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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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※ TSA 抑制 v-Src 癌化細胞生長之研究 ※

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計畫編號：NSC 90－2311－B－040－004－

執行期間：90年 8月 1日至 91年 7月 31日

計畫主持人：馬明琪

共同主持人：

計畫參與人員：

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執行單位：私立中山醫學大學生化所

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行政院國家科學委員會專題研究計畫成果報告

INVESTIGATION OF THE MECHANISMS INVOLVED IN GROWTH INHIBITION OF v-SRC TRANSFORMED CELLS MEDIATED BY TSA

(TSA 抑制 v-Src 癌化細胞生長之研究)

計畫編號：NSC 90-2311-B-040-004
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一、中文摘要

acetylation 是 histone modification 諸多型式中的一種，可以調控 gene transcription。 而 histone acetylase (HAT) 及 histone deacetylase (HDAC) 則為兩個互為拮抗、調控細胞內 histone acetylation 的重要酵素。Trichostatin A (TSA) 是一個 histone deacetylase (HDAC) 的抑制劑。 先前已有報導指出其可抑制多種致癌基因所引發的細胞癌化。 本實驗室在研究 v-Src 細胞癌化的過程中，意外發現 TSA 可以抑制其生長、分裂。 在進一步探究其作用機轉時，我們也觀察到 TSA 處理後的 v-Src transformed cells，其 p97^{Eps8} 的量有顯著下降。 先前我們已指出 *eps8* 是一個致癌基因，且 p97^{Eps8} 是 Src substrate。 故 TSA 有可能透過抑制 p97^{Eps8} 的表現量而具有抑癌能力。
關鍵詞：蛋白表達、v-Src、TSA、Eps8。

Abstract

In order Histone acetylase (HAT) and histone deacetylase (HDAC) are two crucial enzymes determining the structure of chromatin that regulates gene expression. In this study, we observed that TSA, a specific HDAC inhibitor, could effectively inhibit the growth of v-Src transformed cells and abrogated their ability to form colonies in soft agar. By further dissecting the growth inhibition mechanisms, we found that while TSA could reduce the expression of Eps8, no alteration of Src expression and kinase activity was observed. Interestingly, removal of TSA could restore both the cellular growth and the expression of Eps8. Northern and RT-PCR analysis revealed the significant reduction of *eps8* transcript in TSA-treated v-Src transformed cells relative to control. Since v-Src enhanced p97^{Eps8} expression and overexpression of p97^{Eps8} could lead to cellular transformation, thus the observed correlation between TSA-reduced p97^{Eps8} expression and TSA-induced growth inhibition of v-Src transformed cells implicated the involvement of p97^{Eps8} in v-Src-induced transformation. Indeed, expression of dominant negative p97^{Eps8} abolished the transformation caused by v-Src. Based on these findings, we conclude that p97^{Eps8} plays a crucial role in v-Src-induced transformation.
Keywords: protein expression、v-Src、TSA、Eps8。

二、緣由與目的

Eucaryotic DNA is regularly packed into nucleosomes that are folded into higher chromatin fibers (Luo and Dean, 1999). Approximately 146 base pairs of DNA wrapped around a histone octomer containing two molecules each of H2A, H2B, H3, and H4 (Luo and Dean, 1999). And along with histone H1 and linker DNA, the nucleosome core constitutes the fundamental repeating unit of chromatin. A variety of post-translational modifications of histones including phosphorylation, acetylation, methylation, ADP-ribosylation and ubiquitination are reported (Strahl and Allis, 2000), and among them, histone acetylation is the best studied that occurs at specific lysine residues in the amino termini of the core histone (Strahl and Allis, 2000). Histone acetylase (HAT) and histone deacetylase (HDAC) are the two enzymes that regulate the steady-state balance of histone

acetylation in vivo (Struhl, 1998; Brown et al., 2000; Ng and Bird, 2000). Adding acidic acetyl group to lysine residues neutralizes the basic nature of histones and decreases their affinity for DNA. Therefore, the altered nucleosomal conformation increases the accesibility of transcriptional regulators to chromatin templates. Since histones are the major components in "chromatin remolding", their acetylation may also affect other DNA-templated processes including DNA replication, repair, recombination, and chromosome segregation (Aalfs and Kingston, 2000).

