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Mitotic Activation of c-Src Is Suppressed by Csk

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The kinase activity of the proto-oncogene product, c-Src, increases during mitosis through partial dephosphorylation of Tyr527, the negative regulatory site of c-Src. To examine whether or not Csk, a candidate kinase specific for Tyr527, is involved in this regulation, we developed a Balb/c 3T3 cell line overexpressing Csk and a Csk-deficient cell line. The overexpression of wild-type Csk caused significant suppression of the c-Src activity during mitosis. A membrane-targeted Csk, which has an amino-terminal myristylation signal of c-Src, exhibited an effective suppression of the c-Src activity, even though its expression level was lower than that of endogenous Csk. Concomitant with the suppression of the c-Src activation, the level of tyrosine phosphorylation of a cortactin-related protein, a potential substrate of c-Src *in vivo*, was reduced. In contrast, the Csk-deficient cells exhibited constitutive activation of c-Src, which showed no significant change in its activity during mitosis. These results suggest that Csk indeed participates in the regulation of the c-Src activity during mitosis.

Key words: Csk — c-Src — Mitosis — Tyrosine phosphorylation — Cortactin

The proto-oncogene product, c-Src, is a protein tyrosine kinase belonging to the non-receptor class of tyrosine kinases. The c-Src protein is localized at the periphery of the cellular membrane via its N-terminal myristate. Although the function of c-Src still remains elusive, several lines of evidence suggested that c-Src as well as its relatives, e.g. Lck, Fyn and Lyn, can act as a component of the signal transduction machinery.[1,2] Several downstream substrates of c-Src have been identified.[3–5] Some of them were found to be cytoskeleton-associated proteins, such as cortactin (p80/85)[6] and focal adhesion kinase (FAK),[7] implying that c-Src functions in the regulation of cytoskeletal organization. Careful study of c-Src expression pointed to its preferential localization in the endosome, and to co-localization with microtubule structure and the spindle pole during interphase and mitosis, respectively.[8,9] These findings suggest a potential role of c-Src in secretion, protein trafficking and mitotic centriolar organization.

The kinase activity of c-Src is known to be regulated through the phosphorylation of its carboxy-terminal tyrosine residue, Tyr527.[10] The phosphorylation of Tyr527 represses the kinase activity of c-Src, and extensive dephosphorylation or replacement of Tyr527 with Phe causes 10–20 fold elevation of the kinase activity and transforming ability.[10] Thus, it has been believed that the activity of c-Src is regulated by tyrosine phosphatases and tyrosine kinases acting specifically on Tyr527. A membrane-bound tyrosine phosphatase, PTPα, has recently been shown to be an activator of c-Src *in vivo*, but its physiological role remains unknown.[11] A candidate kinase that mediates the repression of c-Src was first found in developing brain.[12–14] The kinase, designated as Csk (carboxy-terminal Src kinase), was able to phosphorylate c-Src specifically at Tyr527 and to repress the activity of c-Src not only *in vitro*, but also *in vivo*.[15] Targeted disruption of the *csk* gene resulted in embryonic lethality between embryonic day 9.5 and 10.5 with a complex phenotype including impaired neural tube formation.[16,17] The Csk-deficient embryos exhibited constitutive activation of c-Src as well as several relatives concomitant with enhanced tyrosine phosphorylation of cellular proteins. These findings demonstrated that Csk is an indispensable kinase involved in the regulation of c-Src.

During mitosis, c-Src is known to be activated through partial dephosphorylation of its regulatory tyrosine Tyr527.[18–21] Concurrent with this activation, c-Src is phosphorylated at serine and threonine residues in its amino terminus by cdc2 kinase or its relatives, though the N-terminal phosphorylation is not directly involved in the activation of c-Src.[22] Thus, it has been suggested that a Tyr527 phosphatase and/or kinase also plays critical roles in the regulation of c-Src during mitosis, although the enzymes remain to be identified.

