant differences between the signals corresponding to SmD1 in samples of different methylation status. By two-dimensional electrophoresis, the differentially detected signals were further pinpointed and putative spots were picked and digested by trypsin. The peptide fragments were analyzed by MS and MS/MS analyses. Interestingly, two proteins (ZNF9 and Carg binding protein 1) contain

typical arginine and glycine (RGG) sequences of the arginine methyltransferase substrates were identified. We prepared recombinant GST-ZNF-9 and GST-Carg-bp1 fusion proteins expressed in *E. coli*. The recombinant proteins can be in vitro methylated by the arginine methyltransferase PRMT1 or RMT1. The confirmation of the proteins differentially detected by anti-Sm due to their methylation status are under investigation.

二、 研究目的與背景

A common feature of autoimmune diseases such as systemic lupus erythematosus (SLE), systemic sclerosis, Sjögren’s disease (SD), and mixed connective tissue disease is the breakdown of tolerance to self antigens, leading to the production of antibodies reactive with multiple self proteins. Recently, the possibility of posttranslational modification to create new self antigens or mask antigens normally recognized by the immune system has been discussed (Utz et al., 2000; Doyle and Mamula, 2001; Doyle and Mamula 2002). Neo-Ags originating from these modified proteins might find their way into the extracellular milieu where they can be taken up by Ag-processing cells and presented to T cells. In experimental systems, this mechanism can initiate an immune response to a protein that is ignored otherwise. Recently, studies have shown that self proteins are cleaved differently in apoptotic cells versus nonapoptotic cells, thus creating new autoAgs. Cells undergoing apoptosis are also subject to a number of protein modifications including, phosphorylation, transglutamination, ubiquitination and citrullination (Utz et al., 2000; Zampieri et al., 2001). Apoptotic cells have been considered a source of autoantigens in the induction of SLE (Utz et al., 1997;). Even though apoptotic cells remain intact, certain antigens that are common targets of autoantibody responses in SLE patients localize to surface blebs of apoptotic cells (Casciola-Rosen et al., 1994) and mice immunized with apoptotic Jurkat cells develop antibodies to multiple autoantigens and autoantigen complexes associated with SLE (Gensler et al., 2001). It is reasonable to speculate that these proteins on the surface of apoptotic cells have undergone post-translational modifications that affect the recognition and processing of Ag thus leading to an immune response.

Protein *N*-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 (Li et al., 1998; Najbauer et al., 1993). Most of the methylarginines in the proteins identified appear to be N^G–monomethylarginine (MMA) and asymmetric N^G, N^G –dimethylarginines (aDMA) in various RNA binding proteins within the Arg-Gly-Gly context (Najbauer et al., 1993). These modifications were catalyzed by the type I arginine methyltransferase (Gary and Clarke, 1998). Another type II methyltransferase helps to modify some other methylaccepting proteins such as myelin basic protein (Baldwin and Carnegie, 1971) and core snRNP SmD1, D3 (Brahms et al, 2000), B/B' and one of the Sm-like proteins LSm4 (Brahms et al., 2001) to form MMA and symmetric N^G, N'^G–dimethylarginine (sDMA).

The type I enzymes include PRMT1 that appears to be the predominant methyltransferase in the mammalian system (Lin et al., 1996, Tang et al., 2000; Pawlak et al., 2000), the zinc-finger containing PRMT3 (Tang et al., 1998), the coactivator-associated arginine methyltransferase CARM1/PRMT4 (Chen et al., 1999) and the newly identified PRMT6 (Frankel et al., 2002). Another PRMT-1-like PRMT gene (HRMT1L3,) with 80% sequence identity with PRMT1 was identified in human and vertebrates but with no reported function yet (Zhang and Cheng, 2003, Hung and Li, 2004). The only type II PRMT identified is the Janus kinase-binding protein JBP1/PRMT5 (Pollack et al., 1999; Roe et al., 2001; Branscombe 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 MBP, a typical type-II substrate, had been demonstrated for the p82/p77 immunoprecipitants (Gros et al., 2003). 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 et al., 2000). The survival of yeast HMT1 or Hsl7 mutants indicates that they are not essential genes.

Interestingly, many type I or type II methylaccepting proteins such as fibrillarin, several hnRNPs, myelin basic protein and SmD1 and D3 are known to be autoantigens of different autoimmune diseases (please see the following table and Boekel and Venrooij, 2002).