Trichostatin A (TSA) was first discovered as an antifungi agent (Tsuji et al., 1976). Later, it was characterized as a noncompetitive inhibitor of histone deacetylase with a Ki value of 3.4 nM (Yoshida et al., 1995). TSA can induce a variety of biological responses including induction of differentiation, cell cycle arrest, and apoptosis (Yoshida et al., 1995). In addition, TSA can also reverse cellular transformation caused by *sis*, *ras* and *src* oncogenes (Sugita et al., 1992; Futamura et al., 1995; Kwon et al., 1998).

As an initially identified substrate for EGF receptor (EGFR), tyrosyl phosphorylation of Eps8 could also be enhanced in response to stimulation by other receptor tyrosine kinases (Fazioli et al., 1993). Further studies indicated that nonreceptor tyrosine kinase such as Src could mediate Eps8 phosphorylation as well (Gallo et al., 1997; Maa et al., 1999). Two Eps8 isoforms ($p97^{Eps8}$ and $p68^{Eps8}$) can be recognized by Eps8 antibody (Fazioli et al., 1993). Although $p68^{Eps8}$ has been speculated as a proteolytic or an alternatively spliced product of $p97^{Eps8}$, its exact coding sequences and function are still unclear. Aberrant overexpression of $p97^{Eps8}$ in cells not only enhanced mitogenic responsiveness to EGF (Fazioli et al., 1993) but also caused cellular transformation (Maa et al., 2001). Like various intracellular signal transducers, $p97^{Eps8}$ contains several protein-protein interaction modules that include an SH3, a putative nuclear targeting sequence, a split PH, a degenerated SH2 and several proline-rich regions (Fazioli et al., 1993; Wong et al., 1994). Through these domains, $p97^{Eps8}$ was reported to associate with a variety of signaling proteins such as Shc (Matoskova et al., 1995), Shb (Karlsson et al., 1995), RN-tre (Matoskova et al., 1996), and E3b1 (Biesova et al., 1997). In addition, the split PH conferred the ability of $p97^{Eps8}$ to associate with plasma membrane in response to serum stimulation that was crucial for $p97^{Eps8}$-induced transformation (Maa et al., 2001). Recently, accumulating evidence indicated that by interacting with E3b1 or RN-tre, $p97^{Eps8}$ was involved in the control of Rac and Rab5 signaling respectively (Scita et al., 1999; Lanzettl et al., 2000). Reflecting its crucial role in signal transduction, the amount of $p97^{Eps8}$ was modulated by growth and differentiation.

In this study, we observed that TSA could inhibit the growth of v-Src transformed cells and effectively reduced the abundance of *eps8* transcripts as well as Eps8 expression. Since $p97^{Eps8}$ was an oncoprotein (Maa et al., 2001) whose expression could be enhanced by v-Src (Maa et al., 1999), the strong correlation between TSA-mediated growth inhibition of v-Src transformed cells and TSA-reduced $p97^{Eps8}$ implicated the participation of $p97^{Eps8}$ in v-Src-induced transformation. The abrogation of foci formation by expression of dominant negative $p97^{Eps8}$ in v-Src transformed cells supported this hypothesis.

三、結果與討論

Identification of TSA as a growth inhibitor of v-Src-transformed cells

Previous studies have indicated that TSA could reverse transformed phenotype caused by multiple

oncogenes (Sugita et al., 1992; Futamura et al., 1995; Kwon et al., 1998). In an attempt to assess the growth response of v-Src transformed cells (IV5) to TSA, we treated cells with different concentration of TSA (0, 1, 10 and 100 ng/ml) for 1, 2 and 3 days. As shown in Figure 1, there was no obvious effect in cells treated with 1 ng/ml TSA as compared to control. However, significant growth inhibition was observed in IV5 cells treated with 10 ng/ml TSA. When TSA concentration reached 100 ng/ml, complete block of cellular growth was detected. Consistently, TSA could repress the colony formation of v-Src transformed cells in soft agar (Figure 2). While 0.1 and 1 ng/ml TSA-treated IV5 cells still retained the ability of anchorage-independent growth, 75 % and 100 % reduction of the colony formation was observed in IV5 cells treated with 5 and 10 ng/ml TSA respectively (Figure 2). These results indicated that TSA was an effective transformation inhibitor for cells expressing v-Src.