In this study, we examined whether or not Csk indeed acts as a Tyr527 kinase during mitosis using cell lines overexpressing or lacking Csk. Also, the regulatory mechanism through Csk was investigated in cell lines expressing a Csk mutant having the myristylation signal of c-Src. Activation of c-Src during mitosis was significantly repressed by the overexpression of Csk, while c-Src was constitutively activated even in G0/G1 phase in the cells lacking Csk. These findings demonstrated that regulation of c-Src during mitosis is mediated by Csk.

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MATERIALS AND METHODS

Cell culture A Csk-deficient cell line was established from Csk-deficient embryos as reported previously (manuscript submitted). To acquire sufficient ability of proliferation, embryos generated by intercrossing of p53-deficient mice with Csk heterozygous mice were used as a source of the cells. Balb/c 3T3 cells and the Csk-deficient cells were grown in Dulbecco-Vogt modified Eagle's medium (DMEM) supplemented with 10% calf serum. Cells (5×10^5) were plated in 100-mm plastic petri dishes (Falcon) and allowed to grow to confluency. Mitotic cells were isolated by incubating the culture in the presence of nocodazole (methyl[5-{2-thienylcarbonyl}-1*H*-benzimidazol-2-yl]carbamate) at a final concentration of 0.4 μg/ml for 8 h. About 50% of the cells showing a rounded shape and mitotic morphology were detached by gentle pipetting up and down with an automatic micropipette, and collected by low-speed centrifugation (1000 rpm). The cell pellet was then washed twice with ice-cold phosphate-buffered saline.

Vector constructions For overexpression of Csk in Balb/c 3T3 cells, Csk cDNA[13] was introduced into pME18Sneo expression vector. The *Sal*I-*Sal*I fragment of Csk cDNA was cut out from pBRCsk, blunt-ended and ligated to a *Mul*I linker. The resulting *Mul*I-*Mul*I fragment was then cloned into the *Mul*I sites of pME18Sneo to yield pMECsk. The cDNA encoding the membrane-targeted Csk (Src/Csk)[23] was kindly provided by Dr. A. Veillete, and it was introduced into the pME18Sneo vector using the *Mul*I linker.

Expression of Csk in the cells Transfection of Balb/c 3T3 cells and the Csk-deficient cells was carried out by the calcium phosphate transfection method.[24] Transformants were selected by incubating the culture in the presence of G418 (GIBCO) at a concentration of 400 μg/ml for over 7 days.

Immunoprecipitation The cells were rinsed twice with ice-cold phosphate-buffered saline and lysed with RIPA buffer (10 m*M* Tris-HCl, pH 7.4, 0.15 *M* NaCl, 1% Nonidet P-40, 1 m*M* EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1 m*M* Na_3VO_4, 50 μM Na_2MoO_4), then the lysate was clarified by centrifugation. c-Src was immunoprecipitated by incubating the lysate (100 μg of protein) with 0.1 μg of an anti-v-Src antibody (Oncogene Science) for 1 h on ice, followed by a 30 min incubation with 1 μg of rabbit anti-mouse immunoglobulin G. Then, 15 μl of 20% (v/v) Pansorbin cell (Calbio) was added and the mixture was allowed to stand for 30 min on ice. Immunocomplexes were collected by centrifugation at 7000 rpm for 1 min at 4°C, and then washed three times with RIPA buffer and further three times with Buffer A (20 m*M* Tris-HCl, pH 7.4, 0.1% Nonidet P-40, 1 m*M* EDTA, 5 m*M* β-mercaptoethanol and 10% glycerol). For an analysis of the tyrosine phosphorylation of cortactin, the latter was immunoprecipitated from the lysate (40 μg of protein) with an anti-cortactin antibody (UBI). The following procedures were carried out according to the method described for immunoprecipitation of c-Src.

***In vitro* kinase reactions** Immunocomplexes of c-Src were washed twice with kinase buffer (20 m*M* PIPES [piperazine-N,N′-bis(2-ethanesulfonic acid]-NaOH, pH 7.0, 10 m*M* $MnCl_2$, 0.5 m*M* Na_3VO_4) and suspended in 20 μl of kinase buffer. Then 2 μl of acid-denatured enolase solution containing 10 μCi of [γ-^{32}P]ATP was added. After incubation for 30 min at 30°C, reactions were terminated by the addition of 10 μl of 3×sample buffer. The phosphorylated proteins were resolved on SDS-10% polyacrylamide gels (SDS-PAGE) and detected by autoradiography.

