行政院國家科學委員會專題研究計畫　成果報告

人類 Ste20 激酵素 Mst3 在細胞中的作用機轉之研究

計畫類別：個別型計畫
計畫編號：NSC91-2311-B-009-003-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立交通大學生物科技研究所

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 11 月 7 日

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『人類 Ste20 蛋白激酵素 Mst3 在細胞中作用分子機轉之研究』

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中文摘要

本研究計畫的主要目的是希望瞭解人類 Ste20 蛋白激酵素 Mst3 在細胞進行凋亡中的角色。Mst3 是一生理活性還不甚明瞭的人類 Ste20 蛋白 Ser/Thr 激酵素，先前我們實驗室與其他合作的實驗室的研究中已經顯示 Mst3 是 caspases 的受質，而 Mst3 受到截斷後會使得細胞產生與凋亡現象密切相關的特徵。再者，從先前的研究中，我們亦發現截斷後的 Mst3 會進行核轉移。進一步研究證實 Mst3 上具有一會導致核移轉的 NLS 片段，及兩段會促成核蛋白外移的核轉出序列（NES）在 Mst3 的 C-端，此一序列的存在會促使蛋白質離開細胞核。然而 Mst3 在核中所扮演的角色目前並不清楚。另外我們亦製備了 Mst3 的高專一性單株抗體 2A8, 此單株抗體只辨認 Mst3 而不會與序列極相似的 SOK1 及 Mst4 產生交互作用。我們已將其用於人體組織切片的研究中，我們發現 Mst3 似乎主要存在於腎臟的腎小管的上皮組織內。

英文摘要

The main objective of this project is to understand the role of human sterile-20-like protein kinase, Mst3, in cells during apoptosis. Mst3 is a human Ste2-like protein serine/threonine kinase of unknown physiological function. Previous studies of our and the collaborated laboratories have shown that Mst3 is the substrate of caspases. The cleavage of Mst3 is correlated with the characteristics of apoptosis. In this project, we further showed that truncated Mst3 kinase could translocate to the nucleus. Further studies were performed to demonstrate that Mst3 contained one nuclear localization sequence (NLS) at amino acids 278-294 and two nuclear exportation sequences (NES) located at the C-terminus of Mst3. The role of Mst3 in nucleus is, however, is unclear so far. A monoclonal antibody mAb2A8 specifically against the N-terminal part of Mst3 were generated in our laboratory. The generated mAb2A8 showed no cross reactivity against SOK1 and Mst4. Preliminary histochemical studies on human kidney tissues showed that Mst3 mainly existed in the epithelium of tubules.

背景介紹

Mst3 (mammalian Ste20-like protein kinase 3) was cloned independently from human HeLa cell cDNA library (Schinkmann and Blenis, 1997) and human gastric cancer cell cDNA library (Huang et al., 2002), respectively. The cDNA of Mst3 encodes a 431-amino acid protein with an expected molecular mass of 50 kDa. It possesses two major functional domains, an N-terminal kinase domain and a C-terminal regulatory domain. Based on the amino acid sequence similarity, Mst3 belongs to the GCK-III or Sps-1 subgroup. The kinase domain of Mst3 is highly homologous to Ste20/oxidant stress response kinase (SOK-1) and Mst4 with a sequence similarity of 88%. Although the physiological function of Mst3 is obscure by now, it has been shown to play a role in cell apoptosis (Huang et al., 2002). 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. Similar result could also be observed in HeLa cells when treated with TNF-α with or without cycloheximide. Using apoptotic Jurkat cell extract and recombinant caspases, the caspase cleavage site was mapped at $AETD^{313}$, which is at the junction of the N-terminal

kinase domain and the C-terminal regulatory domain. Over-expression of either wild-type Mst3 or a truncated mutant inducd a characteristic relate to apoptosis (Huang et al., 2002). 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. Similar to Mst1, the truncated Mst3 was found to translocate and accumulate in the nucleus (Hsu, 2000; Huang et al., 2002). The NLS (nuclear localization sequence) and NES (nuclear exporting signal) motifs were further identified at the C-terminal region of the kinase domain as well as in the C-terminal regulatory domain, respectively (Fig. 4; Lee, W.-S., Hsu, C.-Y. and Yuan, C.-J. unpublished data). These findings suggest that nucleus localization of truncated Mst3 may relate to its activity in inducing apoptosis. Unlike Mst1, Mst3 is not engaged with any known MAPK signaling pathways as demonstrated by report from our and other laboratories (Huang et al., 2002; Schinkmann and Blenis, 1997).

