行政院國家科學委員會補助專題研究計畫 ☑ 成果報告 ☐期中進度報告

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計畫主持人：馬明琪
共同主持人：
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：☑精簡報告　☐完整報告

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中　華　民　國　92 年　10 月　15 日

行政院國家科學委員會專題研究計畫成果報告

STUDY OF THE MECHANISMS RESPONSIBLE FOR CURCUMIN-MEDIATED REDUCTION OF c-SRC EXPRESSION

(curcumin 抑制 c-Src 蛋白表現量機轉之探討)

計畫編號：NSC 91-2311-B-040-002
執行期限：91 年 8 月 1 日至 92 年 7 月 31 日
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一、中文摘要

薑黃素 (curcumin) 是一個歷史悠久廣為人知的天然食用色素，它具有抗發炎、抗氧化、以及抗癌的效果。在本文研究中，我們發現薑黃素可以抑制 c-Src 的蛋白質表現。 為進一步探討其對 Src kinase 活性的影響，我們以 v-Src 癌化之細胞 (IV5) 為研究材料，發現薑黃素處理後的 IV5 細胞， 其 Shc、 cortactin、 FAK 等多種 Src 受質的 酪氨酸磷酸化減少。而在我們 in vitro kinase 的實驗裡，我們也証明了薑黃素可直接抑制 Src 的活性。由於 Src 活性的下降，使 Shc 的 Pi-Tyr-317 減少，進而降低 ERK 的活性，抑制 v-Src 癌化細胞的生長。此外，薑黃素也可直接及間接地抑制 FAK 的活性。藉由降低 cortactin 的酪氨酸磷酸化及 FAK 酵素活性，薑黃素更減緩了 v-Src 癌化細胞的移動 (migration)。據我們的所知，這是第一篇有關薑黃素藉由抑制 Src 及 FAK 的酵素活性，進而抑制細胞生長及移動的報導。

關鍵詞：蛋白表達、curcumin、v-Src、c-Src、FAK。

Abstract

Curcumin (diferulonylmethane) is a well-known agent with anti-inflammatory, antioxidant, and anticarcinogenic properties. In this study, we observed that curcumin could downregulate the expression of c-Src in colon cancer cells. To further study its effect on the kinase activity of Src, C3H10T1/2 fibroblasts expressing v-Src (IV5) were utilized as our study material. Curcumin treatment could inhibit the kinase activity of v-Src, which led to a decrease in tyrosyl substrate phosphorylation of Shc, cortactin, and FAK. Our in vitro kinase experiment revealed that the inhibitory effect of curcumin on Src could be direct. Consistent with the abrogation of Src activity was the reduction of Src Tyr-416 phosphorylation, Src-mediated Shc Tyr-317 phosphorylation, decreased ERK activation, and cell proliferation in v-Src transformed cells. Remarkably, curcumin not only exerted its negative effect on FAK via the disappearance of Src-mediated FAK phosphorylation, but also directly inhibited its enzymatic activity. Concurrent to reduced cortactin tyrosyl phosphorylation and FAK kinase activity was the abolishment of v-Src-mediated cell mobility. To our knowledge, this is the first report indicating that curcumin can retard cellular growth and migration via downregulation of Src and FAK kinase activity.

Key words: protein expression、

curcumin、v-Src、c-Src、FAK。

二、緣由與目的

Curcumin (diferulonylmethane), a popular dietary spice in the East, is a well-known agent with anti-inflammatory, antioxidant, and anticarcinogenic properties (1,2). To date, the inhibitory effects of curcumin on various signaling proteins have been reported (2). Conceivably, through inhibition of these molecules, curcumin can effectively suppress or revert tumor formation and retard metastasis.

The protein tyrosine kinase (TK) is a large and diverse multigene family that plays an important role in a variety of physiologic activities (3). c-Src, encoded by the cellular homologue of v-*src*, is a ubiquitously expressed cytoplasmic TK whose overexpression and enhancement of enzymatic activity have been strongly implicated human tumors (4).

Interestingly, we observed that curcumin could inhibit the expression of c-Src. To further study its effect on the kinase activity of Src, we investigated the biological responses and the molecular mechanisms involved in these responses in cells transformed with v-Src. Notably, we observed that curcumin could directly inhibit the activity of both Src and FAK, which has not been revealed before. And through downregulation of the activity of the activity of these two kinases, curcumin effectively inhibited the proliferation and migration of v-Src transformed cells.