Downregulation of $p97^{Eps8}$ in TSA-treated v-Src transformed cells

Previously, we have demonstrated that overexpression of $p97^{Eps8}$ could lead to cellular transformation (Maa et al., 2001). Since TSA could inhibit the growth of transformed cells, we were interested in its effects on the expression of $p97^{Eps8}$. To answer this question, we analyzed the levels of $p97^{Eps8}$ in v-Src transformed cells treated with different concentrations of TSA for 6 hours. As demonstrated in the dose-response experiment (Figure 3A), while the level of v-Src remained unchanged, significant reduction of $p97^{Eps8}$ was detected in cells treated with 100 ng/ml TSA in comparison with non-treated cells. When the time-response experiment was further carried out to analyze TSA-induced reduction of $p97^{Eps8}$, we observed that IV5 cells treated with 100 ng/ml TSA for 12 and 24 hours exhibited 50 % and 80 % reduction of the expression of $p97^{Eps8}$ respectively (Figure 3B). These findings indicated that TSA could downregulate $p97^{Eps8}$ in v-Src transformed cells. Interestingly, the amounts of the other Eps8 isoform, $p68^{Eps8}$, were also reduced in response to TSA (Figure 3A and 3B).

The expression and kinase activity of v-Src were not affected by TSA treatment

Previously, we have indicated that Src could influence the expression level of $p97^{Eps8}$ (Maa et al., 1999). Thereby, it was possible that through reduction of the amount and kinase activity of Src, TSA could reduce the expression of $p97^{Eps8}$. To check this possibility, the expression level and enzymatic activity of v-Src were determined in TSA-treated IV5 cells. As demonstrated in Figure 3A, in contrast to reduced expression of $p97^{Eps8}$, the levels of v-Src were unchanged in the TSA dose-dependent experiment. At the meantime, we compared the overall pattern of tyrosyl phosphorylated proteins in control and v-Src transformed cells treated with different concentrations of TSA. As shown in Figure 4A, there was no significant change of the p-Tyr profile in control and TSA-treated cells. This suggested that no change of Src activity in response to TSA. To further confirm this point, the level of tyrosyl phosphorylation of cortactin, a substrate for Src, was analyzed. Lysates prepared from control and TSA-treated cells were immunoprecipitated with cortactin antibody, and the precipitated proteins were immunoblotted with p-Tyr antibody and cortactin antibody separately. While similar amounts of cortactin were detected in all lysates (Figure 4B, bottom panel), no alteration of the p-Tyr content of cortactin was observed in control cells and cells treated with various concentrations of TSA (Figure 4B, upper panel). These data confirmed

our speculation that the activity of v-Src was not altered in response to TSA.

Removal of TSA could restore the expression of p97^{Eps8} and cellular growth

Since TSA-inhibited cellular growth of v-Src transformed cells coincided with downregulation of p97^{Eps8}, we speculated that the amount of p97^{Eps8} might correlate to cellular growth. To prove this hypothesis, v-Src transformed cells initially incubated with 100 ng/ml TSA for 24 hours were incubated with TSA-free medium. Then, the growth curve and expression of p97^{Eps8} in these cells were analyzed. We noticed that TSA withdrawal led to the restoration of cellular growth (Figure 5A). Interestingly, while dramatic reduction of p97^{Eps8} was observed in 24 hr TSA-treated cells, significant increase of p97^{Eps8} was detected in v-Src transformed cells after 6 hr TSA removal (Figure 5B). And the longer the cells incubated in TSA-free medium, the more expression of p97^{Eps8} was restored (Figure 5B, upper panel). Similar expression pattern was also applied to p68^{Eps8} in response to presence and absence of TSA (Figure 5B, upper panel). Unlike the Eps8 isoforms, no alteration of v-Src expression was detected in this experiment (Figure 5B, bottom panel) that was consistent with the result of Figure 3A.

Reduced eps8 transcript in TSA-treated v-Src transformed cells

Two *eps8* transcripts of 4.7 and 3.8 kb were previously reported (Fazioli et al., 1993). And while the 4.7 kb mRNA corresponded to the one encoding p97^{Eps8}, the identity of 3.8 kb mRNA was still unclear. Our observation that expression of p97^{Eps8} was downregulated in TSA-treated v-Src transformed cells prompted us to investigate whether TSA-reduced p97^{Eps8} was exerted at the transcriptional level. To answer this question, total RNAs extracted from control and TSA-treated v-Src transformed cells were analyzed by Northern blot. As demonstrated in Figure 6A, the abundance of both 4.7- and 3.8 kb *eps8* transcripts was significantly decreased in TSA-treated cells as compared to control. Further RT-PCR analysis utilizing specific primers for the longer *eps8* transcript, we observed reduced amounts of 4.7 kb *eps8* transcript in response to TSA (Figure 6B). These results implicated that TSA-mediated reduction of p97^{Eps8} was partly attributed by inhibition of *eps8* transcription.