Immunoblot analysis The samples were subjected to SDS-PAGE, and proteins in the gel were electrophoretically blotted onto a nitrocellulose membrane. The membrane was sequentially treated with blocking reagent, the primary antibody and reagent for visualization. At the end of each step, the membrane was washed three times with Tris-buffered saline containing 0.1% Tween 20 (Tween-TBS). To detect c-Src, Csk and cortactin, 1% skim milk in Tween-TBS was used as a blocking reagent. For the detection of phosphotyrosine, the membrane was blocked with Tween-TBS. The primary antibodies used are an anti-v-Src antibody 327 (Oncogene Science, 1/1000 dilution), an anti-Csk antibody (1/2000 dilution), an anti-cortactin antibody (UBI, 1/2000 dilution) and an anti-phosphotyrosine antibody 4G10 (UBI, 1/5000 dilution). For visualizations of the mouse monoclonal antibodies and the rabbit polyclonal antibodies, alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, 1/500 dilution) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Zymed, 1/500 dilution) were used, respectively. The membrane was stained with 5 ml of buffer (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$) containing 0.017% BCIP (5-bromo-4-chloro-3-indolyl phosphate disodium salt 1.5-water; Wako) and 0.033% NBT (nitro blue tetrazolium; Wako).

RESULTS

Suppression of mitotic activation of c-Src by Csk We isolated several Balb/c 3T3 cell lines overexpressing wild-type Csk and a membrane-targeted Csk (Src/Csk), and utilized the clones expressing the highest levels of the Csk proteins. As shown in Fig. 1, the wild-type Csk was expressed about 10 times as much as endogenous Csk, while the expression of Src/Csk was less than that of the endogenous Csk. The kinase activity of Csk in the cells was increased in parallel with the amount of the Csk

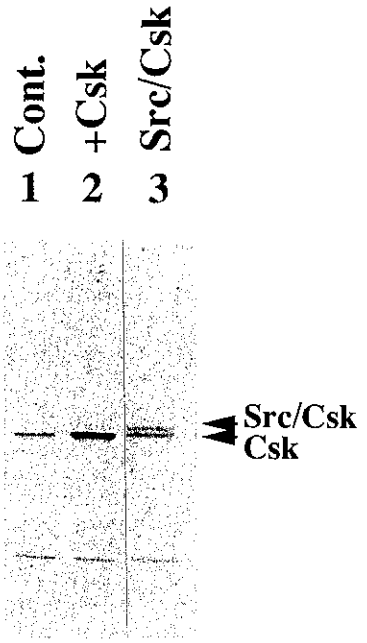

Fig. 1. Expression of wild-type Csk and Src/Csk in Balb/c 3T3 cells. The cell lysates prepared from control Balb/c 3T3 cells (lane 1, Cont.), Csk-overexpressing cells (lane 2, +Csk) and Src/Csk-expressing cells (lane 3, Src/Csk) were subjected to immunoblot analysis with an anti-Csk antibody.

