行政院國家科學委員會補助專題研究計畫成果報告

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人類 Ste20 激酵素，Mst-3,在癌細胞中之特性研究

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計畫主持人：袁俊傑

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執行單位：交通大學生物科技系

中　華　民　國 八十九 年　十　月 三十　日

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，Mst-3,在癌細胞中之特性研究
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主持人：袁俊傑　國立交通大學生物科技系

一、中文摘要

磷脂酵素 A_2 刺激第二型前列腺素合成酵素的生合成的能力可因 staurosporine 的存在具有加成的效果。我們由西方墨點與北方墨點分析的結果，進一步發現 staurosporine 本身即可刺激第二型前列腺素合成酵素在細胞中大量的生合成。為了解轉錄因子在此一作用的角色，我們逐步移除第二型前列腺素合成酵素的啟動子辨識序列，從而發現-188 到+70 的片段在 staurosporine 刺激第二型前列腺素合成酵素的表現中扮演著重要的角色。這一段序列中含有三個啟動子辨識區，即 AP-2、NF-IL6 與 CRE。進一步經由定位突變後，證實 AP-2 及 NF-IL6 可經 staurosporine 刺激而活化，並進而造成第二型前列腺素合成酵素在老鼠成骨細胞中的表現 以 20nM staurosporine 刺激後，發現含有質體 GLB/NA、GLB/NC 及 GLB/AC 的細胞，其蟲螢光素酵素的活性在八小時後達到最高，而在十小時後則急速降低。此一現象不因激酵素 C 的抑制劑 GF 109203X 的加入而有所改變，證明 staurosporine 激發第二型前列腺素合成酵素之生合成與激酵素 C 的訊息傳遞路徑無關。

關鍵詞：胰磷酯質酵素 A_2、胰磷酯質酵素 A_2 受體、NF-IL6、Staurosporine、離氨酸激酵素、環氧化酵素

Abstract

MST3 is a member of GCK-II serine/threonine protein kinase family with unknown physiological functions. It contains a kinase domain at its N-terminus, while a regulatory domain at its C-terminus. Previous studies have shown that MST3 is a cytoplasmic protein and ubiquitously expressed in many species. Recently we found that endogenous MST3 could be specifically cleaved by caspases when incubated with apoptotic Jurkat cell extracts after the treatment of staurosporine or Fas ligation. Similar result was also observed in other cell lines, e.g., Hela and A431 cells. This cleavage could be inhibited by Ac-DEVD-CHO, a potent inhibitor of caspase 3. Using apoptotic Jurkat cell extract and recombinant caspases, we mapped the caspase cleavage site, $AETD^{313}$, which is at the junction of the N-terminal kinase domain and the C-terminal regulatory domain. Overexpression of either wild type or regulatory domain truncated MST3 in cells resulted in morphological changes, e.g., cell shrinkage and nuclear condensation, which were characteristics of apoptosis. These results were also confirmed by DNA fragmentation assay and exogenously expressed â-galactosidase activity assay. In contrast, the cell contained vector only or kinase-dead mutant, MST3-K53R, was morphologically normal. These results strongly support the postulation that MST3 may play an important role in apoptosis. The molecular mechanism underlying this process, however, remains to be studied.

Keywords: PLA2-I, PLA2-I receptor, NF-IL6, Staurosporine, Tyrosine kinase, Cyclooxygenase-2,

二、緣由與目的

Apoptosis (or programmed cell death) is a naturally occurring cell death originated very early in biological evolution and found throughout all animal kingdoms [1,2]. This type of regulation allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage. Thus, the dysregulation of apoptotic cell death may be involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune diseases, neurodegenerative disorders, and viral infection [3-6]. Growing evidence suggests that although apoptosis stimuli vary from cell to cell, there seems to have basic biochemical machinery underlying the process of apoptosis. This basic biochemical machinery involves the activation of a family of Cys-dependent, Asp-specific proteases known as the caspases [7-9]. The activated caspases can cleave the downstream substrates, including components of cellular DNA repair mechanisms, inhibitors of apoptosis, and structural proteins.