四、計畫成果自評

本計畫進行順利，目前已有一篇 manuscript 正在準備中。

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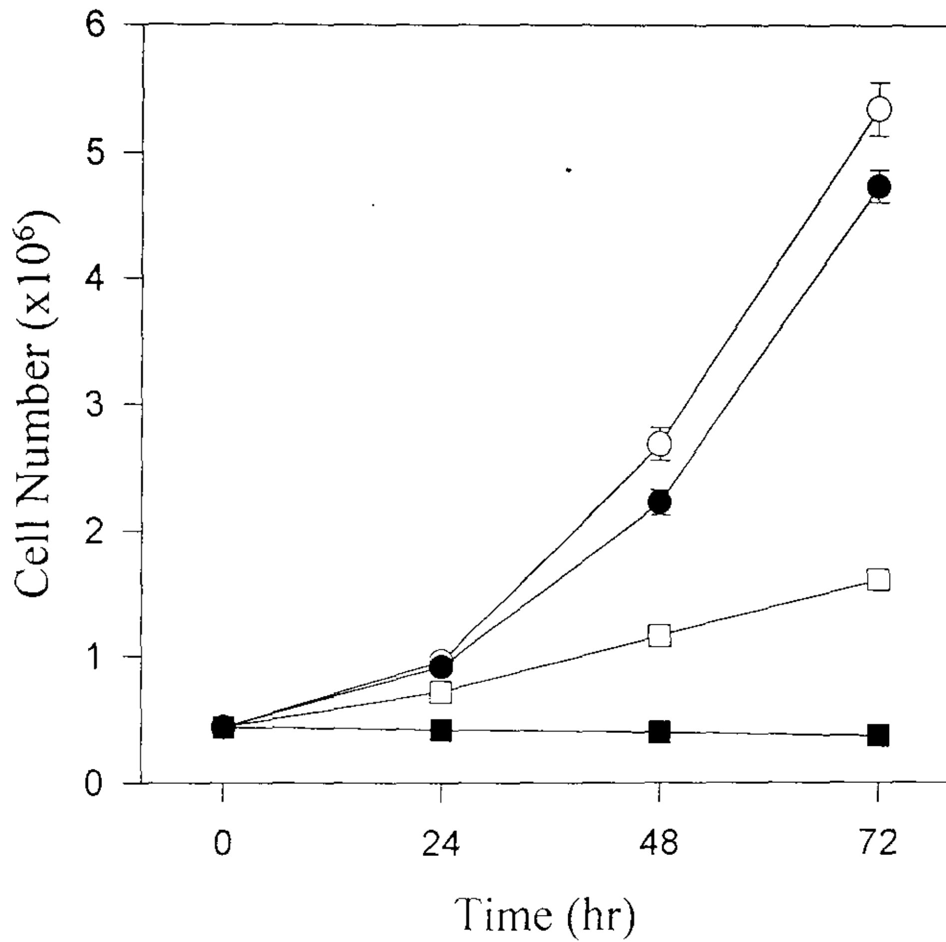


Figure 1. The inhibitory effect of TSA on the growth of v-Src transformed cells. Cells expressing v-Src (IV5) were plated at a density of 3×10^5 cells/60-mm dish and cultivated for 18 hours. At Time 0, different concentrations of TSA (0, 1, 10, 100 ng/ml) were added to the medium and incubated with the cells for 24, 48, and 72 hours. Total number of control and TSA-treated cells were counted and plotted. The results were shown in means ± S.D. of triplicate experiments.

