行政院國家科學委員會專題研究計畫　成果報告

利用斑馬魚為模式生物系統研究蛋白質精胺酸甲基轉移胺基因家族

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一、 摘要

蛋白質精胺酸甲基化近年來已成為一深入研究的轉譯後修飾，和其相關的細胞功能包括訊息傳遞、蛋白質胞內定位分布以及轉錄調控等。至今已有八種人類的蛋白質精胺酸甲基轉移酶 (PRMT) 基因被鑑定出來；我們在斑馬魚中找到這八種基因的異種同源基因，因此在本計畫中我們將以斑馬魚為模式生物系統來闡析 PRMT 基因家族相關成員間之關係。我們會選擇兩組演化上親源相近的基因來研究。PRMT1/PRMT8 有 90%以上序列相同的高度保留性，且在脊椎動度中應可視為同種同源基因；PRMT2/PRMT6 在親源分析上雖同屬一支，但其基因演化保留度均低於其他 PRMT 基因。在本計畫中我們首先分析這 PRMT1 及其他精胺酸甲基轉移酶在斑馬魚中的表現；其次我們分析比較人類及斑馬魚中這 PRMT6 甲基轉移酶的生化活性。

關鍵詞: 蛋白質精胺酸甲基轉移酶，斑馬魚，蛋白體

Abstract

Protein arginine methylation has become one of the most intensively studied posttranslational modification and is involved in various cellular functions including cell signaling, protein subcellular localization and transcriptional regulation. By now there are eight protein arginine methyltransferase (PRMT) genes responsible for the modification identified in human. As we have found orthologues of all eight mammalian PRMT genes in zebrafish, in this project we are going to use the fish as a model system to elucidate the relationship of closely related members in the PRMT family. Specifically, we choose to study two phylogenetically related PRMT pairs in this project: PRMT1 and HRMT1L3 (PRMT8) are highly conserved with more than 90% sequence identity and are likely to be paralogues in vertebrates. PRMT2 and PRMT6, even though can be clustered in one lineage from phylogenetic study, both are evolutionarily more diverged than other PRMTs. In this project firstly we ainvestigated the expression of the PRMT1 gene and the other PRMT genes in different developmental stages of zebrafish. Secondly we obtained and compared the PRMT6 enzyme in human and zebrafish and to analyze their biochemical activity.

Keywords: Protein arginine methyltransferase (PRMT), zebrafish, proteomics.

二、 研究目的與背景

The protein *N*-arginine methyltransferase (PRMT) gene family

Protein arginine methylation is an irreversible posttranslational modification involved in various cellular functions such as signal transduction, protein subcellular localization, transcriptional regulation and protein–protein interactions (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 nine PRMT genes have been identified in the mammalian system and seven of them has direct or indirect evidences to be able to catalyze the transfer of methyl groups from AdoMet to the side chain ω–guanido nitrogens of arginine residues in protein substrates. The PRMT activity can be further divided into type I and type II, depending on the catalyses of formation of asymmetric N^G,N^G –dimethylarginines (aDMA) or symmetric N^G,N'^G–dimethylarginine (sDMA) residues respectively (Gary and Clarke, 1998).

The type I enzymes include PRMT1 (HRMT1L2) that appears to be the predominant methyltransferase in the mammalian system (Lin et al., 1996; Pawlak et al., 2000; Tang et al., 2000), the zinc-finger containing PRMT3 (Tang et al., 1998), the coactivator-associated arginine methyltransferase CARM1/PRMT4 (Chen et al., 1999), PRMT6 (Frankel et al., 2002) and PRMT8 (Lee et al., 2005). Direct evidences of the formation of the type I products catalyzed by these methyltransferases have been reported. The *N*-terminal SH3 domain containing PRMT2/HRMT1L1, even though was the first identified PRMT1 homologue by sequence comparison (Katsanis et al., 1997; Scott et al., 1998), has no direct evidence of PRMT activity. However, it is most likely to be responsible for the methylation of hnRNPE1B-AP5 *in vivo* (Kzhyshkowska et al., 2001) and its potential methyltransferase activity is important for being a coactivator of estrogen receptor (Qi et al., 2002). The human HIV transactivator protein Tat has been shown to be a substrate of PRMT6 and arginine methylation of Tat negatively regulate its transactivation activity (Boulanger et al., 2005). Recently, PRMT6 has been demonstrated to specifically methylate HMGA1a protein both in vitro and in vivo (Miranda et al., 2005; Sgarra et al., 2005). A PRMT-1-like PRMT gene (PRMT8/HRMT1L3) at chromosome 12p13 with 80% sequence identity with PRMT1 was identified in human (Zhang and Cheng, 2003). In a screen of mammalian neural gene, this gene was identified by microarray analyses to be expressed in developing and mature nervous system (Aubert et al., 2003). N-terminal myristoylation of PRMT8 results in its association with the plasma membrane. It has type I PRMT activity, and is tissue-specific, largely expressed in the brain (Lee et al., 2005). The putative methyltransferase Q9DD20 (designated as PRMT9 in the review by (Boisvert et al., 2005)) was reported as a PRMT in Golgi (Wu et al., 2004). However, its amino acid sequence more closely related to uniquinone methyltransferase rather than PRMT thus will not be included in this proposal.

