行政院國家科學委員會專題研究計畫　成果報告

斑馬魚 14-3-3 蛋白的選殖及其生物功能之探討

計畫類別：個別型計畫
計畫編號：NSC93-2311-B-040-009-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學生化暨生物科技研究所

計畫主持人：許立松

計畫參與人員：李惠敏　張智雯

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 10 月 28 日

國科會專題研究計畫成果報告撰寫格式

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國科會基於學術公開之立場，鼓勵一般專題研究計畫主持人發表其研究成果，但主持人對於研究成果之內容應負完全責任。計畫內容及研究成果如涉及專利或其他智慧財產權、違異現行醫藥衛生規範、影響公序良俗或政治社會安定等顧慮者，應事先通知國科會不宜將所繳交之成果報告蒐錄於學門成果報告彙編或公開查詢，以免造成無謂之困擾。另外，各學門在製作成果報告彙編時，將直接使用主持人提供的成果報告，因此主持人在繳交報告之前，應對內容詳細校對，以確定其正確性。

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成果報告繳交之期限及種類（精簡報告、完整報告或期中報告等），應依本會補助專題研究計畫作業要點及專題研究計畫經費核定清單之規定辦理。

二、內容格式：依序為封面、中英文摘要、目錄（精簡報告得省略）、報告內容、參考文獻、計畫成果自評、可供推廣之研發成果資料表、附錄。

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(二)中、英文摘要及關鍵詞(keywords)。

(三)報告內容：請包括前言、研究目的、文獻探討、研究方法、結果與討論（含結論與建議）…等。若該計畫已有論文發表者，可以 A4 紙影印，作為成果報告內容或附錄，並請註明發表刊物名稱、卷期及出版日期。若有與執行本計畫相關之著作、專利、技術報告、或學生畢業論文等，請在參考文獻內註明之，俾可供進一步查考。

(四)頁碼編寫：請對摘要及目錄部分用羅馬字 I 、II、 III……標在每頁下方中央;報告內容至附錄部分請以阿拉伯數字 1.2.3.……順序標在每頁下方中央。

(五)附表及附圖可列在文中或參考文獻之後，各表、圖請說明內容。

(六)計畫成果自評部份，請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

(七)可供推廣之研發成果資料表：凡研究性質屬*應用研究*及*技術發展*之計畫，請依本會提供之表格（如附件二），每項研發成果填寫一份。

三、計畫中獲補助國外或大陸地區差旅費、出席國際學術會議差旅費或國際合作研究計畫差旅費者，須依規定撰寫心得報告（出席國際學術會議者須另附發表之論文），以附件方式併同成果報告繳交，並請於成果報告封面註記。

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使用 A4 紙，即長 29.7 公分，寬 21 公分。

2. 格式

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3. 字體

報告之正文以中英文撰寫均可。在字體之使用方面，英文使用 Times New Roman Font，中文使用標楷體，字體大小請以 12 號為主。

附件一

行政院國家科學委員會補助專題研究計畫 ∨ 成 果 報 告
□期中進度報告

斑馬魚 14-3-3 蛋白的選殖及其生物功能之探討

計畫類別：∨ 個別型計畫　　□ 整合型計畫
計畫編號：NSC　92－ 2311　－　B －040 －　009　－
執行期間：2004　年　08 月　01　日至 2005　年　07　月　31　日

計畫主持人：中山醫學大學生化所許立松老師
共同主持人：
計畫參與人員：　李惠敏、張智雯

成果報告類型(依經費核定清單規定繳交)：∨精簡報告　□完整報告

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□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：