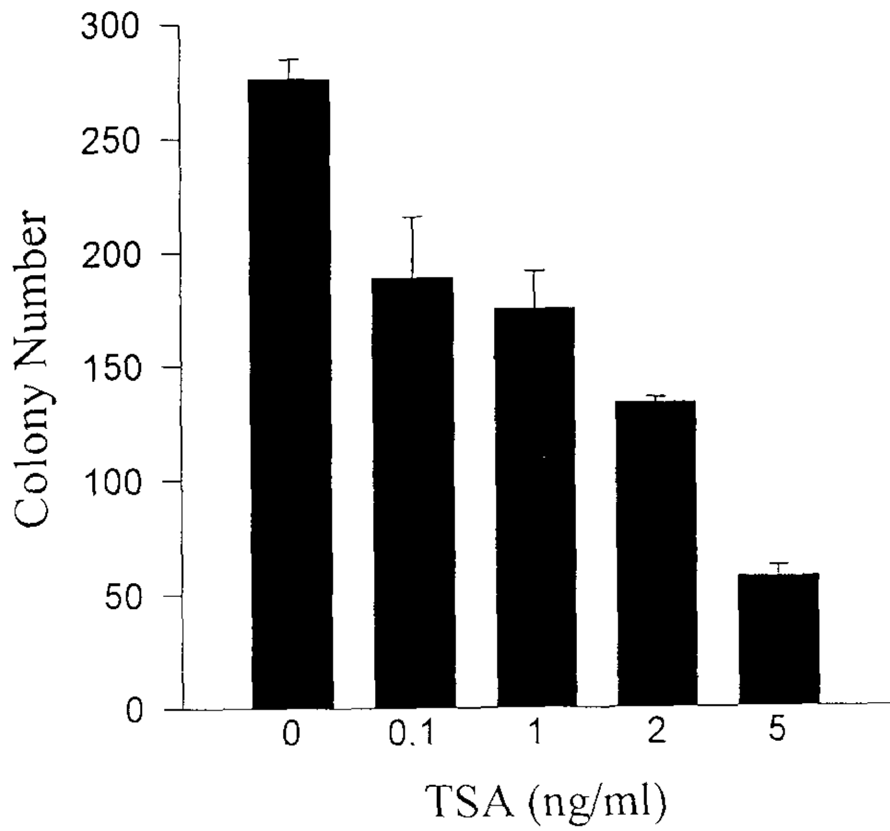


Figure 2. TSA can repress the colony formation of v-Src transformed cells in soft agar. Values for number of colonies in control and TSA-treated groups were the mean ± S.D. of one representative experiment in which 1.5×10^3 cells were seeded per plate in triplicate.