三、結果與討論

Curcumin inhibits the proliferation and v-Src kinase activity in IV5 cells

Since curcumin (Figure 1A) is a well-established food constituent with chemopreventive properties and its effect on Src is poorly described, thus, we utilized cells transformed with v-Src (IV5) to evaluate the possibility that curcumin might inhibit the proliferation of IV5 cells. As shown in Figure 1B, compared to control cells, curcumin significantly inhibited cellular growth of IV5 cells in a dose-dependent manner (the IC_{50} for 24 hr growth inhibition is approximately 15 μM). Because tyrosyl phosphorylation plays a critical role in mitogenesis, we investigated the influence of various concentrations of curcumin on the profile of tyrosyl-phosphorylated proteins in IV5 cells after 24 hr treatment. Whole cell extracts prepared from curcumin-treated and untreated IV5 cells were resolved in SDS-PAGE and analyzed by phosphotyrosine (pTyr) Western immunoblotting. As demonstrated in Figure 2, ~ 20 μM curcumin could cause the decrease in the pTyr content of a number of cellular proteins suggesting that curcumin might inhibit any or multiple tyrosine kinases. Due to no significant change of the pTyr profile in curcumin-treated normal control cells (data not shown), thus, this prompted us to speculate that curcumin might negatively influence v-Src activity. Indeed, reduced Src-Tyr-416 phosphorylation in response to curcumin was observed while no alteration of the amount of v-Src was detected (Figure 3A). When the concentration of curcumin reached ~20 μM, its inhibition of Src phosphorylation

was ~30 % after normalization ($p < 0.05$). To further confirm the reduction of v-Src enzymatic activity following curcumin addition, the level of phosphorylated cortactin in curcumin-treated and untreated IV5 cells was compared. While similar amounts of cortactin were detected in all the samples analyzed, reduced tyrosyl phosphorylation of cortactin following curcumin treatment was dose-dependent (Figure 3B). Further confirmatory evidence for the curcumin-mediated reduction of Src kinase activity was provided by a time-dependent experiment in which Shc Pi-Tyr-317 was significantly diminished in curcumin-treated IV5 cells, though the amount of Shc was not altered (Figure 4). Despite 100 μM curcumin was utilized in the time course study, ~20 μM curcumin was statistically significant to reduce Shc Pi-Tyr-317 (data not shown). And interestingly, we observed that the curcumin-mediated inhibition of Src Pi-Tyr-416 preceding the inhibition of Shc Pi-Tyr-317 (Figure 4).

Curcumin can directly inhibit Src kinase activity

The abrogation of total tyrosyl phosphorylation and the reduced level of tyrosyl-phosphorylated cortactin, Tyr-416 phosphorylated Src, and Tyr-317-phosphorylated Shc in curcumin-treated IV5 cells implied that curcumin could directly or indirectly abolish Src kinase activity. To assess the former possibility, Src immunoprecipitates prepared from IV5 cells were incubated with enolase as an exogenous substrate and [γ-^{32}P]-ATP in the presence or absence of curcumin. As shown in Figure 3C, compared to the control, significantly reduced ^{32}P-labeled Src and enolase were detected in curcumin-incubated samples (IC_{50} = ~50 μM). This finding indicated that curcumin could directly inhibit the enzymatic activity of Src.

Curcumin treatment led to the reduction of ERK phosphorylation

It is well documented that tyrosyl-phosphorylated Shc associates with Grb2/SOS complex and activates Ras, which in turn triggers the Raf -> MEK-> ERK cascade. Since curcumin diminished the level of Shc Pi-Tyr-317, thereby we addressed the point whether the reduced Shc Pi-Tyr-317 might affect ERK activation by determining the ERK activities in control and curcumin-treated IV5 cells. Because MEK-mediated ERK phosphorylation on residues Thr-202 and Tyr-204 increases the enzymatic activity of ERK [5,6], we therefore applied monoclonal antibody specifically recognized these phosphorylated residues of ERK in Western immunoblotting. As demonstrated in Figure 5, a significant dose- and time-dependent reduction of phosphorylated ERK was detected in curcumin-treated IV5 cells as compared to control when the expression of ERK in these cells was normalized.

Curcumin inhibits the kinase activity of FAK

As demonstrated in Figure 2, curcumin treatment decreased tyrosyl phosphorylation