The first 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 in previously recognized in *Schizosaccharomyces pombe* as an Shk1 kinase binding protein skb1 (Gilbreth et al., 1996) and a *Saccharomyces cerevisiae* histone synthetic lethal gene hsl7 (Ma et al., 1996). Another PRMT7 gene p82/p77 is a genetic suppressor element mediating cell sensitivity to DNA-damaging agents. This PRMT was the most outside lineage of the type I branch in our phylogenetic analyses (Hung and Li, 2004). Miranda *et al*. demonstrated that PRMT7 catalyzed the

formation of ω–N^G monomethylarginine in an arginine/glycine containing peptide but not in known protein substrates (Miranda et al., 2004). However, Lee at al. showed that PRMT7 is a new type II protein arginine methyltransferase capable for sDMA modification (Lee et al., 2004). The PRMT8 (AAH64403) designated by Cheng et al (Cheng et al., 2005) is a member of the PRMT family and appears to be most homologous to PRMT7. As it contains specific PRMT features and (HMRT1L3) has been characterized and designated as PRMT8, we suggest that AAH64403 be renamed as PRMT9. No characterization of the gene has been reported yet.

Different mammalian protein arginine methyltransferases are localized in different subcellular compartments and appear to have their own substrate sets and physiological roles. For example, the predominant PRMT1 appears to be located mainly in the nucleus (Tang et al., 1998). Its physiological substrates include histone H2A, many hnRNPs, fibrillarin and Sam 68 (Lin et al., 1996; Tang et al., 1998; Mowen et al., 2001; Lee and Bedford, 2002; Wada et al., 2002). The other widely studied CARM1/PRMT4 were first identified as a coactivator-associated arginine methyltransferase (CARM) localized in the nucleus (Chen et al., 1999). Identified PRMT4 substrates include histone H3 (Chen et al., 1999), p300/CBP (Xu et al., 2001), PABP1 (Lee and Bedford, 2002) and an mRNA –stabilizing protein HuR (Li et al., 2002). The JBP1/PRMT5 substrates include myelin basic protein (Pollack et al., 1999; Branscombe et al., 2001; Rho et al., 2001), SmD1, D3 (Brahms et al., 2000), Sm B/B', Lsm4 (Brahms et al., 2001) and coilin (Hebert et al., 2002). Even though cytoplasmic localization of PRMT5 has been suggested (Rho et al., 2001; Frankel et al., 2002), the interactions of PRMT5 with complexes involved in transcriptional regulations (Fabbrizio et al., 2002; Kwak et al., 2003; Pal et al., 2003) indicate that certain portion of the protein should be present in the nucleus also.

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

Nine PRMT genes (DART1-9) have been identified in *Drosophila* (Boulanger et al., 2004). However, only DART1, 4, 5, and 7 are the putative homologues of mammalian PRMT1, 4, 5, and 7. Other DART family members have closer resemblances to PRMT1 but do not have identifiable mammalian homologous. In our study, orthologues of the eight human PRMT genes in two fish model systems pufferfish *Fugu rubripes* and zebrafish *Danio rerio* were identified based on their amino acid sequence homology (Hung and Li, 2004). In addition, the PRMT genes are found in a simplified form with two family members (PRMT2 and HRMT1L3/PRMT8)

missing in *Ciona intestinalis*, a plausible approximation to the ancestral chordates for the origin of vertebrates and chordate. Paralogous vertebrate genes such as gene families for cell signaling (such as the fibroblast growth factor) and transcription factors (such as SMAD) often match to a single gene in *C. intestinalis* (Dehal et al., 2002). Interestingly, the missing PRMT1 (HRMT1L2) homologue HRMT1L3/PRMT8 has identical exon junctions and share high sequence identity with PRMT1 in human and fish. It is likely that PRMT8 occurred by gene duplication of PRMT1 after the divergence of the vertebrate ancestors from other chordates and can be viewed as a paralogue of PRMT1 in the vertebrates. It is interesting that we could not identify PRMT2 in *Ciona* also. We found that phylogenetically PRMT6 and PRMT2 converged in one lineage parallel to the PRMT1/3 lineage. The two PRMTs are the least conserved PRMT from fish to human, with about 50% sequence identity. Whether the two PRMT genes are evolved at a higher rate is an interesting issue. However, the *N*-terminal SH3 domain before the catalytic core of PRMT2 and the characteristic one coding exon of PRMT6 is conserved from fish to human. Surprisingly, we found that *Ci* PRMT6 contains six exons, indicating that the one coding exon character of PRMT6 is specific only in the vertebrates. It is attractive to propose that PRMT2 is evolved from a duplication of a common ancestor of both PRMT6 and PRMT2 in the early vertebrates but are diverged with faster rates than other PRMT genes.