中　華　民　國　94 年　10 月　29　日

中文摘要：

目前已有許多實驗證實 14-3-3 家族蛋白可和含有磷酸化 serine 或磷酸化 threonine 位於 RSXp (S/T) XP or RX (Ar/S) XpSXP 序列的蛋白質結合(X 代表任何胺基酸而 Ar 為有苯環的胺基酸)，14-3-3 蛋白可和很多的蛋白例如：蛋白質激酶、蛋白質水解酶以及穿越細胞膜接受器結合進而調節它們的生物功能。目前已知 14-3-3 蛋白可與磷酸化的 BAD 結合進而抑制細胞凋零現象，相同的，14-3-3 蛋白也可使磷酸化的 FKHRL1 蛋白滯留在細胞質中進而抑制了 FKHRL1 依賴性的細胞凋零現象。至今 14-3-3 蛋白已在不同的生物體內如：酵母菌、果蠅、哺乳類動物中發現並且擁有高度的相似性。在此研究，我們首先以電腦比對方式尋找出斑馬魚中 14-3-3 蛋白的 DNA 序列，斑馬魚的 14-3-3 beta 基因含有 1413 鹼基可以轉錄出 242 胺基酸序列，且和人類的蛋白有極高的相似度。利用反轉錄酶-聚合酶連鎖反應，我們偵測出 14-3-3 beta 再早期胚胎發育一直到成年皆有表現，而且在腦部組織的表現量為最高。利用原位雜交方式(in situ hybridization)來觀察 14-3-3 蛋白在不同的斑馬魚發育過程中的表現模式亦得到相同結果，顯示 14-3-3 beta 可能在斑馬魚的腦部發育扮演一個重要角色。

關鍵字：14-3-3、斑馬魚、表現模式

英文摘要：

Accumulating reports have been shown that 14-3-3 family proteins can interact with peptides containing a phosphoserine or phosphothreonine within RSXp (S/T) XP or RX (Ar/S) XpSXP sequence (X represents any amino acids and Ar denotes aromatic amino acids). A wide range of proteins including protein kinase, protein phosphatase, and transmembrane receptors were reported to interact with 14-3-3. Through interaction with other proteins, 14-3-3 functions in the regulation of various biological processes. 14-3-3 proteins can inhibit apoptosis through interacting with phospho-BAD. Similarly, 14-3-3 proteins interact with and tether phosph-FKHRL1 in the cytoplasma and inhibit FKHRL1-mediated apoptosis. To date, several 14-3-3 isoforms are isolated from various species including yeast, fruit fly, and mammalian cells and shared high homology. In this study, we first searched for zebrafish 14-3-3 (z14-3-3) using in silico method, we have isolated the z14-3-3 beta which contains 1413 bp and can translate into 242 amino acids and shared highly homologous to human ortholog. RT-PCR analysis indicated that 14-3-3 beta was expressed from two cell stage to adult stage with highly expressed in brain region. Consistent with RT-PCR data, 14-3-3 beta was found to be mainly expressed in brain by in situ hybridization analysis. Our results suggested that 14-3-3 beta may play an important role in brain development of zebrafish.

KEYWORD：14-3-3、zebrafish、expression pattern

Introduction：

To date, at least seven isoforms of 14-3-3 proteins were identified from vertebrates (1-3). It has been shown that related isoform of 14-3-3 from diverse species including vertebrates, invertebrates, plants, and yeast share high sequence homology (4). The 14-3-3 proteins were identified to be signaling molecules to interact with peptides containing a phosphoserine or phosphothreonine within RSXp (S/T) XP or RX (Ar/S) XpSXP sequence (X represents any amino acids and Ar denotes aromatic amino acids) (5, 6).

A wide range of proteins including protein kinase, protein phosphatase, and transmembrane