Protein kinases emerge as the direct substrates and effectors of caspases [10,11]. For example, caspase-mediated proteolytic degradation inactivated two anti-apoptotic protein kinases, Raf-1 and Akt, as suggested by Willdman and colleagues [12]. Proteolytic cleavage of ATM generated a kinase inactive protein and prevented DNA repair and DNA damage signaling [13]. Cleavage of focal adhesion kinase (FAK) by caspases interrupts the assembly of the focal adhesion complex, resulting in cell death [14,15]. In contrast, caspase cleavage in response to apoptotic stimuli might activate other type of kinases. This type of kinases includes MEKK-1 [16,17]. PAK2/hPAK65 [17-19], PKC isoforms δ [20-22] and θ [23], PKN [24], and MST1 [25,26]. In each case, caspase cleavage generated a constitutively active kinase by removing inhibitory domains from the proteins. Importantly, the active fragments are then acting as signals propagating the apoptosis processes. These observations imply that protein phosphorylation may play an essential role in apoptotic signal transduction.

Mst3, a 52 kDa protein, is one of mammalian Ste20-like serine/threonine protein kinase (Mst) family [27]. Mst3 is a ubiquitously expressed protein serine/threonine kinase with unknown physiological functions. Mst3 belongs to the Sps-1 subgroup of Ste20-like protein kinases consisting of the N-terminal kinase domain and a C-terminal regulatory domain. The kinase domain of Mst-3 is most relating to Ste20/oxidant stress response kinase (SOK-1) with a sequence identity of 88%. Mst protein kinase family plays an important role in cell apoptosis [28]. For example, Mst1, a ubiquitously expressed serine/threonine protein kinase, can be cleaved and activated by caspase3 during apoptosis. Overexpression of either full length Mst1 or its truncated mutant in BJAB cells induced morphological changes characteristic of apoptosis [25]. Mst2 differentially expressed in kidney, skeletal muscle and placental tissues. Recently the cleavage of MST2 associated with anticancer drug-induced apoptosis as reported previously [28,30]. The single report showed that Mst3 is not involved in any known signaling pathways [27]. It is likely that Mst-3 is involved in a novel-signaling pathway.

Recently, MST3 was found to be readily cleaved by caspase3 as well as caspase8 through *in vitro* study and generated two fragments with sizes of around 40 kDa and 13 kDa, respectively. The cleavage of endogenous MST3 was also demonstrated in Jurkat cells treated with Fas antibody or Staurosporine. Similar results could be observed in Hela cells when treated with TNF-α with or without cycloheximide. The cleavage of MST3 followed the same time courses as that for activation of caspase activity. This process could be specifically blocked by caspase 3 and caspase 1 inhibitors, Ac-DEVD-CHO, Ac-YVAD-CHO, respectively. Using apoptotic Jurkat cell extract, the caspase cleavage site, in MST3 at $AETD^{313}G$. These observations implicated that MST3 may be involved in the process of apoptosis. The mechanism underlying this

process, however, is unknown so far. We propose that the proteolytic cleavage of MST3 may activate its kinase domain and contribute to the characteristics of apoptosis.

三、結果

Overexpression of Mst-3 results in characteristics of apoptosis- To elucidate the role of Mst-3 in apoptosis, we generated the fusion proteins of enhanced green fluorescent protein (EGFP) and Mst-3s. GFP is often used as a reporter to monitor gene expression, protein-protein interaction, vesicles trafficking and protein localization in vivo. In this study, EGFP may help us to tell whether expression of Mst-3 is the main cause of cell apoptosis. Several EGFP-tagged Mst-3s were generated by insertion of Mst-3 cDNAs into a pEGFP plasmid (Clontech), termed EGFP-Mst-3^{WT} (with full length wild type Mst-3, a.a. 1-431), EGFP-Mst-3^{KR} (with a kinase-dead Mst-3, i.e., Lys^{53} to Arg mutation), EGFP-Mst-$3^{WT\Delta 314}$ (a C-terminal region a.a. 314-431 removed Mst-3), and EGFP-Mst-$3^{KR\Delta 314}$ (a truncated kinase-dead mutant), resprectively (Fig. 2). These vectors were then transiently transfected into the human embryonic kidney 293 (HEK293) and HeLa cells, respectively. Thirty-six hours following transfection, cells expressing either EGFP/Mst-3^{WT} or EGFP/Mst-$3^{WT\ \Delta\ 314}$ displayed a profoundly shrunken morphology and nuclear condensation as determined by green fluorescence and DAPI staining. In contrast, cells expressing EGFP/Mst-3^{KR} or EGFP/Mst-$3^{KR\ \Delta\ 314}$ were morphologically normal. These results demonstrated that expression of the Mst-3 kinase domain induces morphological change characteristic of apoptosis.

