行政院國家科學委員會補助專題研究計畫成果報告 6989

REGULATION OF c-SRC KINASE ACTIVITY BY PI3-KINASE

(PI3-kinase 調節 c-Src 活性的探討)

計畫類別：☑個別型計畫　□整合型計畫

計畫編號：NSC 89－2320－B－040－019－

執行期間：88 年 8 月 1 日至 89 年 7 月 31 日

計畫主持人：馬明琪

共同主持人：

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執行單位：私立中山醫學院生化所

中　華　民　國　89 年　10 月　18 日

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一、中文摘要

v-Ras 癌化的細胞，其細胞架構較諸正常細胞產生了巨大的變化，且整體蛋白的 tyrosyl phosphorylation 較正常細胞有明顯增加。本實驗室進一步研究結果，發現在 v-Ras 癌化的細胞，其 c-Src 的 kinase activity 是增加的。而 Wortmannin 處理下的 v-Ras 癌化的細胞，其 c-Src kinase activity 是下降的。有趣的是 c-Src 的 substrate，$p97^{Eps8}$，是介於 Ras 和 Rac 之間的 mediator。而我們發現 $p97^{Eps8}$ overexpression 會造成 cell transformation。

關鍵詞：Ras，c-Src，PI3K，$p97^{Eps8}$

Abstract

Studies of v-Ras transformed cells revealed their altered cytoskeletal network and enhanced tyrosyl phosphorylation of total cellular proteins relative to normal cells. Further studies indicated that the activity of c-Src was increased in cells expressing v-Ras and decreased c-Src activity was observed in Wortmannin-treated v-Ras transformed cells. Interestingly, the substrate of Src, $p97^{Eps8}$, was an important mediator between Ras and Rac. We observed that overexpression of $p97^{Eps8}$ can lead to cellular transformation.

Keywords: Ras，c-Src，PI3K，$p97^{Eps8}$

二、緣由與目的

Ras is a 21 kDa small GTP-binding protein with intrinsic GTPase activity. Previous studies have shown that GTP-bound Ras can induce cell proliferation more efficiently than GDP-bound Ras; thus, through cycling between an active GTP-bound form to an inactive GDP-bound form, Ras can function as a molecular switch regulating cell growth and other functions (1,2). A single point mutation happens at the 12 amino acid position (Gly -> Val) impairs the intrinsic GTPase activity of Ras. Due to its loss of GTPase activity, this mutated Ras maintained the GTP-bound state constitutively and caused cell transformation (3,4).

To date, a variety of extracellular stimuli such as mitogens, cytokines, phorbol esters and LPA have been reported to induce the activation of Ras (1). And the recruitment of SOS, the Ras guanine nucleotide exchange factor, to the membrane turns out to be the major mechanism leading Ras to its activation. Subsequent signaling to the nucleus from activated Ras involves the direct interaction of a serine/threonine kinase, c-Raf, with Ras and downstream activation of a cascade of serine-threonine kinases, culminating with MAP kinases (5). MAP kinases have been shown to translocate from the cytosol to the nucleus, where they are capable of phosphorylating transcriptional factors, thus completing the signaling pathway originated from the plasma membrane.

Elevated tyrosyl phosphorylation was detected in v-Ras transformed cells.

However, to date, the responsible tyrosine kinase(s) as well as proteins with their tyrosyl phosphorylation enhanced in response to v-Ras have not been described. To address this point, cells expressing oncogenic Ras were examined. Increased overall tyrosyl phosphorylation in v-Ras transformed cells suggests the activation of a tyrosine kinase(s). Indeed, enhanced c-Src activity was detected in v-Ras transformed cells relative to that in normal cells. Interestingly, reduced c-Src activity was observed in wortmannin-treated v-Ras transformed cells but not in normal cells. These results suggest that through a wortmannin-sensitive signaling pathway, v-Ras can induce the activation of c-Src.

Eps8 was initially identified as a substrate for EGFR whose tyrosyl phosphorylation was increased in response to EGF stimulation. Further studies indicated that Eps8 could also be phosphorylated by other receptor tyrosine kinases as well as Src tyrosine kinase. Recent studies conducted in Eps8-/- fibroblasts revealed that Eps8 was a mediator between Ras and Rac. To study the implication of Eps8 in cellular transformation, cells overexpressing $p97^{Eps8}$ were generated. Compared to normal parental cells, cells overexpressing $p97^{Eps8}$ exhibited increased ability to form foci in culture dish and tumors in mice.

三、結果與討論

Elevation of c-Src kinase activity by the expression of v-Ras. To confirm the activation of c-Src in v-Ras transformed cells, detergent lysates prepared from normal and v-Ras transformed cells were immunoprecipitated with Src antibody and the immunoprecipitates were divided into two equal parts. One part was subjected to the in vitro kinase assays in the presence of enolase. The remaining part was resolved on SDS/PAGE and immunoblotted with Src antibody. As shown in Figure 1, while similar amounts of c-Src were detected in both cell lines, increased 32P-incorporated enolase and c-Src was observed in v-Ras transformed cells. This observation indicates that c-Src is activated by the expression of oncogenic Ras.