receptors were reported to interact with 14-3-3 (7,8). Through interaction with other proteins, 14-3-3 functions in the regulation of various biological processes, for example, neurotransmitter synthesis, neuronal development, apoptosis, and cell growth control (9-12). Upon binding to ASK1 (apoptosis signal-regulating kinase 1), 14-3-3 inhibits the kinase activities of ASK1 and reduces the stress-inducing apoptosis (13). In general, BAD functions as to induce apoptosis through binding and inhibition of antiapoptotic effects of Bcl-X_L/Bcl-2 (14). After phosphorylation of AKT at Ser136, BAD produced a potential 14-3-3 binding motif. When bound with 14-3-3, BAD loss the ability to interaction with Bcl-X_L/Bcl-2 and unable to induce apoptosis (15). Two Ser residues (S259 and S621) of Raf-1 fit the consensus sequence for 14-3-3 binding, but the role of 14-3-3 in regulation of Raf-1 activity is not clear yet (16). However, it has been proposed that 14-3-3 is needed to maintain Raf-1 in an inactive form in resting state and to stabilize an active conformation of Raf-1 in the present of GTP-bound Ras. The interaction of Raf-1 with 14-3-3 has been demonstrated to facilitate Ras-dependent Raf-1 activation (16-18). Emerging documents have been demonstrated that distinct 14-3-3 participates in different develop stage of Xenopus, Drosophila and parasites (19-21). Tien and co-workers have shown the expression pattern of Drosophila 14-3-3 epsilon (d14-3-3 epsilon) (21). In the syncytial blastoderm, d14-3-3 epsilon was found to localize in nuclei while d14-3-3 epsilon gradually become membrane-bound during cellularization (21). Study using microinjection of R18 peptide, an inhibitor of 14-3-3 proteins, has revealed that 14-3-3 was required for mesodermal specification in Xenopus (20). Blocking the function of 14-3-3 resulted in embryos with axial patterns defects and reduced expression of mesodermal marker genes in Xenopus (20).

Method and materials

Fish

Zebrafish (*D. rerio*) were raised and maintained at 28℃ on a 14h light/ 10 dark cycle according to previous described. Different develop stages were detected according to the Zebrafish book.

RT-PCR of zebrafish 14-3-3 beta

Total RNA was isolated from different stages and from a various tissues using TRIzol reagent. Five mg of total RNA will be converted into first strand cDNA by Moloney murine leukemia virus reverse transcriptase according to the manufacturer's recommendations (Superscript II, Life Techonologies). Using cDNA as templates, 35 cycles of PCR (94℃ for 1 min, 50℃ for 1 min, and 72℃ for 1 min) will be performed with indicated oligonucleotides as primers. Amplification of elongation factor will be used as an internal control: sense primer (5'-GCTCAAGGAGAAGATCG-3') and antisense primer (5'-TCAAGCATTATCCAGTCC-3').

Whole mount in situ hybridization

Sense and anti-sense riboprobes for 14-3-3 beta will be made using in vitro transcription by T7 RNA polymerase in the presence of digoxigenin-labeled UTP. Whole-mount in situ hybridization will be performed as previous described.

Result and discussion

To determine the role of 14-3-3 proteins in zebrafish development, first, we have attempted to isolated zebrafish 14-3-3 proteins. We have used human 14-3-3 beta protein sequence to

perform BLAST search using TBLASTN program for sequence similarity analysis, respectively. Several expression sequence tag (EST) clones containing the full coding region were obtained. DNAs derived from these EST clones were subjected for sequence analysis. Zebrafish 14-3-3 beta (z14-3-3 beta) composed 1413 bp that contains an open reading frame of 242 amino acids (Fig.1).

To address the temporal and spatial expression of 14-3-3 beta, we have performed RT-PCR analysis. Total RNA derived from different stages were converted into cDNA and performed PCR using designed primers for 14-3-3 beta, the 14-3-3 beta was expressed in two hours after fertilization and thereafter. We also found that 14-3-3 beta was expressed in all tissues and mainly expressed in the brain tissue (Fig. 2).

To determine the exact expression tissues of 14-3-3 beta, we have performed in situ hybridization. The sequences composed entire coding region were labeled and subjected to in situ hybridization with different stages of zebrafish. Base on our results, it also shown that 14-3-3 beta was expressed in the brain region (Fig. 3).