of a number of proteins, especially 125-kDa protein, in IV5 cells. Since FAK is a putative Src substrate with a similar molecular weight, it is likely that curcumin reduces tyrosyl phosphorylation of FAK. To prove this hypothesis, lysates prepared from curcumin-treated and untreated IV5 cells were immunoprecipitated with anti-FAK antibody and analyzed by SDS-PAGE with either anti-pTyr or anti-FAK immunoblotting. As expected, while a similar amount of FAK was present in each FAK immunoprecipitate analyzed, a significant decrease of tyrosyl phosphorylation of FAK was observed when the concentration of curcumin reached 20 μM ($p< 0.01$) (Figure 6B). And consistent with what has been described previously (i.e., that Src-mediated phosphorylation results in FAK activation), FAK activity measured by the level of FAK Tyr-397 phosphorylation was greatly reduced in curcumin-treated IV5 cells (Figure 6B). Again, this curcumin-mediated downregulation of FAK activity was not only time-, but also dose-dependent (Figure 6A). To further study whether curcumin can directly affect FAK enzymatic activity, FAK immunoprecipitates prepared from IV5 cells were incubated with GST-397Y, which contains FAK Tyr-397 and its neighboring sequence as an exogenous substrate, and [γ-^{32}P] ATP in the presence or absence of curcumin. As demonstrated in Figure 6C, the ^{32}P-incorporation of FAK and GST-397Y was greatly diminished in curcumin-treated samples in a dose-dependent manner (IC_{50} = ~34 μM). This finding suggests that curcumin can directly abrogate FAK kinase activity.

Curcumin abolished fibronectin-mediated cell migration in IV5 cells

FAK is a component promoting focal-contact turnover and its interaction with Src is crucial for integrin-induced cell motility [7]. Since curcumin could inhibit the enzymatic activity of both Src and FAK, thereby its effect on integrin-mediated cell mobility was attempted. To address this question, a modified Boyden chamber assay was performed to determine the fibronectin-induced chemotaxis in the presence or absence of curcumin. As demonstrated in Figure 7, curcumin could reduce the migration of IV5 cells toward fibronectin in a dose-dependent manner. This observation implies that curcumin can inhibit integrin-mediated cell migration.

四、計畫成果自評

本計畫進行順利，報告所提出的實驗成果已經被 Biochemical Pharmacology 所接受。

五、參考資料

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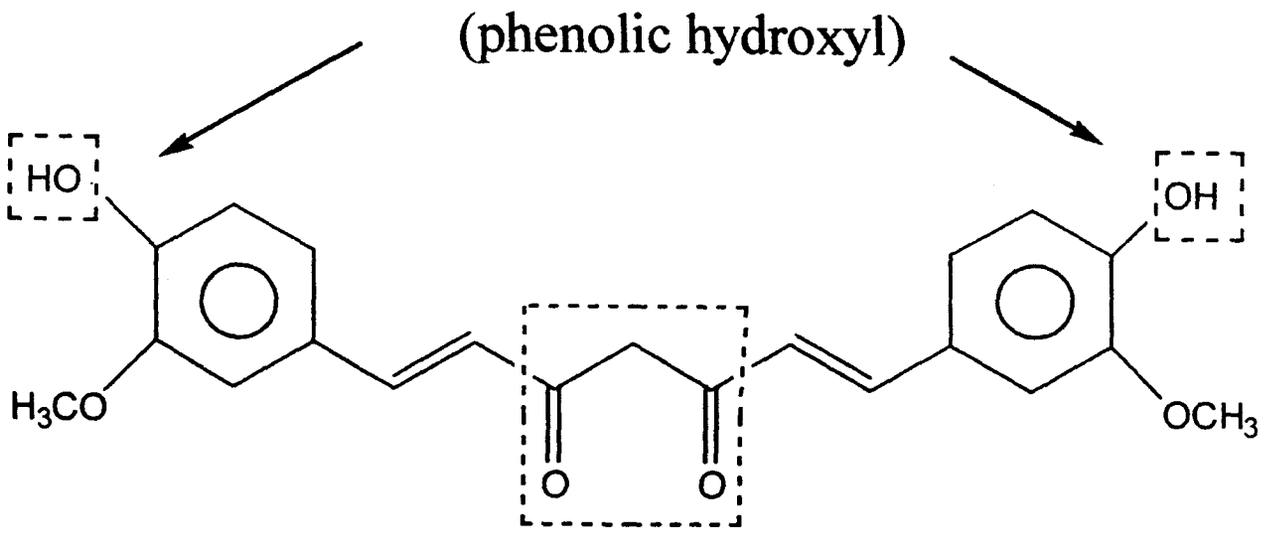
A



(β-diketone)