Autoantigen/ arginine methyl	autoimmune disease	methylarginine accepting protein
Fibrillarin	scleroderma	aDMA
Nucleolin	SLE (systematic lupus erythematosus)	aDMA
HnRNPA1	connective tissue disease (SLE, RA, MCTD)	aDMA
Sm D1, D	SLE	sDMA
Myelin basic protein	multiple sclerosis	sDMA

Ten different autoantisera recognize only the sDMA peptide of SmD1 and D3 but not unmethylated or aDMA peptides (Brams et al., 2000), indicating methylarginine modification can be important for autoantibody recognition. Furthermore, peptides with aDMA modification were identified as natural MHC class I ligand, indicating that specific cytotoxic T-cell response against cells presenting aDMA modified cells can be elicited (Yague et al., 2000). Whether abnormal arginine methylation can be correlated with the formation of autoimmune disease worth further investigation. In this project we are going to investigate the involvement of type I and type II protein arginine methylation in autoimmune disease, most specifically SLE. Even though by now there were no reports on type I modified aDMA residues as specific sites for autoantibody recognition, it is rather widely distributed in known autoantiagens involved in a wide spectrum of autoimmune disease. Different from phosphorylation that is irreversible, arginine protein methylation is irreversible and most likely to occur soon after protein synthesis, thus it probably can be viewed as a normal process of protein maturation. However, involvement of protein arginine methylation with signal transduction has been suggested. How specific arginine modification in the nuclear protein such as Sm D1 will elicit the production of specific autoantibodies is an interesting question. Moreover, the arginyl residues not only can be modified by methylation, in some autoantigens they undergo citrullination by peptidylarginine deiminase (Moscarello, 2002). In multiple sclerosis, MBP methylation has been reported to reduce arginine deimination, which has been directly related to the disease. Citrulination of fillagrin has been shown to be the key factor in rheumatoid arthritis (Girbal-Neuhauser et al., 1999). Filaggrin is a large protein with mutiple RGGR sequences dispersed in its 3870 amino acid residues (Gan et al., 1990). It is interesting whether this protein can be methylated, and whether SmD with sDMA modification might undergo citrullination in some fraction. We will cooperate with Project 4 on this issue.

Finally, posttranslationally modified self-antigens may play a role in regulating autoimmune disease through suppression of autoreactive T cells. St. Louis *et al.* found that a palmitoylated PLP peptide (139-151) completely suppressed, or considerably reduced the acute and chronic relapsing stages of EAE in SJL mice. Adoptive transfer of lymphocytes from mice treated with the palmitoylated PLP peptide also delayed the onset of disease in recipient mice. These studies offer the intriguing idea that posttranslationally modified peptides can act as altered peptide ligands, and thus may be useful in the treatment of autoimmune diseases (Louis et al., 2001).

三、 結果與討論

In our first year study, we performed western blot analyses using anti-Sm autosera from three different SLE patients against AdOx-treated (proteins presumably at hypomethylation state) and untreated (proteins at normal methylation states) HeLa cell extracts. Reduced signals between molecular mass of 18 to 21 kDa and about 31 kDa were consistently detected from cell extracts treated with AdOx compared to the ones without AdOx treatment by one-dimensional SDS-PAGE analyses. However, there were no significant differences between the signals corresponding to SmD1 in samples of different methylation status. By two-dimensional electrophoresis, the differentially detected signals (Fig. 1) were further pinpointed and putative spots were picked and digested by trypsin. The peptide fragments were analyzed by MS and MS/MS analyses. Interestingly, two proteins (ZNF9 and CArG binding protein) contain typical arginine and glycine (RGG) sequences of the arginine methyltransferase substrates were identified. We thus obtained the ZNF9 and CarG BP cDNA clones from RZPD (German) and construct pGEX4-ZNF-9 and pGEX-CarG BP to express GST-fusion proteins. Recombinant GST-ZNF-9 and GST-CArG BP fusion proteins were expressed in *E. coli* and purified (Fig. 2a and 2b). Whether these proteins can be methylated were investigated by in vitro methylation with either recombinant protein arginine methyltransferase (PRMT1) or (RMT1) or HeLa total cell extract as the enzyme source. Both CArG BP and ZNF9 can be methylated by the methyltransferases in HeLa cell extract or recombinant type I protein arginine methyltransferase such as PRMT1 and RMT (Fig. 3). We also examined if the anti-Sm antisera can recognize these two proteins. As shown in Fig. 4, the anti-Sm can recognize these two recombinant proteins as low as 100 ng. Whether methylation of these two proteins will affect (increase) the recognition by anti-SM is under investigation.