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ACCGGAGAGCAAGAAGGGTACGGGACATAAATCGTATCTTTTTAGCGTTTAAAATCA
TCGAAAACACGGTTCAAAACTGTAAAAATGGACAAGAGCGATCTGGTGCAGAAAGCC
                           M  D  K  S  D  L  V  Q  K  A
AAGCTAGCCGAACAGGCCGAGCGGTATGATGACATGGCAGCAGCAATGAAAGCTGTG
K  L  A  E  Q  A  E  R  Y  D  D  M  A  A  A  M  K  A  V
ACTGAAGGTGGTGTGGAGCTTTCCAATGAAGAGCGCAACTTGCTCTCTGTGGCTTAC
T  E  G  G  V  E  L  S  N  E  E  R  N  L  L  S  V  A  Y
AAGAATGTGGTGGGTGCCCGGCGCTCATCCTGGCGCGTCATCTCAAGCATTGAGCAG
K  N  V  V  G  A  R  R  S  S  W  R  V  I  S  S  I  E  Q
AAGACCGAGGGGAATGAGAAGAAGCAGCAGATGGCTCGCGAGTATCGTGAAAAGATC
K  T  E  G     N  E  K  K  Q  Q  M  A  R  E  Y  R  E  K  I
GAGACCGAACTACAGGACATTTGCAGTGATGTGCTGGGTCTTTTGGAGAAGTACCTC
E  T  E  L  Q  D  I  C  S  D  V  L  G  L  L  E  K  Y  L
ATTGCCAATGCCTCTCAAGCAGAGAGCAAGGTTTTTTACCTGAAAATGAAAGGCGAT
I  A  N  A  S  Q  A  E  S  K  V  F  Y  L  K  M  K  G  D
TACTATAGATATTTATCTGAGGTAGCATCCGGAGAGTCCAAGGCCACCACGGTTGAG
Y  Y  R  Y  L  S  E  V  A  S  G  E  S  K  A  T  T  V  E
AACTCTCAGAAGGCTTACCAGGATGCTTTTGACATAAGCAAGAAGGACATGCAGCCC
N  S  Q  K  A  Y  Q  D  A  F  D  I  S  K  K  D  M  Q  P
ACGCACCCTATACGGTTGGGTCTTGCCCGCAACTTCTCCGTTTTTTACTATGAGATC
T  H  P  I  R  L  G  L  A  R  N  F  S  V  F  Y  Y  E  I
CTCAACTCTCCTGAGAATGCCTGCCAACTTGCCAAGACGGCTTTTGATGAAGCCATT
L  N  S  P  E  N  A  C  Q  L  A  K  T  A  F  D  E  A  I
GCTGAGCTTGACACTGTAAATGAGGACTCCTACAAAGACAGCACCTTGATCATGCAG
A  E  L  D  T  V  N  E  D  S  Y  K  D  S  T  L  I  M  Q
CTTCTAAGGGACAACCTCACTCTTTGGACATCAGAAAACCAGGGTGAAGAAGCAGGG
L  L  R  D  N  L  T  L  W  T  S  E  N  Q  G  E  E  A  G
GAAAACGAAAACTAGGGGAGCCCTGATCTCCACACACCCACATACATTCATCTTAAA
E  N  E  N
TACACAAAAGGCTTCCATCAACTCTCCCACCTCATTCCACCCAACACCACTCACCCTTCTCTATT
TAGTTCCCAGCCCTGCTCCTCTATACCCTTTGAATGATGATCAGCACCCGCAGGAAGACGCTCAA
CGAAACCCTTTAAACAGATCCCCAGCGGCTGTAGAGGGGGTGGGTTGGCTCACAGGGTTATACGC
TGACGTTATAGCAGGTCCAATAATTTTGTTTGTGAATTTAATCTGTACTGATTATCAATCCCTGT
TCTTTATGTGCGTCTGTGTGACTGTAAGTGTGCGCGTGCCACTAACCAAAAGTAACTGTAGTGGC
TGTACGTTTGTGCTTGAAAGTGCGAGTGAATGTATTGGGGATCAGAGGTTGCGAAATCTAATTGT
CCTCCTTGATAATTCCCTTTGTTTGACTATAGCAGGCTGTGCATTTTCCTTTTTTTCTTTATCTC
TCTCTCTCTCTCTTTTTTTTAGGATAATTCATCTGTATTTACAGGTTTTATTGTATGGTTATTAA
ACTGGCAGTTTATTTTCCCCAAAAAAAAAAAAAAAAA
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Fig. 1 **The nucleotide and deduced amino acids sequences of 14-3-3 beta.**

