行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※

※　　　　　　　　　　　　　　　　　　　　　　　　※

※　人類蛋白激酵素 Mst3 的生理功能與作用機轉之研究　※

※　　　　　　　　　　　　　　　　　　　　　　　　※

※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC 92－2311－B－009－005

執行期間：九二年八月一日至九三年七月三十一日

計畫主持人：袁俊傑

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□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

執行單位：國立交通大學生物科技系

中　華　民　國九十三年十月二十八日

行政院國家科學委員會專題研究計畫成果報告
國科會專題研究計畫成果報告撰寫格式說明
Preparation of NSC Project Reports

計畫編號：NSC 92－2311－B－009－005
執行期限：92 年 8 月 1 日至 93 年 7 月 31 日
主持人：袁俊傑
執行機構及單位名稱:國立交通大學生物科技系

一、中文摘要

本計畫的主要目的是希望瞭解人類 Ste20 蛋白激酵素 Mst3 的生理功能。Mst3 是一人類 Ste20 蛋白家族的 Ser/Thr 激酵素，先前在我們已證實 Mst3 是 caspases 的受質，而 Mst3 受到截切不但使細胞產生凋亡現象亦會造成核轉移之現象。這些發現證明 Mst3 可能在細胞凋亡的過程中扮演著重要的角色。在本實驗中我們進一步利用分子技術建構了數株與綠螢光蛋白融合之 Mst3 突變株，並應用螢光顯微鏡觀察綠螢光在細胞中的分佈狀況。由此一系列實驗我們發現 Mst3 上同時具有 NLS 及 NES 兩種訊息序列，而 Mst3 在細胞質及細胞核中的分怖便取決於這兩序列活性的消長有絕對的關係。另外我們亦發現大量表現 Mst4 可造成乳癌細胞 MCF-7 的凋亡，此一凋亡現象的發生可能是經由 JNK 的訊息傳遞路徑。

關鍵詞：Mst、激酵素、細胞凋亡、Jun N-端蛋白激酵素

Abstract

The main objective of this project is to elucidate the physiological functions of mammalian sterile 20 (Ste20)-like protein kinase 3, Mst3. Mst3 is a member of human Ste2-like serine/threonine protein kinases with unknown physiological function. Previous studies have shown that Mst3 is the substrate of caspases. The cleavage of Mst3 exhibited not only the characteristic of apoptosis, but also the nuclear translocation. These findings suggest that Mst3 may play an important role in the process of apoptosis. In this study we further generated several EGFP-tagged, C-terminal truncated Mst3 mutants. The green fluorescence the fusion proteins was monitored under fluorescent microscope. Interestingly, we found that Mst3 might contain both NLS and NES regions that dynamically control the subcellular distribution between cytoplasm and nucleus. Furthermore, Mst4 was also found to induce the apoptosis blast cancer cell line MCF-7 via a JNK-dependent pathway.

Keywords: Mst, Kinase, Apoptosis, JNK

二、緣由與目的

Ste20 (Sterile-20)-like family kinases are the newly emerging sensory protein kinases that play important roles in regulating various cellular events including matting, regulation of apoptosis, regulation of cell growth, and rearrangement of the cytoskeleton leading to cell-shape change and cell motility [1-5].

Until now, about 30 Ste20-related protein kinases have been identified in mammals, *Drosophila*, *Caenorhabditis elegans* and other organisms. Based on the relative location of kinase domain and functional properties, mammalian Ste20-like protein kinases can be categorized into two subfamilies, PAK and GCK. PAK (p21-activated kinase) subfamily contain a C-terminal kinase domain and an N-terminal regulatory domain, which binds p21 small G-protein, Cdc42/Rac. The yeast Ste20 [3] and human p21-activated kinases 1-4

(hPAK1-4) [1,5] belong to this class. The GCK (germinal center kinase) family members, however, are distinguished from PAK family kinases in that they have a kinase domain at their N-terminus instead of at the C-terminus. The GCK family represents a rather large family of protein kinases with over twenty members identified in humans thus far [6].

Mst protein kinases belong to the GCK-type human Ste20-like protein kinase family, homologous to the budding yeast Ste20 Ser/Thr kinase throughout their kinase domains. Four members in Mst family, Mst1/Krs2, Mst2/Krs1, Mst3, and Mst4/MASK, have been found and cloned so far [7-12]. The amino acid sequence alignment of all four Mst members have revealed that Mst protein kinases are highly related to each other with amino acid similarities ranging form 53% to 88% [10]. Although all Mst family kinases are members of GCK kinase family, they belong to two different GCK sub-families. Mst1/Krs2 and Mst2/Krs1 belong to GCK-II subfamily, while the closely related members Mst3 and Mst4/MASK are in GCK-III subfamily [6].