結果與討論

Identification of NES domains in Mst3

Although we have identified the NLS domain in MST3, The nuclear localization of EGFP-$Mst3^{WT\triangle 314}$ may also controlled by nuclear export signal (NES). To examine this possibility, we used leptomycin B (LMB), an inhibitor of Crm1-mediated nuclear exporting process, to see whether LMB can change the cellular distribution of Mst3. The result showed that EGFP-$Mst3^{WT}$ was mainly distributed in the cytoplasm of the HeLa cells. After treatment with or without LMB, EGFP-$Mst3^{WT}$ started accumulating in the nucleus. This result suggests that Mst3 may contain a NES domain as proposed earlier. In order to find out the proposed NES region of Mst3, we generated several EGFP-tagged C-terminal truncated Mst3 mutants, termed EGFP-$MST3^{WT\Delta 416}$ (a.a. 416-431 was removed), EGFP-$Mst3^{WT\Delta 385}$ (a.a. 385-431 was removed), EGFP-$Mst3^{WT\Delta 336}$ (a.a. 336-431 was removed). EGFP-$MST3^{WT\Delta 416}$, similar to EGFP-$Mst3^{WT}$, still remains in the cytoplasm, while EGFP-$Mst3^{WT\Delta 385}$ and EGFP-$Mst3^{WT\Delta 336}$ translocated to the nucleus. This result suggests that the amino acid sequences between 416 and 385 and between 335 and 386 of Mst3 may contain potential NES domains.

Generation of monoclonal antibody for Mst3

We expressed GST-Mst3 fusion proteins for two reasons: i) GST-$Mst3^{WT\triangle 314}$ 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-$Mst3^{WT\triangle 314}$, 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-$Mst3^{WT\triangle 314}$ was, then, used as an antigen for the preparation of monoclonal antibody specific for Mst3 N-terminal domain.

After immunization, hybridoma preparation, and screening, a specific monoclonal antibody for Mst3 N-terminal, clone 2A8, was obtained. The binding specificity of mAb 2A8 was initially tested on ELISA, followed by Western blotting by using GST and GST-Mst3 fusion proteins, GST-$Mst3^{WT\triangle 314}$, GST-$Mst3^{1\text{-}85}$, and GST-$Mst3^{244\text{-}313}$, as targets. We found that mAb 2A8 could recognize GST-$Mst3^{WT\triangle 314}$ with high affinity, whereas GST and GST-$Mst3^{244\text{-}313}$ could barely be detected. The GST-$Mst3^{1\text{-}85}$ could only be detected by mAb 2A8 on ELISA method. Further study showed that mAb 2A8 could distinguish Mst3 from two closely related Ste20-like protein kinases, Mst4 and SOK1. Both Mst4 and SOK1 exhibit around 88% amino acid sequence homology to Mst3.

Immunohistochemical studies of human tissues

In collaborating with anatomical pathologists, we performed imunohistochemical studies on several human tissues, such as placenta, kidney, and skin. The preliminary results showed that human Mst3 does exist in the organs tested. This result is consistent with the results observed in Northern blotting analysis. Interestingly, we found that Mst3, instead of distributing in every tissue, only concentrated in certain tissues in the organs tested. In kidney, for example, Mst3 is mostly concentrated in the epithelial cells of distal convoluted tubules and collecting ducts and expressed in less amount in proximal convoluted tubules. In contrast, no Mst3 was seen in the glomeruli and other interstitial areas.

The time course of Mst3 induced cell apoptosis

Overexpression of wild type Mst3 induced cell apoptosis with a time-dependent manner. 36 hours after transfection the survival rate of HeLa cells reduced to about 50%. The TUNEL assay also showed that the overexpression of full length Mst3 significantly induced DNA fragmentation in cells compared with that of control cells.

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成果自評

The major goal of this project is to understand the role of mammalian Ste20-like protein kinase Mst3 in the process of cell apoptosis. Although the physiological functions of Mst3 is still not fully understood, the results obtained in this project make us believe that Mst3 is one the mediators in the process of apoptosis. Furthermore, we have identified a NLS and two NES regions in Mst3. The nuclear translocation may signal and amplify the apoptotic response. Most importantly, we generated a highly specific monoclonal antibody 2A8 for Mst3. Preliminary immunohistochemical study on human kidney tissues by using this monoclonal antibody showed that Mst3 may specifically distributed in the epithelium of tubules of the kidney. We expect to use this monoclonal antibody to explore the physiological functions of endogenous Mst3.