In the final stage of apoptosis, chromatin can be fragmented by nucleases, while the plasma membrane still remains intact. Hence the DNA ladders become one of characteristics of apoptotic cells. To demonstrate that Mst-3 does involve in the process of apoptosis, we transiently expressed EGFP-Mst-3^{WT} and its truncated mutants, EGFP-Mst-$3^{WT\ \Delta\ 314}$ and EGFP-Mst-$3^{KR\Delta 314}$, in HEK293 cells. Thirty six-hour following transfection the small molecular weight DNA was isolated and analyzed on a 2% agarose gel. As shown in Fig. 4A, overexpression of EGFP-Mst-3^{WT} and EGFP-Mst-$3^{WT\ \Delta\ 314}$ in HEK293 cells resulted in DNA fragmentation. In contrast, the cells expressing EGFP only or EGFP-$MST3^{KR\ \Delta\ 314}$ were shown no DNA fragmentation at all (Fig. 3A). This result further confirmed the role of Mst-3 in apoptosis.

The extent of cell death could also be determined by the activity of exogenous β-galactosidase expressed in cells. Only the viable cells can hold the expressed the β-galactosidase inside the cell. The β-galactosidase activity is presented as relative units with the activity from cells expressing LacZ only defined as 1. To find out the effect of Mst-3 and its mutant in apoptosis, HEK293 cells were co-transfected the pCMV/LacZ reporter vector with various EGFP-Mst-3 plasmids, i.e., EGFP-Mst-3^{WT}, EGFP-Mst-$3^{WT\Delta 314}$, and EGFP-Mst-$3^{KR\Delta 314}$. The cells expressing LacZ vector only were used as the control. Compared with control, the cells expressed EGFP-Mst-3^{WT} and EGFP-Mst-$3^{WT\ \Delta\ 314}$ exhibited significantly low β-galactosidase activities, about 55% and 28% of control, respectively (Fig. 3B). In contrast, cells expressing EGFP alone or EGFP-Mst-$3^{KR\ \Delta\ 314}$ fusion protein showed normal β-galactosidase activity compared with that of control.

Truncation results in nuclear localization of Mst-3- Since the C-terminal to the kinase domain contains a nuclear localization signal (NLS), we also study the subcellular distribution of Mst-3 in the cell. Four EGFP-tagged Mst-3 fusion proteins, EGFP-Mst-3^{WT}, EGFP-Mst-$3^{WT\ \Delta\ 314}$, EGFP-Mst-3^{KR}, and EGFP-Mst-$3^{KR\Delta 314}$, were transiently expressed in both 293T and HeLa cell lines. As shown in Fig. 4, EGFP-Mst-3^{WT} and EGFP-Mst-3^{KR} were predominantly present in the cytoplasm (Fig. 4A and 4B). This observation is consistent with the finding of Schinkmann and Blenis [27]. Interestingly, the truncated forms of Mst-3, EGFP-Mst-$3^{WT\Delta 314}$ and EGFP-Mst-$3^{KR\Delta 314}$,

were localized mainly in the nucleus (Fig.4C and 4D). This result suggests that a nuclear localization signal (NLS) sequence in indeed located within amino acids 1-313 of Mst-3. The truncation may somewhat expose the NLS motif for nuclear transportation. Although wild type Mst-3 was demonstrated to present predominantly in the cytoplasm [27], the discovery of nuclear translocation of truncated Mst-3 promote us to investigate the proposed NLS sequence in Mst-3.