Mst3 (mammalian Ste20-like protein kinase 3) was cloned independently from human HeLa cell cDNA library [10] and human gastric cancer cell cDNA library [12], respectively. The cDNA of Mst3 encodes a 431-amino acid protein with an expected molecular mass of 50 kDa. Although the physiological function of Mst3 is obscure by now, it has been shown to play a role in cell apoptosis [12]. The caspase-dependent cleavage of Mst3 was demonstrated by using cell extracts from apoptotic Jurkat cells. The cleavage of Mst3 could be inhibited by Ac-DEVD-CHO, a potent inhibitor of caspase 3. Over-expression of either wild-type Mst3 or a truncated mutant induced a characteristic relate to apoptosis [12]. DNA fragmentation assay and exogenously expressed β-galactosidase activity assay further confirmed the role of Mst3 in apoptosis. In contrast, cells containing control vector only or kinase-dead mutant, $Mst3^{K53R}$, were morphologically normal. These results strongly support the postulation that Mst3 plays an important role in apoptosis.

三、結果與討論

The expression of GST-Mst3 fusion proteins - We expressed GST-Mst3 fusion proteins for two reasons: i) GST-$Mst3^{WT\ 刮\ 314}$ would be used as antigen for the preparation of monoclonal antibody specific against the N-terminal region of Mst3 and ii) GST-Mst3 will be used in the pull-down assay to isolate the intracellular associated protein. Various expression vectors for GST-Mst3 fusion proteins, GST-$Mst3^{WT\ 刮\ 314}$, GST-$Mst3^{1\text{-}85}$, and GST-$Mst3^{244\text{-}313}$, were constructed. These fusion proteins were overexpressed and purified on a glutathione agarose column as demonstrated on a 10% SDS-polyacrylamide gel. The purified GST-$Mst3^{WT\ 刮\ 314}$ was, then, used as an antigen for the preparation of monoclonal antibody specific for Mst3 N-terminal domain.

Leptomycin B Inhibits Nuclear Export of Mst3 - To examine that whether Mst3 contains NES, the EGFP-$Mst3^{WT}$ transiently expressed-HeLa cells were treated with 10 ng/mL leptomycin B (LMB), an inhibitor of the Crm1-mediated export. As the result, the originally cytoplasmic-localized EGFP-$Mst3^{WT}$ began to accumulate in the nucleus after LMB treatment. This result suggests that Mst3 may contain a NES-like sequence, which is functionally active and that the Crm1/exprotin-dependnet nuclear exporting mechanism may be responsible for the nuclear export of the full-length Mst3.

NES controls the subcellular localization of Mst3 - The amino acid sequence alignment revealed that the C-terminal regulatory domain of Mst3 may contain two Leu/Ile-rich sequences that are characteristic of NES signals. Therefore, the C-terminal regulatory domain of EGFP-tagged Mst3 was serially deleted and transiently expressed in HeLa cells. The result showed that green fluorescence began to accumulate in the

nucleus when the regions between amino acids 386-416 and 337-385 were lost. This result demonstrates that the C-terminal regulatory domain of Mst3 contains the NES. To further identify the NES in Mst3 various EGFP-tagged C-terminal fragments of Mst3 were constructed and transiently expressed in the HeLa cells. Interestingly, a significant amount of the $Mst3^{312-431}$- or $Mst3^{335-431}$-tagged EFGP was found to accumulate in the cytoplasm, while $Mst3^{385-416}$-tagged EGFP remained in the nucleus. These results suggest that Mst3 may contain a NES, which is in the region between amino acids 335 and 385. These results were published recently [13].

Overexpression of Mst4 in MCF-7 cells resulted in cell death - Compared with Mst3, Mst4 overexpression could induce significant cell death (~50%) of human breast cancer cell line MCF-7. Two other chimeric protein kinases, Mst4/N-Mst3/C and Mst3/N-Mst4/C also exhibited strong potential of inducing cell death of MCF-7. Further study showed that overexpression of Mst4 in MCF-7 cells could induce an extensive DNA fragmentation, a characteristic of apoptosis. This result suggests that Mst4, similar to Mst3, involved in the process of apoptosis.

Mst4-indiced cell apoptosis was demonstrated by TUNEL assay - TUNEL (TdT-UTP Nick End Labeling) assay is sensitive in detecting the DNA fragmentation process at early apoptotic stage. The MCF-7 cells were transiently overexpressed $Mst4^{WT}$, and $Mst4^{KR}$ and subjected to TUNEL assay. The $Mst4^{WT}$-overexpressed MCF-7 cells exhibited extensively brown granule deposit in the nucleus, while almost no brown granule deposit was seen in cells with pcDNA3.1 or with $Mst4^{KR}$.