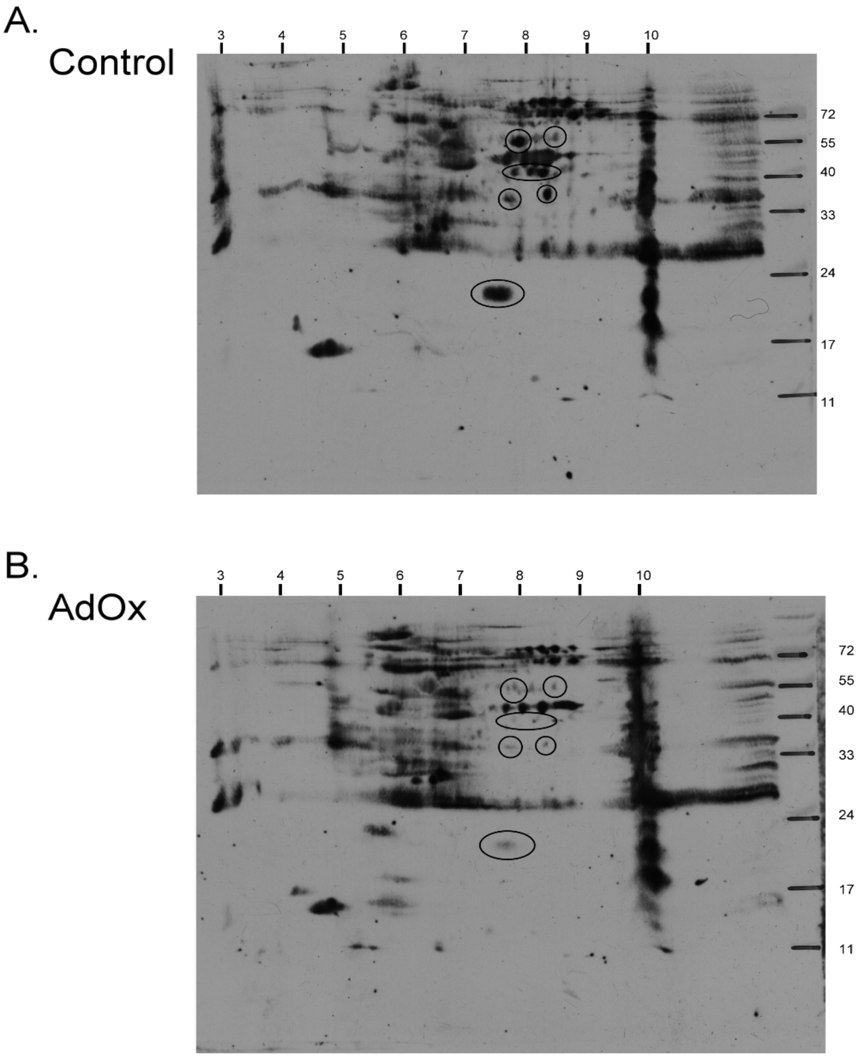

Fig. 1. Differential recognition of proteins due to methylation status by anti-Sm autoantibodies analyzed by two-dimensional electrophoresis. HeLa cell extracts (containing 250 g of total protein) were prepared from cells grown in the absence (A) or presence (B) of 20 M of AdOx for 24 h. The cell extracts were separated by SDS-PAGE and transferred onto NC membrane. Western blot analysis was performed anti-Sm autoantibody from SLE patient 1. The signals that are differentially recognized by the antisera were circled.

(A)