Wortmannin treatment can inhibit the activation of c-Src in v-Ras transformed cells. It is important to understand which mechanism(s) can lead to the activation of c-Src in v-Ras transformed cells. To date, GAP, NF1, c-Raf, and PI3-kinase are the known effectors and regulators that interact with active GTP-bound Ras. Previous studies described by Tameh et al demonstrated that PtdIns(3,4,5)P3 (the product of PI3-kinase) was capable of associating with the SH2 domain of c-Src, thereby, PtdIns(3,4,5)P3 may be involved in the regulation of c-Src kinase activity. To test this hypothesis, normal and v-Ras transformed cells grown in 10% FCS-containing media were treated with different amounts of wortmannin for 2 hours. Then the in vitro kinase assays of c-Src immunocomplexes prepared from each group were carried out. Unfortuantely, under this condition, decreased c-Src kinase activity was not detectedin both cells in response to wortmannin treatment. Since FCS contained different mitogens, it was likely that through the FCS-induced signaling

pathways, c-Src can still be activated. To minimize the possible interfering effects, normal and v-Ras transformed cells were preincubated overnight with 0.5% FCS-containing medium before wortmannin treatment. Later, the kinase activity of c-Src immunocomplexes prepared from wortmannin-treated or –nontreated samples were analyzed. As shown in Figure 3A, wortmannin treatment can cause more than 50 % decrease of c-Src activity and 100 nM wortmannin can totally wipe out the elevated kinase activity of c-Src. To further study how the activity of c-Src is affected by wortmannin, normal and v-Ras transformed cells pre-incubated with serum-minus media were treated with 100 nM wortmannin for 0, 0.3 and 5 hours. While no significant alteration of c-Src activity was detected in wortmannin-treated normal cells, 5 hr wortmannin treatment can reduce 50% c-Src kinase activity observed in v-Ras transformed cells (Figure 2B).

Generation of cell lines overexpressing $p97^{Eps8}$. $p97^{Eps8}$ is a putative substrate of Src family kinases (γ). To study its implication in tumorigenesis, cells overexpressing $p97^{Eps8}$ (Wt) were generated by retroviral infection method. By comparison of the growth property of control cells and cells overexpressing $p97^{Eps8}$, we observed that Wt cells retained the ability to form foci in culture dishes relative to negative control cells (Figure 3). To further analyze the tumorigenic ability of $p97^{Eps8}$ overexpressors, the cells recovered from $p97^{Eps8}$-induced foci were inoculated into mice. In the meantime, cells expressing v-Src (IV5) were also inoculated into mice as a control. As demonstrated in Table 1, all the mice inoculated with $p97^{Eps8}$ overexpressors developed tumors with diameter of 0.5 cm within 20 to 30 weeks. In comparison, cells expressing v-Src (IV5) produced tumors of similar size in less than two weeks (Table 1). These findings indicated that overexpression of $p97^{Eps8}$ could lead to cellular transformation, though it was far less potent than that of v-Src.

四、計畫成果自評

本計畫進行順利，目前已有一篇 manuscript 投稿、另一篇 manuscript 正在準備中。

五、參考資料

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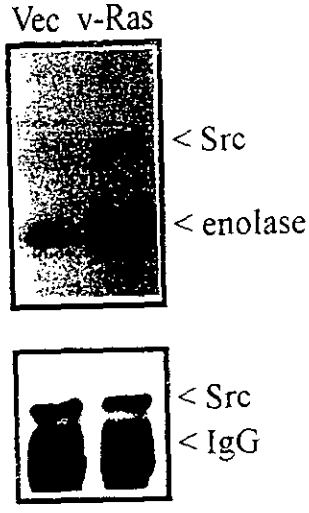

Figure 1. Increased c-Src kinase activity in v-Ras transformed cells. Equal amounts of lysates (500 μg) prepared from control and v-Ras transformed cells were immunoprecipitated with Src antibody. Half the c-Src immunocomplexes were assayed for in vitro kinase activity (upper panel). Half the c-Src immunocomplexes were resolved with SDS/PAGE and Western blotted with Src antibody (lower panel).

