行政院國家科學委員會專題研究計畫　成果報告

GCK-II 家族 Ste20 蛋白激酵素 Mst3 及 Mst4 的生物功能與作用機轉之比較

計畫類別：個別型計畫
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Preparation of NSC Project Reports

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一、中文摘要

本計畫的主要目的是希望瞭解人並比較蛋白激酵素 Mst3 及 Mst4 的生化特性及作用機轉。在本實驗中我們發現 Mst4 造成 MCF-7 及 HeLa 細胞凋亡的機轉與 JNK 有關。進一步實驗發現 Mst3 是一粒線體蛋白，而造成細胞凋亡的過程中則與 caspases 無關。Mst3 是一粒線體蛋白的假設，由免疫螢光染色、西方墨點法及電子顯微鏡等實驗得到證實。進一步實驗我們發現 Mst3 會同時與粒線體中的促凋亡蛋白質 AIF 及 endoG 結合，Mst3 亦會在抗癌藥 staurosporine 的刺激下而從粒線體中轉移到細胞核內。Mst3 核移轉的角色目前未知。另外我們亦發現 Mst3 會在自然產的胎盤 cytotrophoblasts 細胞中大量產生，此一現象的發生似乎與 oxidative stress 有關。

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 and compare the molecular mechanism of mammalian Ste20-like protein kinase 3 and 4, Mst3 and Mst4. Mst4 was demonstrated to induce the apoptosis in MCF-7 and HeLa cells. Interestingly, different from Mst3, Mst4 was found to be involved in JNK signaling pathway. Further studies showed that Mst3 was resided in mitochondria and could induce the cell apoptosis via a caspase-independent pathway. The localization of Mst3 in mitochondria was demonstrated by immunofluorescence staining, western blotting and electronic microscopic monitoring. In mitochondria, Mst3 was further shown to associated with pro-apoptotic proteins, AIF and endoG. Interestingly, Mst3 could translocate from mitochondria to nucleus upon staurosporine treatment. The role of nuclear translocation of Mst3 remains to be elucidated. In this project, we also demonstrated that Mst3 was overexpressed in cytotrophoblasts of human placenta during spontaneous delivery. The induction of Mst3 seemed to be an oxidative stress-dependent manner.

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 [6].

Mst protein kinases belong to the GCK-type human Ste20-like protein kinase family. 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 have revealed that Mst protein kinases are highly related to each other with amino acid similarities ranging form 53% to 88% [10].

Mst3 (mammalian Ste20-like protein kinase 3) is 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. Recently, we found that Mst3 might contain both NLS and NES regions that dynamically control the subcellular distribution between cytoplasm and nucleus [13].

三、結果與討論

Mst3 induces apoptosis via a caspase-independent pathway –Caspases are the central executioners of most classical apoptotic pathways and are suggested to involve in Mst3-mediated apoptosis in Jurkat cells [12]. To elucidate the role of caspases in Mst3-induced apoptosis universal caspase inhibitor z-VAD-fmk was used in an exogenous β-galactosidase assay, in which pSV-LacZ and $Mst3^{WT}$ or its mutants were co-transfectd into the HeLa cells. Notably, $Mst3^{WT}$-induced cell death could not be inhibited by 50 μM zVAD-fmk. Both pcDNA3.0 vector and $Mst3^{KR}$ showed no significant effect on HeLa cells and were not affected by caspase inhibitor. The result suggests that Mst3 may induce apoptosis of HeLa cells in a caspase-independent manner.

Neither JNK nor p38/MAPK was involved in Mst3-induced apoptosis

Mst1 or Mst2 has been shown to induce apoptosis through the JNK/SAPK and p38/MAPK pathways [9]. Although Mst3 was shown to induce cell apoptosis through a pathway independent of any known MAPK pathway [12], we want to further confirm this observation using exogenous β-galactosidase assay in the presence of SP600125, an inhibitor for JNK or SB220025, an inhibitor for p38/MAPK. As expected Mst3-induced apoptosis of HeLa cells was not affected by JNK inhibitor, SP600125 and p38/MAPK inhibitor, SB220025, confirming the previous observations [12].

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 M JNK inhibitor SP60015, whereas, p38 MAPK inhibitor could not effectively inhibit Mst4-induced apoptosis at as high as 1 M.

Mitochondrial localization of Mst3 by Immunofluorescent study- The subcellular localization of endogenous Mst3 was investigated by immunofluorescence staining of HeLa cells. The mitotracker, a mitochondrial marker, was used in this study. The results show that Mst3 co-localizes with the mitotracker, AIF and endoG, indicating

the presence of Mst3 in mitochondria. The reason for the presence of Mst3 in mitochondria is, however, remain elucidated.

Mitochondrial localization of Mst3 by Western blot analysis and electronic microscopic study - The existence of endogenous Mst3 in various cellular fractions, including cytosol, mitochondria and nucleus, was further determined by Western blotting. As expected, we found that endogenous Mst3 was not just present in the cytoplasm but also existed in the mitochondria. Consistent with previous observation, without stimulation, only little Mst3 is present in the nucleus. The existence of Mst3 in mitochondria was also proved by electronic microscope.

Staurosporine induces nuclear localization of Mst3 - Earlier work has shown that the association of Mst3 with AIF and endoG may be essential for regulation of apoptosis. AIF and endoG were shown previously to translocate to nucleus in response to apoptotic signal. The distribution between cytoplasm and nucleus of Mst3 could be dynamically controlled by its NLS and NES regions [13]. Interestingly, we demonstrated that Mst3 and other apoptosis regulating factors, such as AIF and EndoG, might promptly translocate from mitochondria to nucleus upon the treatment of 0.5 μM staurosporine at 37ºC for 3 h. The subcellular distribution of other mitochondrial proteins, such as cytochrome C and Bcl-2, were not changed by stauroaporine.

Mst3 was expressed in cytotrophoblast of human placenta at term - It has been shown that prior term labor a substantial population of trophoblasts of human fetal membrane underwent apoptosis. The apoptosis was thought to be induced by oxidative stress with the evidence that women in active labor exhibited high level serum hydroperoxides. Thus, oxidative stress is postulated to be involved in initiating the labor process. To understand the molecular mechanism underlying the oxidative stress-induced trophoblast apoptosis we checked the signs of oxidative stress, DNA fragmentation, caspase 3 activation and Mst3 expression in the human full term and selective cesarean placenta and 6~8 weeks endometrium spacemen by Immunohistochemistry. Mst3 is a human Ste20-like protein kinase that may play a role in apoptosis in response to apoptotic signals. Interestingly, compared with cytotrophoblasts in the first-trimester of pregnancy and selective cesarean placenta, we found that the cytotrophoblasts underwent an extensive apoptosis in a full term placenta. Furthermore, the cytotrophoblasts at full term placenta contained high level Mst3 and nitrotyrosine, a marker of oxidative stress, which were undetectable in trophoblasts of first-trimester and of selective cesarean placenta.

四、計畫成果自評

The major goal of this project is to compare the molecular mechanism of Mst3 and Mst4-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. We showed for the first time that Mst3 is mainly present in mitochondria, where it associates with pro-apoptotic proteins, AIF and endoG. This observation can partially explain the observation that Mst3 induces a caspase-independent cell apoptosis. Mst3 was further showed to be overexpressed in cytotrophoblasts of human placenta during spontaneous delivery. The role of facilitating placenta delivery after fetus delivery is postulated upon this observation. 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.

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