Fig. 2. Expression and purification of GST-fusion CarG BP or ZNF9. 將含有 pGEX -CArG BP(A) or pGEX-ZNF9 的 DH5α菌株於 LB/Amp medium 中 37℃培養 16 小時後，以 1:100 比例倒入含有 100ug/ml 的 300ml 培養液中於 37℃培養。直到菌液生長濃度達到 OD260nm 約 0.6-0.8 之間時，加入 IPTG 使其最終濃度爲 1mM，在 37℃培養 4 小時。之後將菌液離心後，以 20ml 蛋白萃取緩衝液 (0.15M NaCl ; 0.1M Tris pH8.0 ; 5% glycerol ; 1mM EDTA ; 1mM PMSF ; 1% Triton X-100 ; 1mM DTT ; 2 mg/ml lysozyme) 溶解菌體。經過超音波震盪、離心後，將收集到的上清液與 Glutathione-Sepharose 4B 均勻混合，於 4℃進行管柱純化。首先先收集所有通過管柱的液體（Flow through），之後以 10mlPBS 沖洗管柱並收集濾液 (Wash) ，最後加入 7ml Elution buffer (10uM glutathione ; 100uM Tris pH8.0 ; 0.12M NaCl ; 0.1% Triton X-100) ，以 1ml 分管收取 (E1~E7) 。從 Flow through、Wash 及 elute 完所收集的各管中，分別取 15ul 以 12.5% SDS-PAGE 分析，確認純化的成果。

ibrillarin、ZNF9 及 CArG BP2.25ug，分別加入 30ug HeLa 細胞萃取液、5ul RMT1、2ul PRMT1 後，再加入 1.5 μCi ^{3}H-SAM，於反應試劑 (50mM sodium phosphate pH7.5) 中 37℃作用一小時，利用 12.5% SDS-PAGE 進行電泳分析。經過 Coomassie blue 染色、去染液（25% methanol ; 10% acetic acid) 脫色後，膠體浸泡於 EN^{3}HANCE 中一小時、水中 30 分鐘，最後將處理後的膠抽乾後在-80℃下以 X 光片顯影三天。

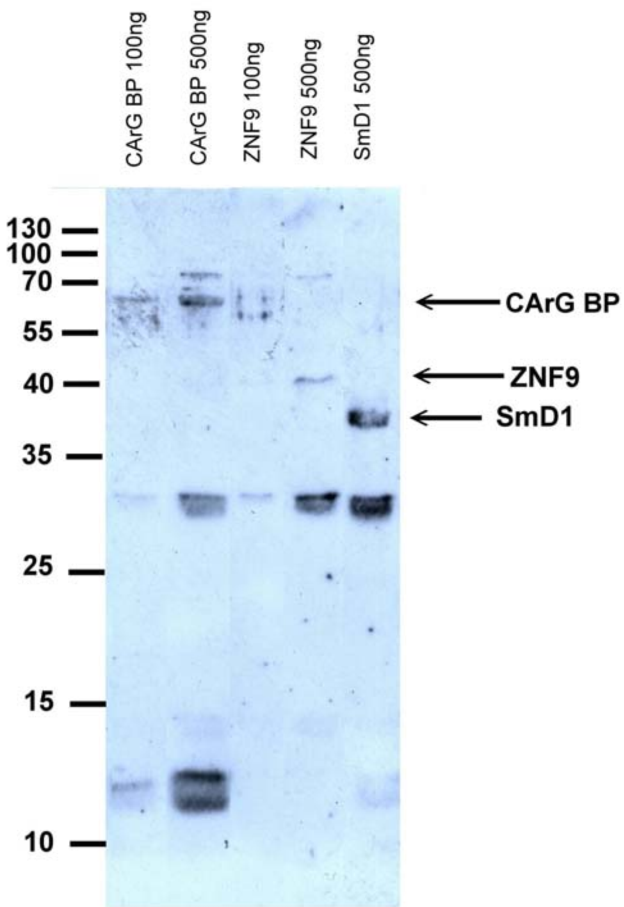

Fig. 4. 以 anti-Sm autoantisera 對 3 種重組蛋白作 Western blot

以 3 種重組蛋白進行 12.5% SDS-PAGE 後，作 Western blot(1 抗：anti-Sm(1：200)，2 抗：anti-human IgG-HRP(1：20000))。 Anti-Sm autoantisera(1 號病人)能對 3 種 GST-fusion protein(CArG BP, ZNF9, SmD1)產生辨識。

四、成果自評

In the first half of the three-year project, we are glad that we were able to obtain some interesting results to contribute the connection of autoimmune disease SLE with protein methylation, mainly arginine methylation. We have identified two putative protein-arginine methyl-accepting proteins that have not been reported to be autoantigens or protein-arginine methyl-accepting protein. Manuscript based on this part of work will be prepared soon after some further confirming experiments. We also studied the distribution of PRMT1 protein, protein arginine methyltransferase activity and methylarginine containing protein in tissues of NTZ or age-matched normal mice. We are going to examine the apoptosis conditions established in other component projects for protein arginine methylation.

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