Analyses of PRMT substrates or arginine methylaccepting proteins

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). The arginine methylaccepting substrates were identified and studied through different approaches. Some proteins, for example, fibrillarin (Lischwe et al., 1985), nucleolin (Lapeyre et al., 1986), and hnRNPA1 (Rajpurohit et al., 1994) were identified to contain asymmetric dimethylarginines through direct biochemical analyses by amino acid analyses or Edman sequencing. More proteins with similar arginine and glycine rich motifs, mostly RNA binding proteins such as herpes simplex virus ICP27 protein (Mears and Rice, 1996), FGF2 (Klein et al., 2000) and EWS (Belyanskaya et al., 2001), were further identified to be methylaccepting substrates. After identification of the PRMT genes, proteins without typical arginine and glycine rich sequences have been found to contain methylarginines. For example, in PABPII protein the methylation site has been identified on the RXR motif (Smith et al., 1999) while the methylarginine in STAT1 is located in the EIRQY context (Mowen et al., 2001); however, arguments on STAT1 methylation has been published (Komyod et al., 2005). The other approach to systematically study methylaccepting proteins is to make cells deficient in methyltransferase activity either by genetic approaches (Frankel and Clarke, 1999; Pawlak et al., 2000; Yadav et al., 2003) or by chemical treatment (Najbauer et al., 1993; Huang et al., 2002). The members on list of the arginine methylaccepting substrates for specific PRMT enzymes as reviewed by

Bedford et al. (Bedford and Richard, 2005) are growing very fast.

More recently, mass spectrometry has been demonstrated to be an excellent system to elucidate this type of posttranslational modification. Rappsilber et al (Rappsilber et al., 2003) introduced a novel method that can detect arginine dimethylated peptides and distinguish between the isomeric symmetric and asymmetric position of the methyl groups by parallel precursor ion scanning mass spectrometry in positive ion mode. They showed that dimethylarginine containing peptides give rise to five characteristic fragments in the positive ion mode: m/z 46.06 and 71.06 as well as a neutral loss of 45.05 for asymmetric dimethylation and m/z 71.06 as well as neutral loss of 70.05 for symmetric dimethylation. Gehrig et al. performed similar experiments (Gehrig et al., 2004) to study the fragmentation pathways of N^G -methylated and unmodified arginine residues in peptides examined by ESI-MS/MS and MALDI-M. Electrospray ionization tandem mass spectra revealed that dimethylammonium (m/z 46) is a specific marker ion for aDMA and a precursor ion scanning method utilizing this fragment ion was developed. The neutral losses of mono- and dimethylamines permit the differentiation between aDMA and sDMA. Furthermore, Brame et al. observed abundant characteristic ions at $[M+nH-31]^{n+}$ and $[M+nH-70]^{n+}$ in spectra of symmetrically methylated peptides and at $[M+nH-45]^{n+}$ in spectra of asymmetrically methylated peptides (Brame et al., 2004). They speculate these ions arise from neutral loss of monomethylamine, dimethylcarbodiimide, and dimethylamine, respectively. Instead of the precursor ion search, they performed neutral loss scanning of the +2 and +3 peptides. This method can provide a rapid means to screen for and characterize dimethylarginine sites.

Questions to be answered about arginine methylation

Why so many different methyltransferases are required to modulate different biological functions in the vertebrates and how the methyltransferases are evolved are interesting questions. As suggested by the interacting partners and the substrates of various PRMTs, arginine methylation has been implicated to play roles in signal transduction, protein subcellular localization, RNA processing, transcriptional regulation as well as DNA repair(Boisvert et al., 2005). Arginine methylation has long been considered as stable posttranslational modification. However, peptidyl arginine deiminase (PAD4) has been demonstrated to be able to reduce arginine methylation of histone (Cuthbert et al., 2004; Wang et al., 2004) by converting arginine and monomethylarginines to citrulline. This leads to the possibility of reversibility of arginine methylation. Interestingly, a new proposal of a protein protection function of arginine methylation was suggested recently. Methylation of arginine residues in long-lived, nonenzymatically active proteins such as histones and hnRNP proteins may shield the proteins from attacks of dicarbonyl containing molecules such as methylglyoxal from common side-product of glycolysis and other metabolic pathways (Fackelmayer, 2005).