Identification and characterization the NLS domain of Mst-3- Upon sequence alignment, we found that Mst-3 contains a bipartite basic charge-rich sequence, ^{278}KKTSYLTELIDRYKRWK^{294}, which is homologue to the nuclear localization signals (NLS) of nucleoplasmin and human Pro-Tα (Table 1). NLS is required for proteins to translocate from the cytoplasm to the nucleus. There is no general consensus sequence for NLS so far. However, upon the amino acid sequence homology the NLS sequences can be classified into two major classes, i.e., a single cluster of basic amino acids (such as, NLS of SV40 T-antigen), and the bipartite type. The bipartite type is composed of two sets of adjacent basic amino acids separated by a stretch of approximately 10-12 amino acids. To further characterize the role of NLS domain in the subcellular distribution of Mst-3, we subcloned a sequence (a.a. 235-313) covering the NLS of Mst-3 through PCR and fused to the C-terminal of EGFP gene, termed EGFP- NLS^{Mst-3} (Fig. 5). Upon transfection, we found that the green fluorescence present predominantly was in the nucleus of 293T cells (Fig. 6). Further studies were performed by deleting the C-terminal region of truncated Mst-3 (Fig. 5). The mutagenesis of NLS domain in truncated Mst-3 was also carried out to further confirm the existence of NLS sequence (Table 2). Similar to EGFP-Mst-3$^{WT\ \Delta\ 314}$, EGFP-Mst-3$^{WTÄ314(A291/A292)}$ mutant was mainly localized in the nucleus. The fusion protein EGFP-Mst-3$^{WT\ \Delta\ 314(A278/A279)}$, however, was present in both the cytoplasm and nucleus. Similarly, EGFP-Mst-3$^{WT\ \Delta\ 314(Ala4)}$ also localized in the cytoplasm. The replacement of K278 and K279 in NLS domain with Ala changed the distribution of truncated Mst-3 from nucleus to cytoplasm was evidenced.

四、討論

The mammalian STE20-related kinases represent a rapidly growing kinase family. The type-I mammalian STE20-related protein kinases, such as hPAK1-4, contain a p21 Ras-like-GTPase binding region at their N-terminal regulatory domain. The binding of p21 Ras-like-GTPase may induce autophosphorylation and activation of this type of protein kinases. The type-I STE20-like protein kinase, hPAK1 has been demonstrated to activate JNK/SAPK and p38 pathway [32-34] and hPAK2 has been shown that involved in the programmed cell death [18]. The type-II mammalian STE20-like protein kinases that contain a kinase domain at N-terminus, comprise at least 10 members, including Mst1, Mst2, Mst3, kinase homologous to STE20 (KHS) [35], HGK [36], STE20/oxidant stress response kinase-1 (SOK-1) [37], germinal center kinase (GCK) [38,39], and human STE20-like protein kinase (hSLK) [40]. Some members of the type-II STE20-like protein kinase, such as GCK and Mst1, are highly responsive to cellular stress. For example, GCK could be activated by inflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α) [38]. Mst1, on the other hand, could be induced by Fas-antibody and staurosporine and involved in the process of apoptotic cell death [25]. Ste20 is a component of the pheromone-response pathway in budding yeast and plays a role in mating in Saccharomyces cerevisiae [41]. Several mammalian Ste20-like protein kinases have been identified so far, such as PAK (p21 activated kinase) [42,43], GCK (germinal center kinase) [38], HPK (hematopoietic progenitor kinase) [44], KHS (kinase homologous to Ste20/Sps1) [45], Mst1 (mammalian Ste20-like kinase) [35], SOK-1 (Ste20/oxidant stress response kinase) [37], and Krs1/2 (kinase responsive to stress) [46]. These Ste20-like kinases are likely to be cellular sensors in response to numerous

stimuli, including inflammatory cytokine, ischemia, ultraviolet radiation, DNA-damage, and heat shock [47].