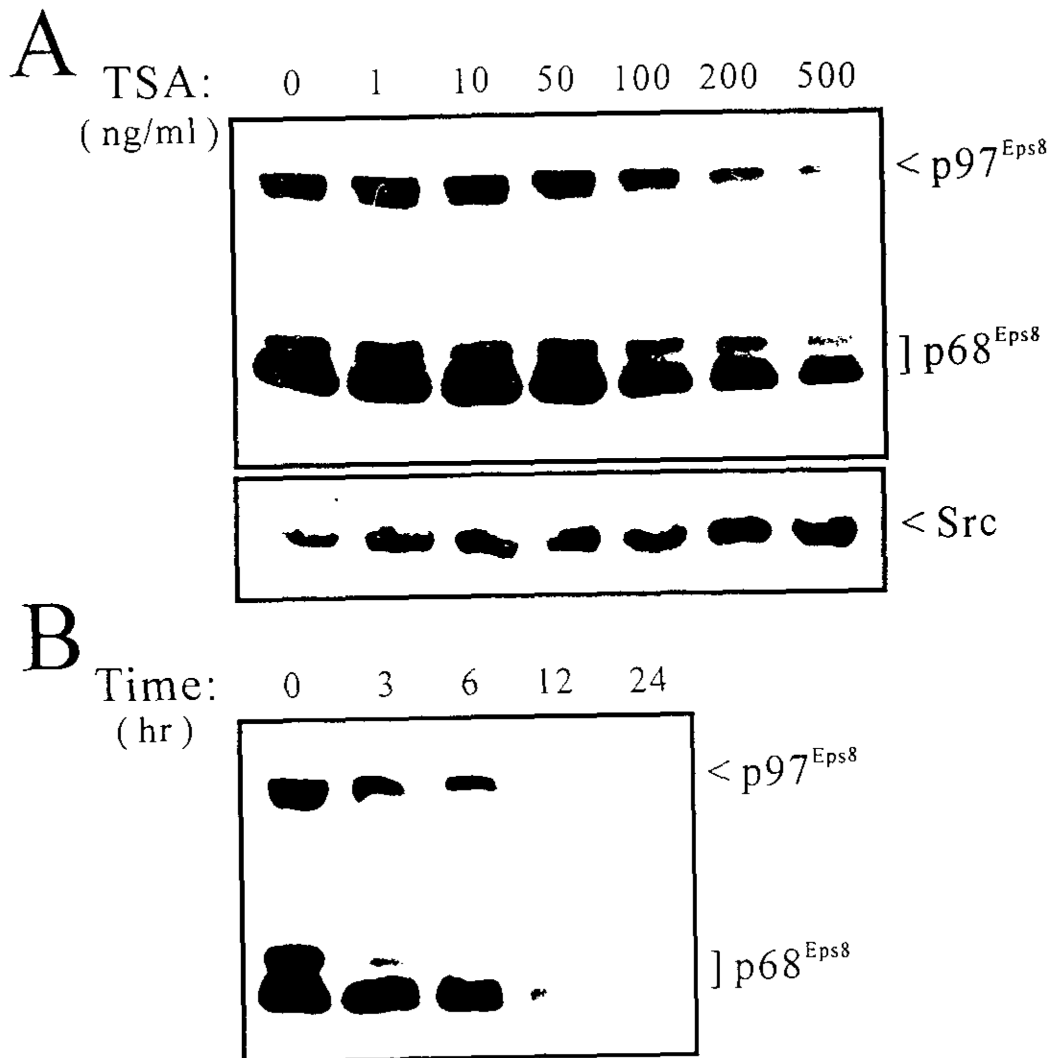


Figure 3. Reduced expression of p97[Eps8] in response to TSA. (A) The dose-response curve of TSA-reduced p97[Eps8]. Cells expressing v-Src (IV5) were treated with various concentration of TSA for 24 hr. Equal amounts (100 μg) of cellular lysates were analyzed directly by anti-Eps8 for Western immunoblot. The Src amount was demonstrated at the bottom panel as an internal control. (B) The time-response curve of TSA-reduced p97[Eps8]. IV5 cells were treated with TSA (100 ng/ml) for different period of time. Lysate proteins (100 μg) from each group were examined by direct Western immunoblot analysis for p97[Eps8] protein levels.