B

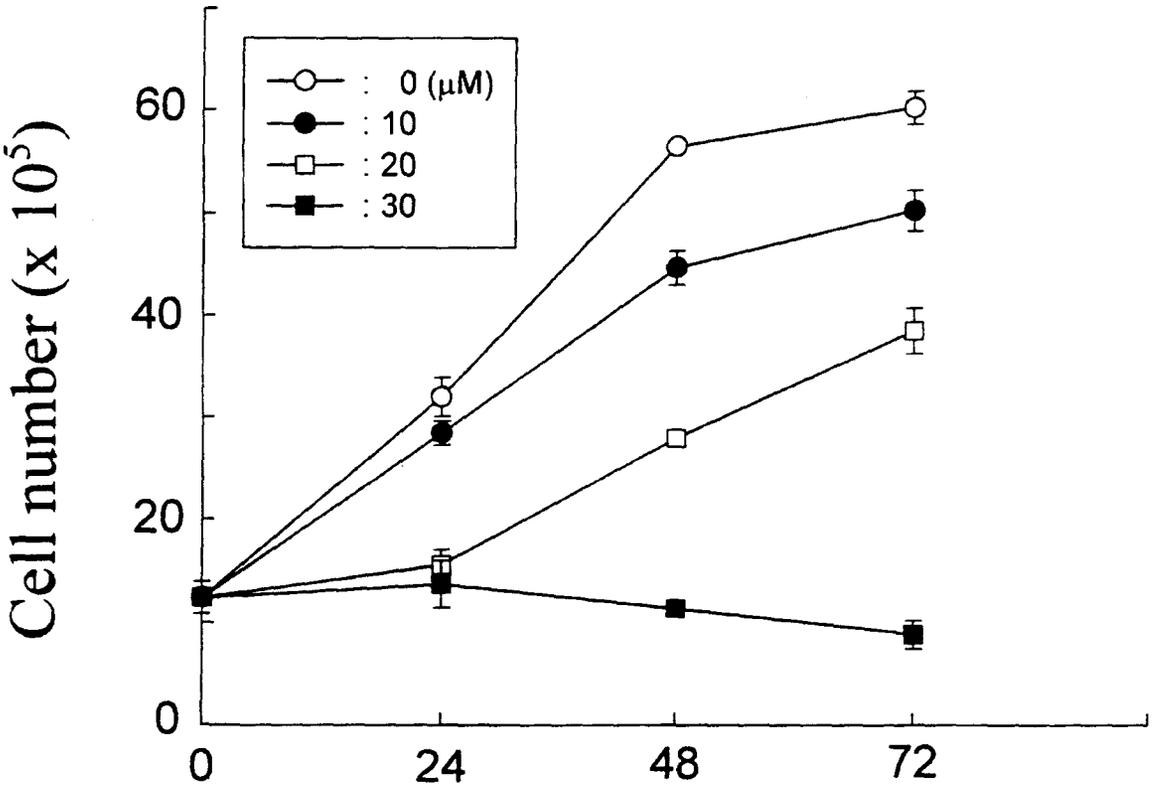


Fig. 1

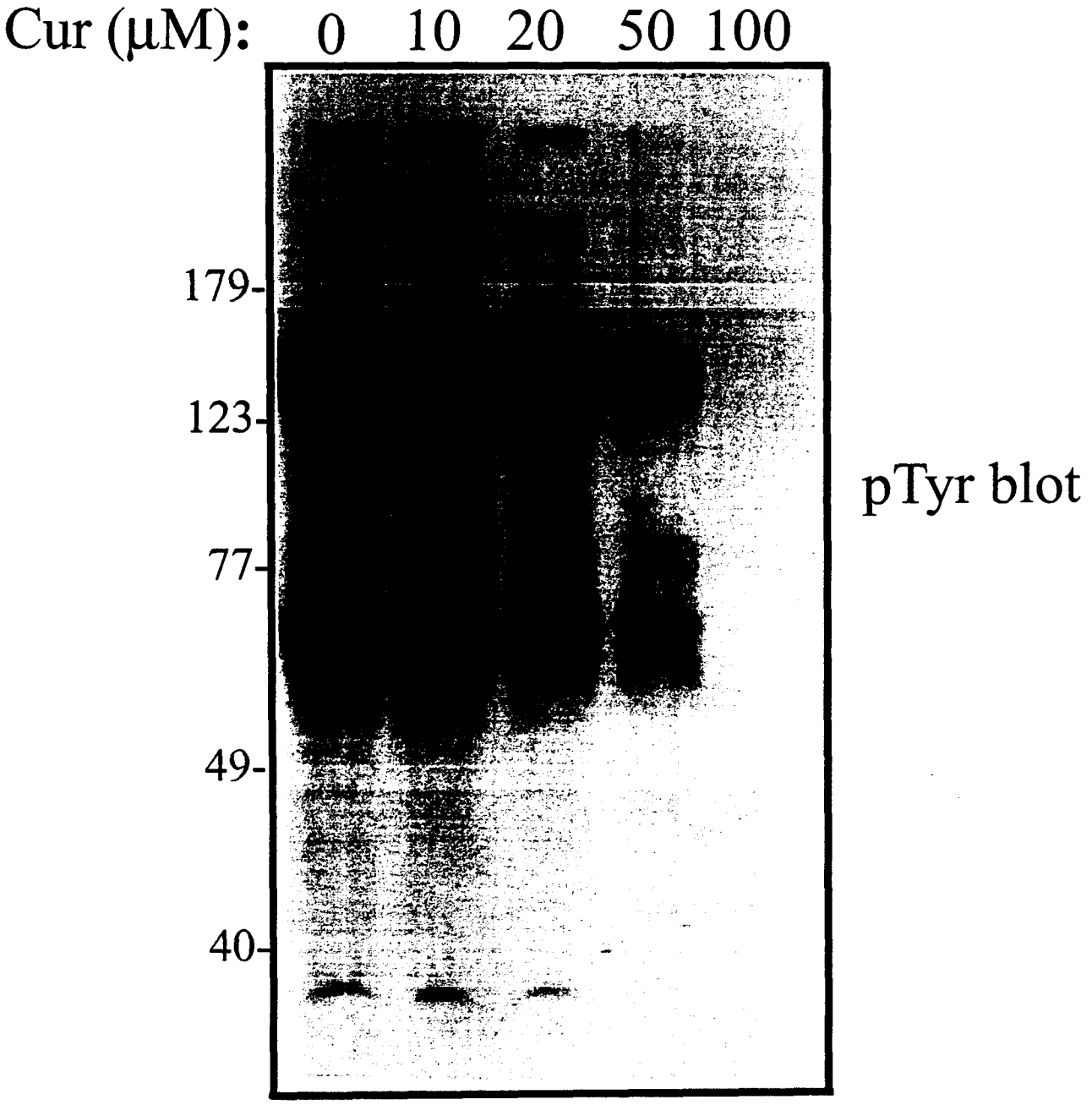


Fig. 2

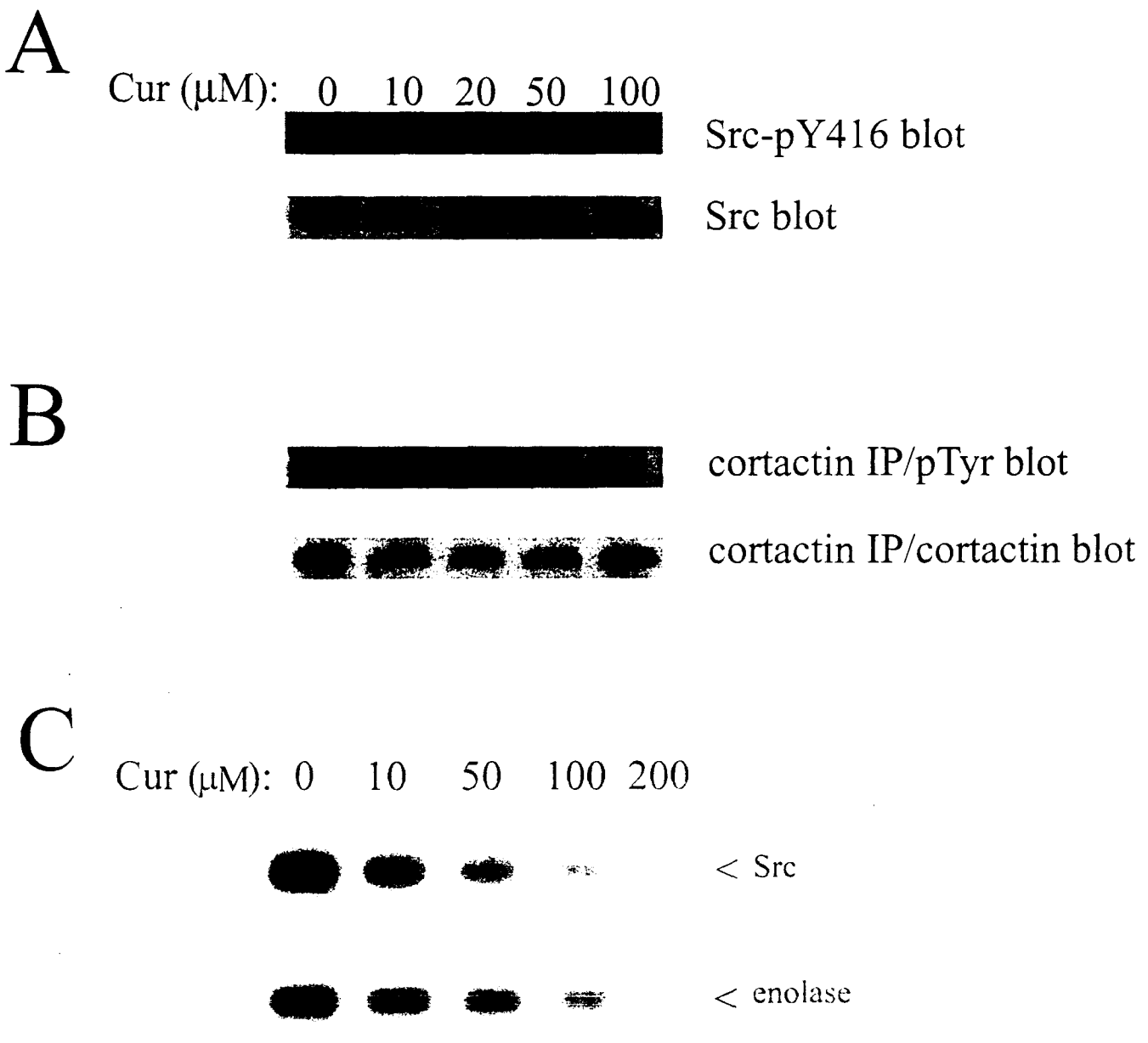