Previous studies have shown that Mst3 could be cleaved by caspases 3, 7, and 8 to generate 40 kDa and 13 kDa fragments during apoptosis. The site for the cleavage of caspase 3, 7, and 8 was located at D^{313} ($AETD^{313}G$), which is at the junction of the N-terminal kinase domain and C-terminal regulatory domain of Mst3. To further demonstrate the role of Mst3 in apoptosis, we overexpressed wild type and the truncated Mst3 in HEK293 cells as well as in HeLa cells, respectively. Based on the results from morphological change, DNA fragmentation, and β-galactosidase assay, we concluded that overexpression of wild type and truncated MST3 were sufficient to trigger apoptosis in both HEK293 and Hela cells. In contrast, the kinase-dead mutant of Mst3 (Lys^{53} was replaced with Arg) EGFP-$MST3^{KR\triangle 314}$, EGFP-$Mst3^{KR}$, as well as EGFP alone did not induce any change in alone experiments. Since, activity of β-galactosidase was lower in cells expressing truncated Mst3 than that of cells expressing full-length wild type Mst3. In DNA fragmentation assay, we further observed that both wild type and the truncated Mst3 could induce DNA fragmentation, a typical characteristic of apoptosis in both cell lines.

In previous studies, we found that the truncation of the C-terminal regulatory domain could drive Mst3 from the cytoplasm to the nucleus. This result suggests that (i) there might be a NLS domain in the N-terminus of Mst3 and (ii) the removal of C-terminus might expose the NLS domain. In this study, we determined the localization of Mst3 in three different cell lines (HeLa, HEK293, and 293T cells) by fusing EGFP with various constructs of Mst3. As shown in Fig.4, full-length MST3s (either wild type or KR mutant) were predominantly localized in the cytoplasm. The truncated Mst3s were, however, translocated into the nucleus. Based on analysis of the primary amino acid sequence of Mst3, we found a potential nuclear localization signal (NLS) sequence in Mst3, the C-terminus of the kinase domain, ^{278}KKTSYLTELIDRYKRWK^{294}.

The active transport of proteins between nucleus and cytoplasm is an important process in eukaryotic cells. The majority of proteins required for the nucleus functions are transported through the recognition of NLS domain by importins [48]. The NLS of Mst3 is highly homologous to that of human ProT-α (KKAAEDDEDDDVDTKKQKTD) [49] and nucleoplasmin (KKPAATKKGQAKKKK) [50]. Deletion analysis of NLS on Mst3, suggested that NLS of Mst3 is necessary for the translocation of truncated Mst3. Further study of NLS sequence on Mst3 through site-directed mutagenesis identified two amino acid residues in NLS, i.e., Lys^{278} and Lys^{279}, were important for nuclear localization of truncated Mst3. The nuclear localization of truncated Mst3 was blocked partially by substitution of amino acid residues Lys^{278} and Lys^{279} with Ala. In contrast, the replacement of Lys^{293} and Arg^{294} with Ala did not affect the distribution of Mst3.

According to our results, we hypothesize that Mst3 may involve in the process of programmed cell death through the activation of caspases. The cleavage of the c-terminus of Mst3 may somewhat expose the nuclear localization signal motif of Mst3 and result in the nuclear translocation. Nuclear localization of truncated Mst3 may be essential for Mst3 for initiating apoptosis, although its significance remains to be studied.

Recently, Mst3 was shown to be phosphorylated by Akt (Protein Kinase B) in in vitro study. This protein kinase can be activated by insulin and various growth and survival factors. Akt functions to promote cells survival by inhibiting phosphorylating BAD, a proapoptotic Bcl-2 family, and apoptosis. It was demonstrated that Akt inactivated Bad by phosphorylation of BAD and effectively blocked BAD-induced cell death [51]. It is possible that the phosphorylation of MST3 by Akt might inactivate MST3 and hence inhibiting apoptosis. It will be interesting to understand the relationship between PI3-kianse–Akt

pathway and the apoptotic-inducing pathway of MST3 in the further.

五、成果自評

The major goal of this project is to understand the role of mammalian Ste20-like protein kinase 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, the translocation of Mst3 was observed and confirmed through fluorescent microscope. The nuclear translocation may signal and amplify the apoptotic response. Since full-length wild type Mst3 can also induce certain amount of cell death, other regulation, i.e., phosphorylation, can not be ruled out in our hypothesis. Therefore, cleavage and activation of the ubiquitously expressed Mst3 may be a common phenomenon during apoptotic signaling.

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