Mst4-induced MCF-7 apoptosis is a JNK- but not p38 MAPK-dependent process - Interestingly, we found that the Mst4-induced apoptosis of MCF-7 cells is a JNK-dependent manner. The p38 MAPK, on the other hand, is not involved in this process. The Mst4-induced MCF-7 apoptosis could be totally reversed by 1 贡 M JNK inhibitor SP60015, whereas, p38 MAPK inhibitor could not effectively inhibit Mst4-induced apoptosis at as high as 1 贡 M.

四、計畫成果自評

The major goal of this project is to elucidate the molecular mechanism of Mst3-mediated cell apoptosis and find out the physiological function of Mst4. Although the whole picture of the molecular mechanism underlying the Mst3-induced apoptosis is remained to be elucidated, the results obtained in this project could partially explain how Mst3 acted to mediate the apoptosis of cell. First, we showed the existence of NES in the C-terminal regulatory domain of Mst3 that might counteract the activity of previously found NLS signal. This result may explain the fact that Mst3 can translocate from cytoplasm to the nucleus upon the caspase cleavage. A breakthrough in the study of role of Mst4 in cells we is that we found that Mst4 may also play a role in regulating the apoptosis of a cancerous cell line MCF-7. In contrast to Mst3, Mst4 may mediate cell apoptosis through a JNK signaling pathway. In the future, we hope to consult these results and develop new experiments to further elucidate the molecular mechanisms underlying the Mst3- and Mst4-mediated cell apoptosis.

五、參考文獻

[1] Bagrodia, S. and Cerione, R. A. (1999) Pak to the future. *Trends Cell Biol.* **9,** 350-355.

[2] Kriakis, J. M. (1999) Signaling by the germinal center kinase family of protein kinase. *J. Biol. Chem.* **274,** 5259-5262.

[3] Leberer, E., Dignard, D., Harcus, D., Thomas, D. Y., and Whiteway, M. (1992) The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein 刍讪 subunits to downstream signaling components. *EMBO J.* **11,** 4815-4824.

[4] Sells, M. A. and Chemoff, J. (1997) Emerging from the Pak: the p21-activated protein kinase family. *Trends Cell Biol.* **7,** 162-167.

[5] Sells, M. A. (1999) Pictures in cell biology: Pak1 kinase activity affects the character of cell. *Trends Cell Biol.* **9,** 355.

[6] Dan, I., Watanabe, N. M., and Kusumi, A. (2001) The Ste20 group kinases as regulators of MAP kinase cascade. *Trends Cell Biol.* **11,** 220-230.

[7] Creasy, C. L. and Chernoff, J. (1995) Cloning and characterization of a human protein kinase with homology to Ste20. *J. Biol. Chem.* **270,** 21695-21700.

[8] Dan, I., Ong, S.-E., Watanabe, N. M., Blagoev, B., Nielsen, M. M., Kajikawa, E., Kristiansen, T. Z., Mann, M., and Pandey, A. (2002) Cloning of MASK, a novel member of mammalian Germinal Center Kinase-III subfamily, with apoptosis-inducing properties. *J. Biol. Chem.* **277,** 5929-5939.

[9] Lin, J.-L., Chen, H.-C., Fang, H.-I., Robinson, D., Kung, H.-J., and Shih, H.-M. (2001) Mst4, a new Ste20-related kinase that mediates cell growth and transformation via modulating ERK pathway. *Oncogene* **20,** 6559-6569.

[10] Schinkmann, K. and Blenis, J. (1997) Cloning and characterization of a human Ste20-like protein kinase with unusual cofactor requirements. *J. Biol. Chem.* **272,** 28695-28703.

[11] Taylor, L. K., Wang, H. C., and Erikson, R. L. (1996) Newly identified stress-responsive protein kinase Krs-1 and Krs-2. *Proc. Natl. Acad. Sci. USA* **93,** 10099-10104.

[12] Huang, C.-Y. F., Wu, Y.-M., Hsu, C.-Y., Lee, W.-H., Lu, T.-J., Lai, M.-D., Lu, T.-J., Huang, C.-L., Leu, L.-H., Shih, H.-M., Fang, H.-I, Robinson, D. R., Kung, H.-J., and Yuan, C.-J. (2002) Caspase Activation of Mammalian Sterile 20-like Kinase 3 (Mst3): Nuclear translocation and induction of apoptosis. *J. Biol. Chem.* **277,** 34367-34374.

[13] Lee, W.-S., Hsu, C.-Y., Wang, P.-L., Huang, C.-Y. F., Chang, C.-H., and Yuan, C.-J. (2004) Identification and Characterization of the Nuclear Import and Export Signals of the Mammalian Ste20-like Protein Kinase 3 (Mst3). *FEBS Lett.* **572,** 41-45.