A
Cur (μM): 0 10 20 50 100
Src-pY416 blot
Src blot
B
cortactin IP/pTyr blot
cortactin IP/cortactin blot
C
Cur (μM): 0 10 50 100 200
< Src
< enolase

Fig. 3

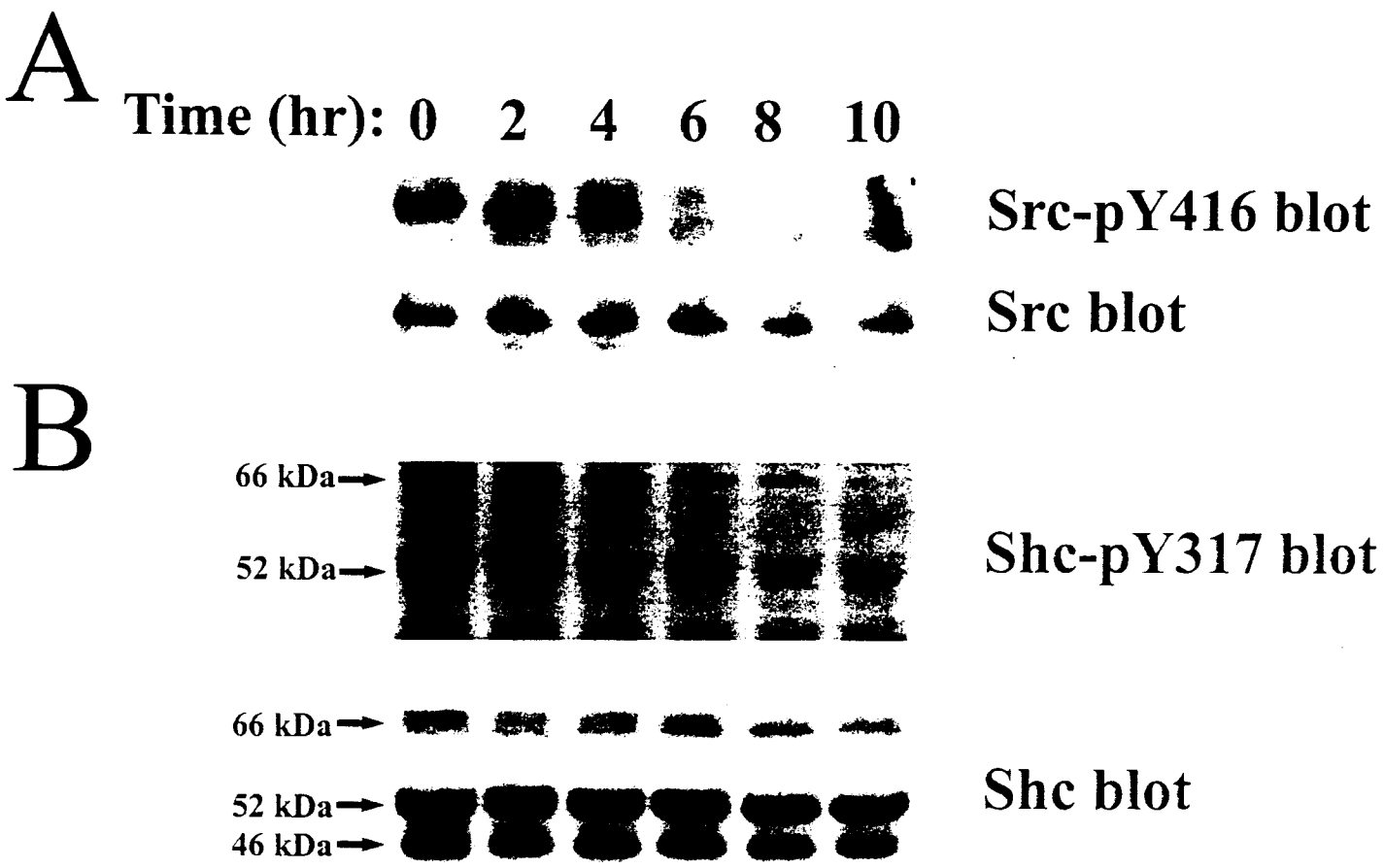
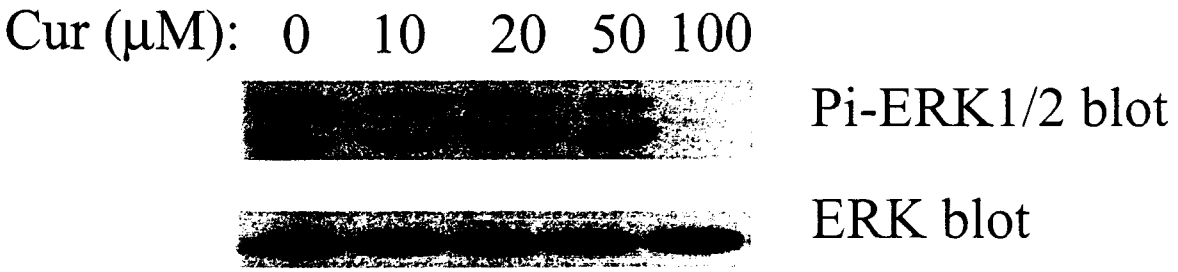