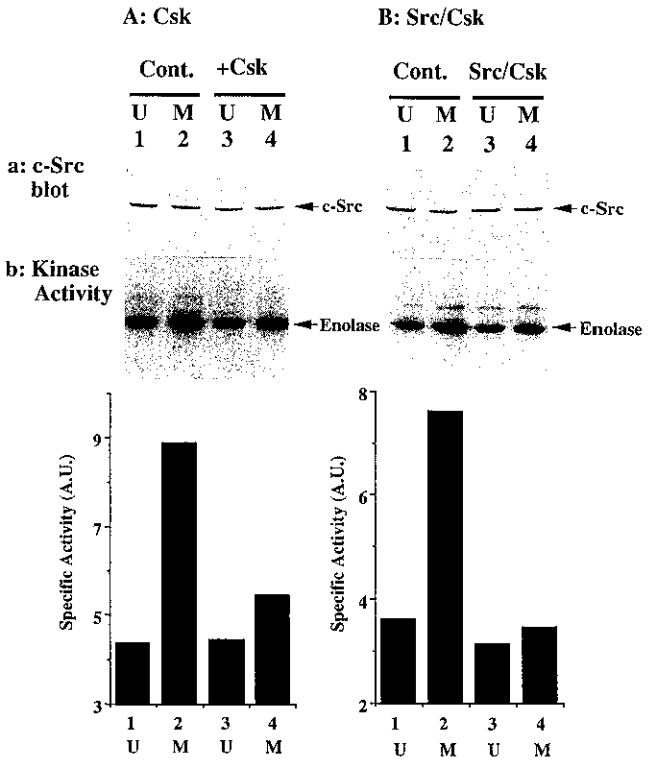

Fig. 2. Suppression of mitotic activation of c-Src by the expression of Csk. A) The cell lysates were prepared from unsynchronized (U) (lane 1) and mitotic (M) (lane 2) control Balb/c 3T3 cells, and unsynchronized (lane 3) and mitotic (lane 4) Csk-overexpressing cells, and aliquots were analyzed for c-Src expression by immunoblotting with an anti-Src antibody (a). The c-Src protein was immunoprecipitated from the lysates, and its kinase activity was determined with enolase as an endogenous substrate (b). Radioactivity incorporated into enolase was divided by the intensity of the band of c-Src, and the calculated specific activity of c-Src is shown in arbitrary units (A.U.) in the lower panel. B) Unsynchronized (lane 1) and mitotic (lane 2) control Balb/c 3T3 cells, and unsynchronized (lane 3) and mitotic (lane 4) Src/Csk-expressing cells were lysed, and the expression of c-Src (a), the kinase activity of the immunoprecipitated c-Src (b) and the specific activity of c-Src (lower panel) were analyzed by the same procedure as described above.

protein (data not shown). The c-Src protein was immunoprecipitated from the cell lysates prepared from unsynchronized Balb/c 3T3 cells (Fig. 2A lane 1), mitotic cells (lane 2), unsynchronized cells overexpressing Csk (lane 3) and mitotic cells overexpressing Csk (lane 4), and the kinase activity of c-Src was determined with enolase as an exogenous substrate. As can be seen in lanes 1 and 2, the specific activity of c-Src increased two- to three-fold in the control mitotic cells. In the Csk-overexpressing cells, the level of c-Src activation during mitosis (about 1.3-fold) was less than that in the control cells. The expression of Src/Csk showed a similar suppressive effect on the c-Src activity to that of wild type Csk (Fig. 2B), even though its expression level was lower than that of endogenous Csk (Fig. 1). A Csk-deficient cell line (fibroblast-like cells) was established from the Csk-deficient embryos (manuscript submitted). In these Csk-deficient cells, c-Src exhibited an enhanced specific activity (about 15-fold) (Fig. 3 lane 1), but further activation of Src in mitotic cells were hardly detected (lane 2). On the other hand, in a cell line expressing Csk at a comparable level to that of normal cells, generated by transfection of Csk cDNA into the Csk-deficient cells, the mitotic activation of c-Src was restored (lanes 3 and 4).

Tyrosine phosphorylation in the mitotic cells Owing to the activation of c-Src, some target proteins for c-Src were expected to become phosphorylated in the mitotic cells. As shown in Fig. 4B, however, the level of tyrosine phosphorylation detected with an anti-phosphotyrosine antibody was rather reduced in the mitotic cells. The decreases in the major bands seen in the unsynchronized cells around 120 kDa, 70 kDa and 60 kDa, which corresponded to $p125^{FAK}$, paxillin and c-Src respectively, were most evident. The identities of these proteins were