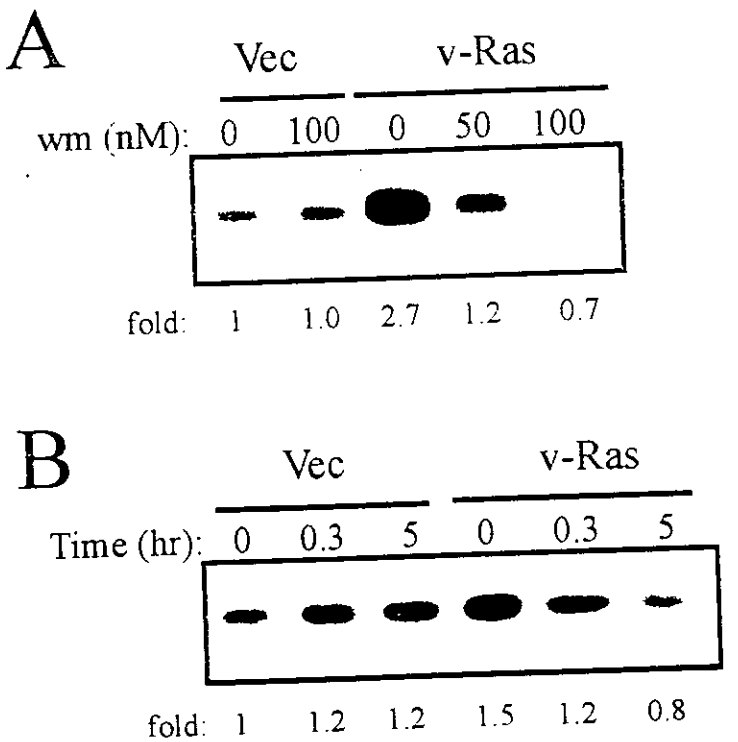

Figure 2. Wortmannin can inhibit the activation of c-Src in v-Ras transformed cells. Serum starved control and v-Ras transformed cells were non-treated or treated with wortmannin and the enzymatic activity of c-Src immunocomplexes prepared from each group was analyzed. (A) Cells are treated with different wortmannin concentration for 30 min. (B) Cells are treated with 100 nM wortmannin for different period of time. The c-Src activity in non-treated control cells is chosen to have a value of 1, the relative c-Src activity of other samples are then determined and shown at the bottom of each panel.

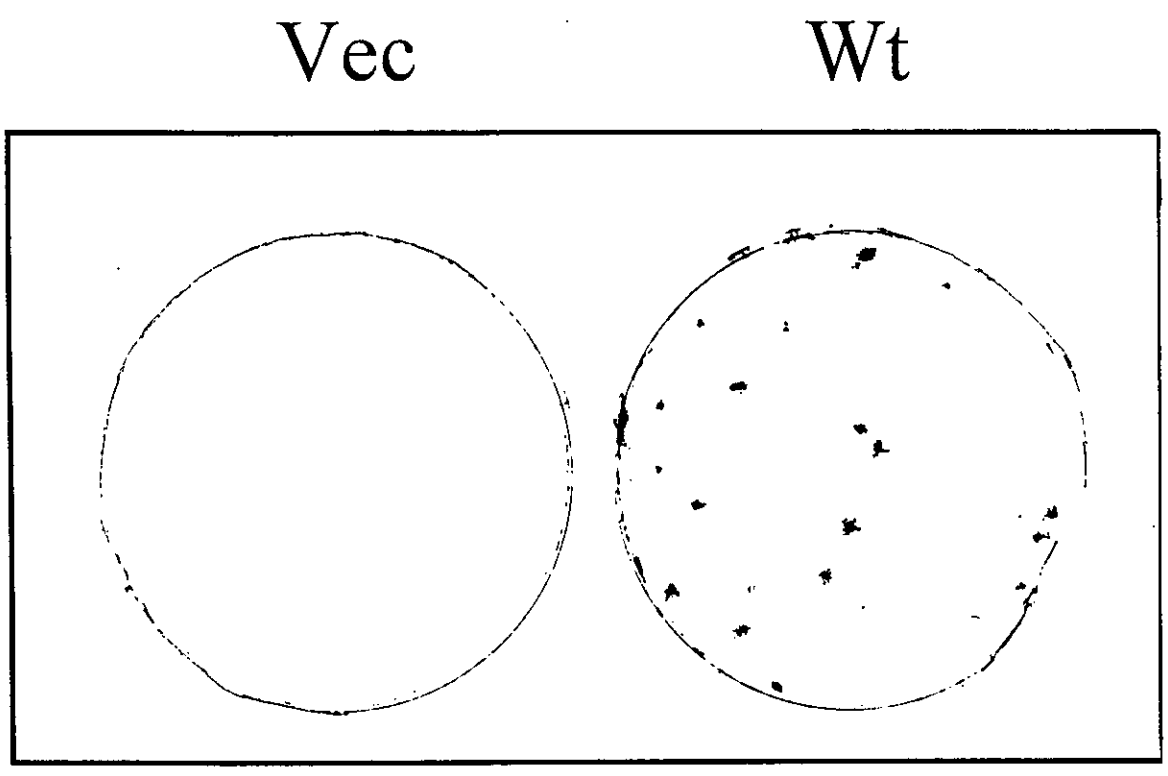

Figure 3. Enhanced foci formation in cells overexpressing $p97^{Eps8}$. Equal numbers of cells overexpressing $p97^{Eps8}$ (Wt) and control cells (Vec) were cultured for around four weeks and the transformed foci were observed.

Table 1. Tumorigenicity of $p97^{Eps8}$ overexpressors recovered from $p97^{Eps8}$-induced foci.

Cell line	No. of tumors/no. of mice injected[a]	No. of weeks for tumors to reach 0.5 cm[a]
$p97^{Eps8}$ overexpressors	12/12	20 to 30
v-Src transformed cells (IV5)	12/12	< 2

[a] These results were calculated from three independent experiments.