A
Time (hr): 0 2 4 6 8 10
Src-pY416 blot
Src blot
B
66 kDa
52 kDa
Shc-pY317 blot
66 kDa
52 kDa
46 kDa
Shc blot

Fig. 4

A


Cur (μM): 0 10 20 50 100
Pi-ERK1/2 blot
ERK blot

B

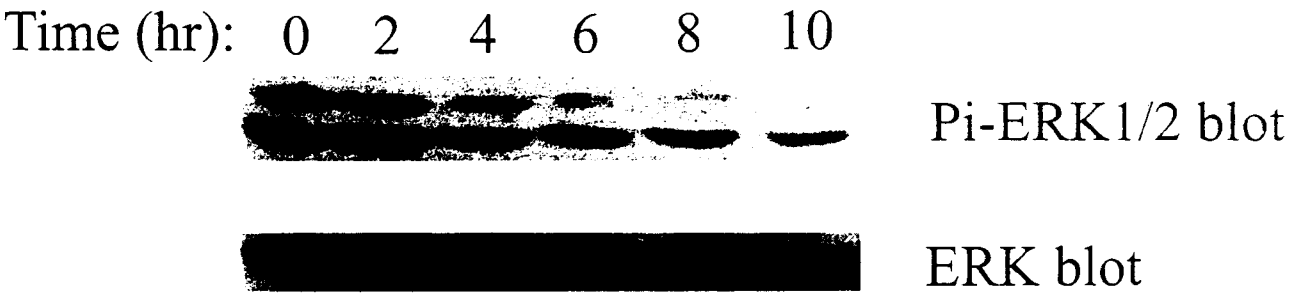
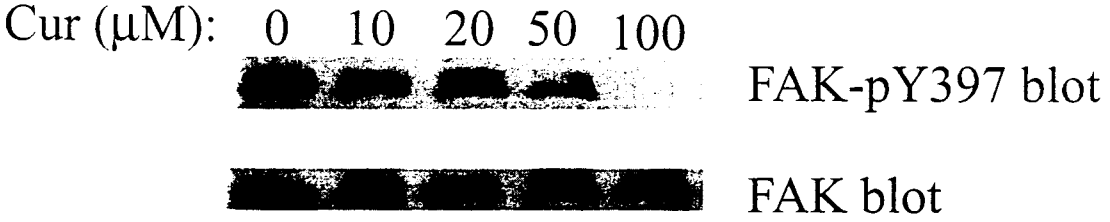
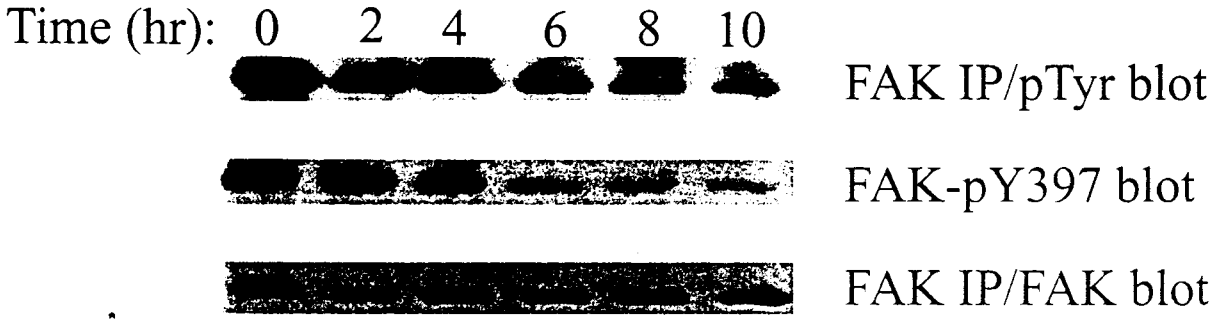