Basically, the genomic structures of PRMT orthologues are conserved from fish to human. The conservation indicates that these genes are conserved in vertebrates and the divergence of these PRMT genes probably occurred before the origin of vertebrates. As

shown in Table1, zebrafish is also the biological system with the complete set of PRMT genes as in mammals. Other biological systems such as *Drosophila malangaster* or *C. elegans* appear to have different sets of PRMT genes. Zebrafish is a model organism that is amenable to genetic manipulations and has been widely used in developmental studies. Since the PRMT genes are highly conserved from fish to human, we suggest that zebrafish would be a valuable model system close to human to study protein arginine methylation.

Diversities of the substrates modified by PRMT complicated the understanding of the function of arginine methylation. Part the PRMT enzyme substrate relationships have been established in cellular systems (Bedford and Richard, 2005). Thus in this project we will use zebrafish as a model system to study some of the interesting issues raised above. Firstly, we will study the most predominant and evolutionally most conserved PRMT1 in zebrafish. As the PRMT1 knock-out mice die early during embryogenesis (Pawlak et al., 2000), we will prepare PRMT1 knock-down in zebrafish. Since the embryos are transparent and can be directly observed under microscope, we can observe the changes in the PRMT1 knockdowns at different early developmental stages. As the PRMT8 gene is so close related to PRMT1, we will investigate this gene in this proposal parallel to the PRMT1 studies. We will then analyze if PRMT8 can complement (completely or partially) in the PRMT1 knockdown and vice versa. We can also use proteomic tools that have been established in our lab to analyze the methylaccepting substrates of individual PRMT in the gene knock–down systems. Another type I PRMT pair we will analyze is PRMT2 and PRMT6. We will perform similar analyses as for PRMT1/PRMT8 to study these two evolutionarily less conserved PRMT pairs(Hung and Li, 2004). By now there were no suitable models to analyze the over all picture of the PRMT enzyme-substrate relationships and the putative cross-PRMT inter-relationships, we are confident that the zebrafish system we are going to develop will provide an excellent system to approach these critical issues.

Even though proteome-based analytical platform has been proposed to be one of the three important technologies for high-throughput screens using zebrafish, this area of analyses has not been developed in zebrafish yet (Love et al., 2004). As our laboratory is experienced in proteomic analyses, this is a great opportunity for us to lead in the development of zebrafish to a system for proteomic as well as for posttranslational modification. In addition, we are going to search for the orthologues of known mammalian PRMT substrate proteins in zebrafish. Besides, proteins with typical short GR or GGR repeats that contains methylarginines will be *in silico* identified as putative methylaccepting proteins. The putative methylation sites, PI and molecular mass of the candidate proteins will be obtained and be compared with the experimental results.

三、 結果與討論

From *in silico* data mining we have identified and designated eight PRMT genes in zebrafish. We have obtained zebrafish cDNA clones of PRMT1, 3, 4, 5, 6, 7 from RZPD. PRMT8, which ahs no commercially available

clone, were successfully cloned by RT-PCR. We have subcloned the cDNAs and designed primers for probe synthesis for in situ hybridization. We have examined the expression of some PRMT proteins in fish by western blot analyses (Hung et al., 2004). We have detected the PRMT1 RNA expression in all developmental stages (0, 6, 12, 24, 48, 60 and 72 hrs) by RT-PCR. The expression of the PRMT1 transcripts appeared low at the 0 and 2 hr time points but increased afterwards. By western blot analyses, we found that at 24 and 36 hrs, specific lower molecular weight isoforms of PRMT1 are present. We are setting up to further analyze this specific putative developmental stage specific isoform of PRMT1.

We have the GST-human PRMT 1, 2 fusions in our laboratory. We have obtained the zebrafish PRMT1 cDNA clone. Moreover, we have successfully cloned the human, zebrafish and *fugu* PRMT6 gene as GST fusion protein for further biochemical analyses. These enzymes can methylate recombinant mouse fibrillarin protein as well as a peptide with the HIV Tat protein sequence known to be able to be methylated by PRMT6 (Boulanger et al., 2005). PRMT6 automethylation was reported for the human PRMT6 (Frankel et al., 2002). We found it can be observed in zebrafish PRMT6 but not *fugu*PRMT6. Comparing the sequences, we suspect specific N-terminal sequence in human as well as zebrafish but not *fugu* sequence might be the automethylation site. Different constructs with point mutations and deletions have been constructed for further biochemical analyses.

四、 成果自評

Although this project was proposed as a three-year projected, we only got one-year support. However, we are glad that we were able to obtain some interesting results to contribute the understanding of arginine methylation in zebrafish. We havefound interesting PRMT1 expression patterns and are about to further illustrate the automethylation activity of PRMT6.

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