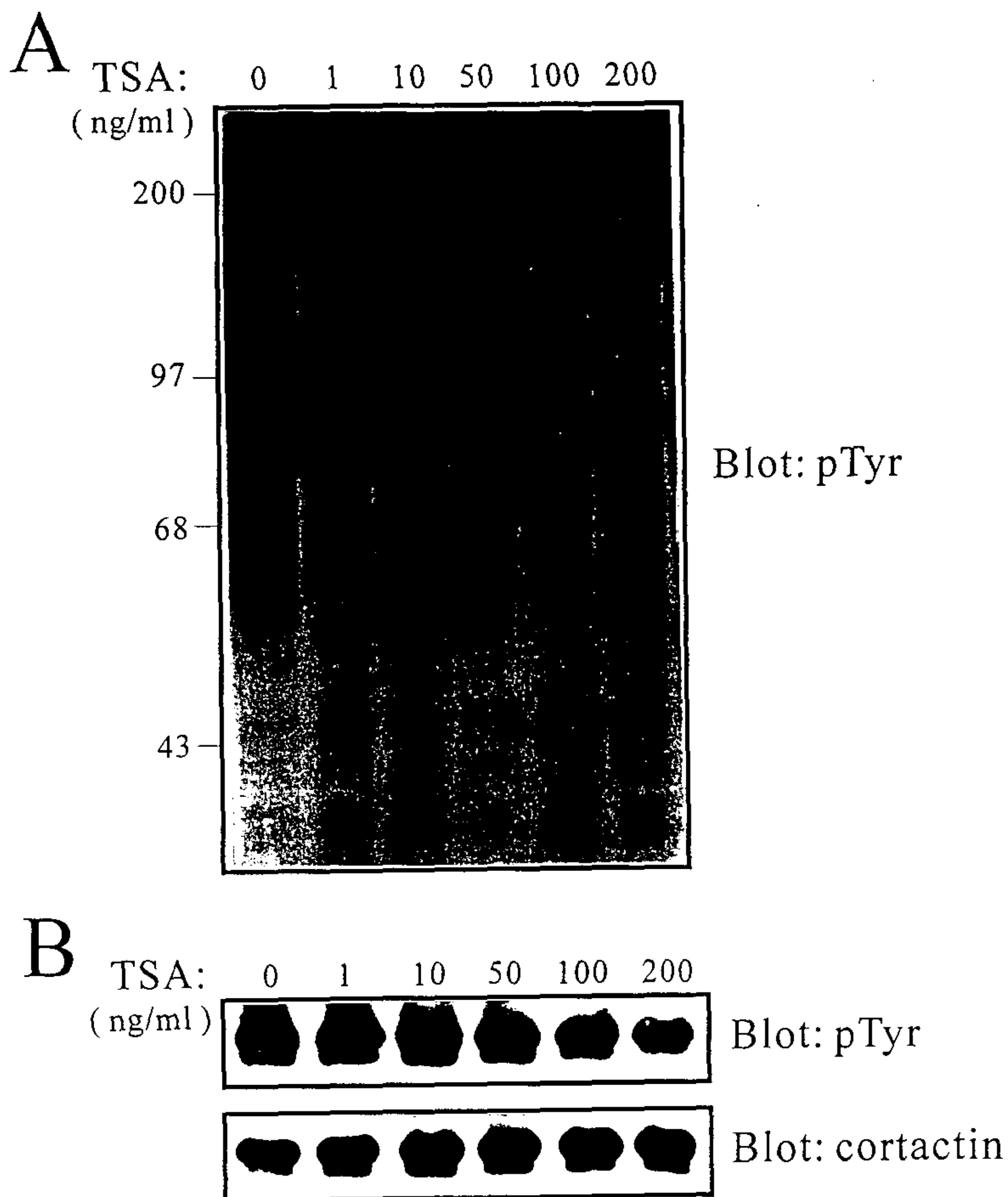
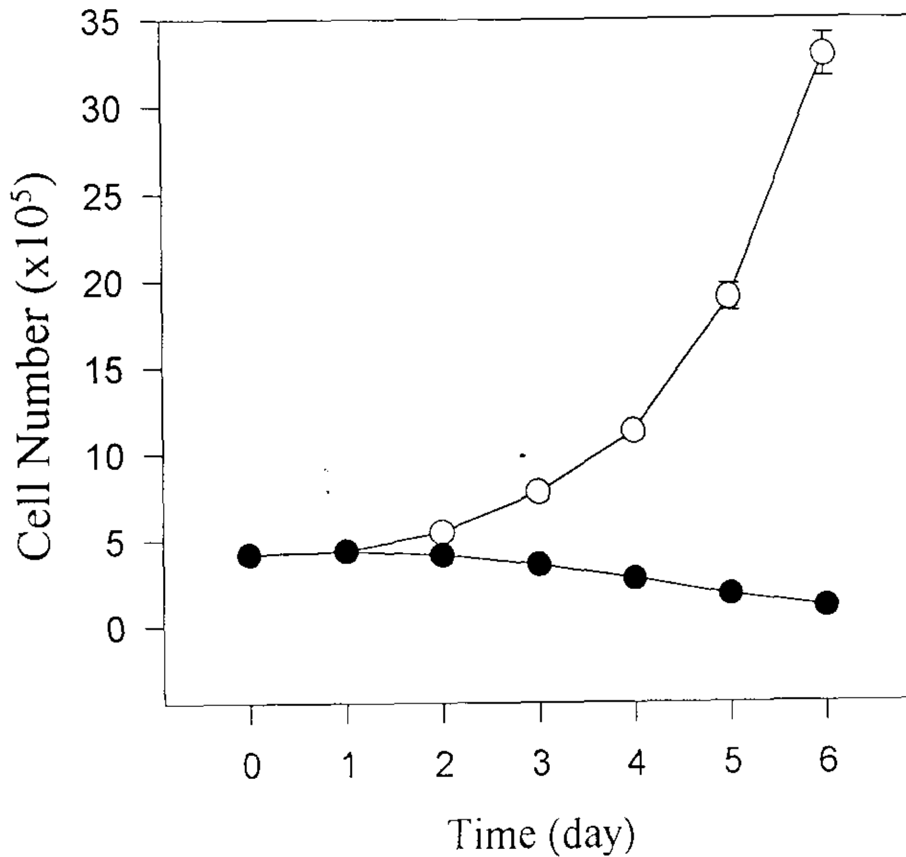


Figure 4. No alteration of Src kinase activity in response to TSA. (A) 1V5 cells were treated with various concentration of TSA for 24 hr. Equal amounts (100 mg) of cellular lysates were analyzed directly by anti-pTyr antibody. (B) Equal amounts of lysates (500 mg) were prepared and immunoprecipitated with anti-cortactin antibody. The immunocomplexes were analyzed by Western immunoblotting with anti-pTyr antibody (upper panel) or anti-cortactin antibody (bottom panel).