Time (hr): 0 2 4 6 8 10
Pi-ERK1/2 blot
ERK blot

Fig. 5

A


Cur (μM): 0 10 20 50 100
FAK-pY397 blot
FAK blot

B


Time (hr): 0 2 4 6 8 10
FAK IP/pTyr blot
FAK-pY397 blot
FAK IP/FAK blot

C

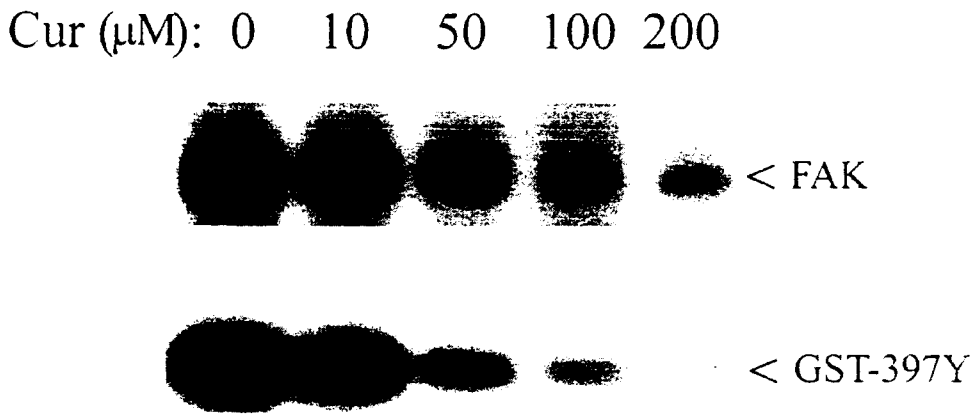

Cur (μM): 0 10 50 100 200
< FAK
< GST-397Y

Fig. 6

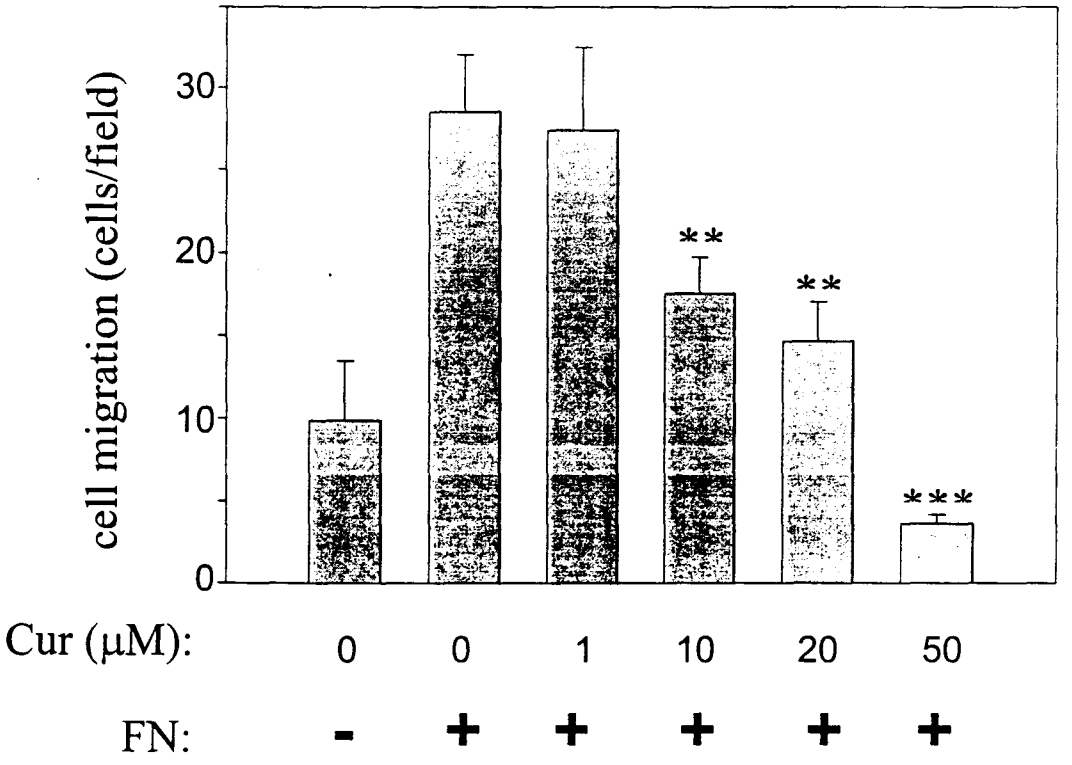


Fig. 7