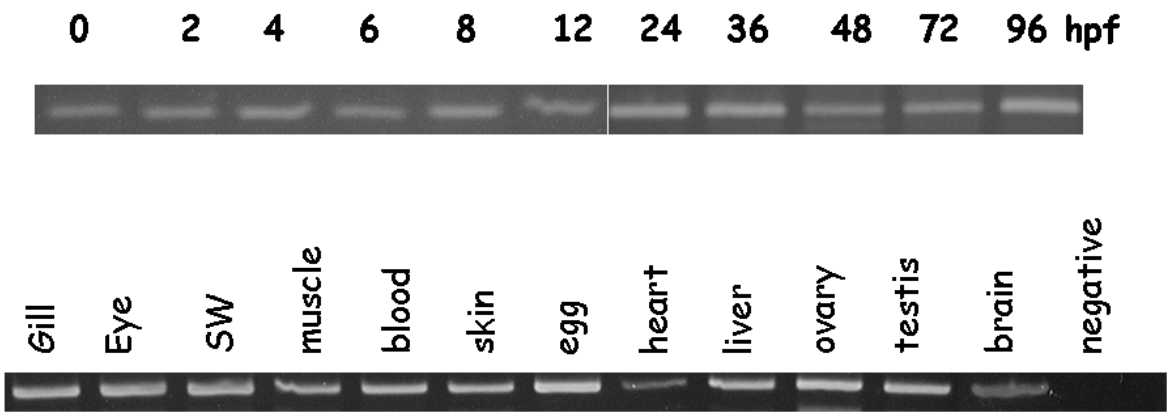

Fig. 2 **The temporal and spatial expression of 14-3-3 beta.** Total RNA from different stages (upper panel) and tissues (lower panel) were converted into cDNA and performed PCR. The fragments were separated by 3% agarose gel and staining with ethidium bromide.

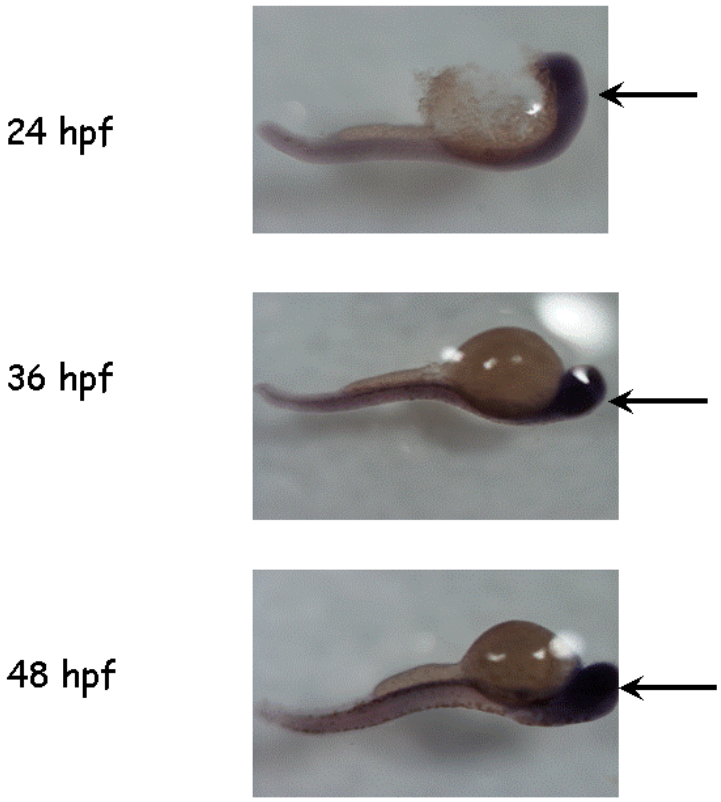

Fig. 3 **The in situ hybridization analysis of 14-3-3 beta of zebrafish.** Anti-sence ribonucleotide was used to undergo in situ hybridization with 24, 36, and 48 hour post fertilization zebrafish. Arrow indicated heavy stain in the brain region.