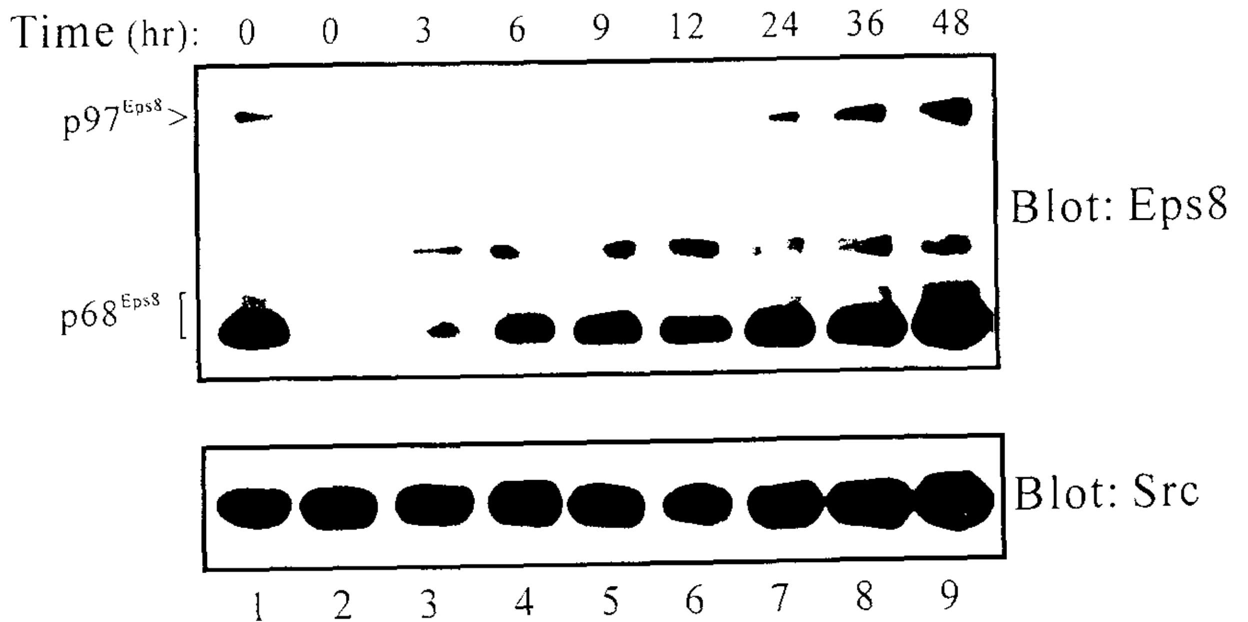


Figure 5. Restoration of p97Eps8 after TSA withdrawal. IV5 cells were incubated with or without 100 ng/ml TSA for 24 hr. Then, the TSA-treated cells were incubated with fresh medium for different periods of time as indicated. Equal amounts (100 μg) of total cell lysates were analyzed directly by anti-Eps8 (upper panel) and anti-Src (bottom panel) Western immunoblots.

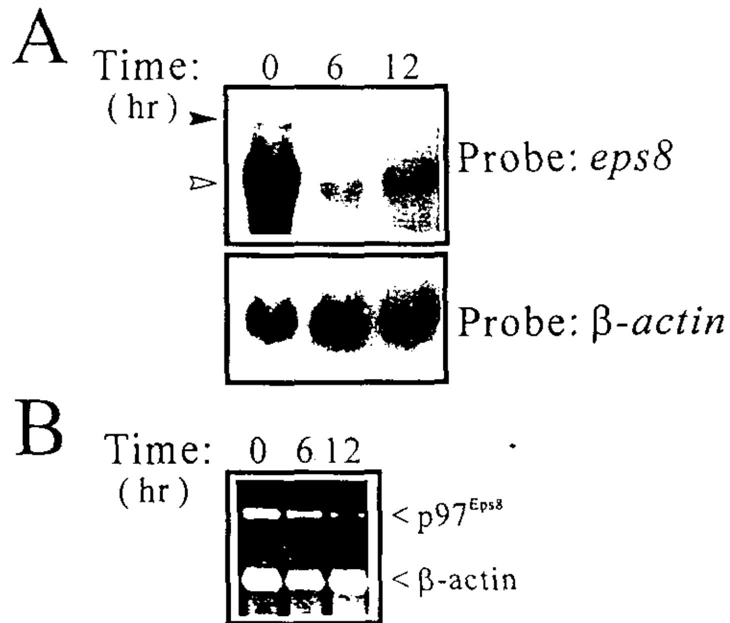


Figure 6. Reduced *eps8* transcript in response to TSA. IV5 cells were non-treated or treated with 100 ng/ml TSA for 6 and 24 hr. RNAs (30 μg/lane) extracted from these cells were examined by Northern analysis. Filters were hybridized with probes for *eps8* and GAPDH.