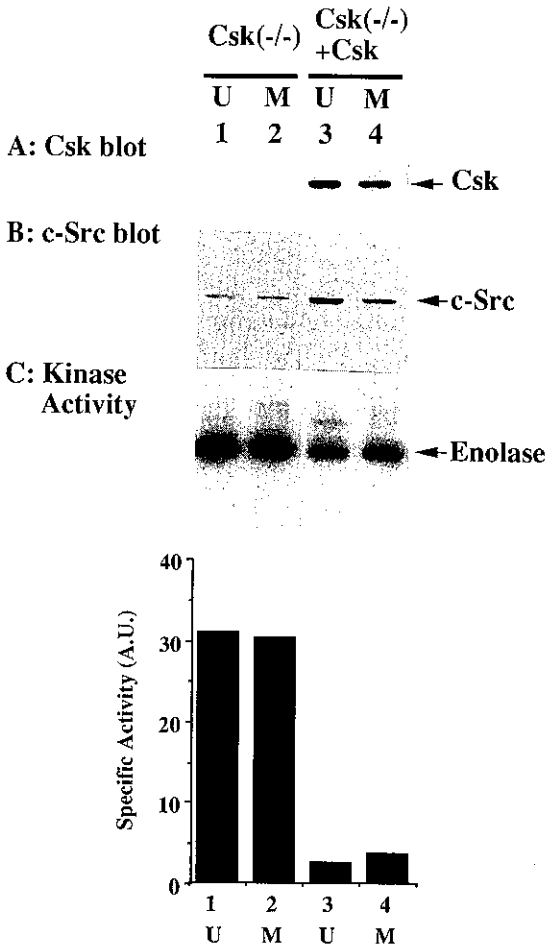

Fig. 3. Constitutive activation of c-Src in the Csk-deficient cells. The cell lysates were prepared from unsynchronized (lane 1) and mitotic (lane 2) Csk-deficient cells (Csk(−/−)), and unsynchronized (lane 3) and mitotic (lane 4) Csk-expressing cells (Csk(−/−)+Csk). The expressions of Csk (A) and c-Src (B) were determined by immunoblot analysis with an anti-Csk antibody and anti-Src antibody, respectively. The kinase activity of the immunoprecipitated c-Src was determined with enolase as a substrate. The lower panel shows the calculated specific activity of c-Src.

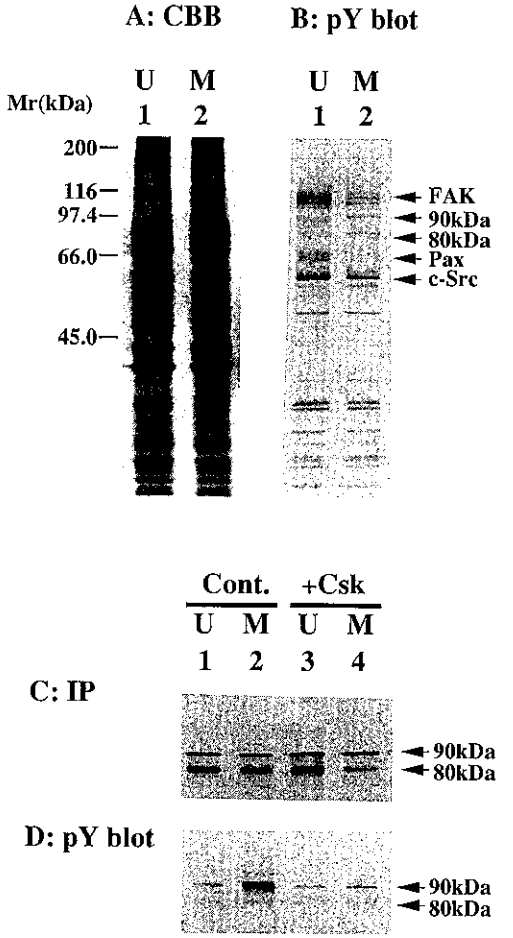

Fig. 4. Tyrosine phosphorylated proteins in the unsynchronized and mitotic cells. The cell lysates were prepared from unsynchronized (lane 1) and mitotic (lane 2) Balb/c 3T3 cells. The lysates were resolved on an SDS-PAGE gel, and the proteins were stained with Coomassee Brilliant Blue (A). The molecular sizes of marker proteins are shown on the left of the gel. The tyrosine-phosphorylated proteins were detected by immunoblot analysis with an anti-phosphotyrosine antibody (B). The locations of $p125^{FAK}$ (FAK), 90 kDa protein, 80 kDa protein, paxillin (Pax) and c-Src are shown by arrows on the right of the gel. The cell lysates were prepared from unsynchronized (lane 1) and mitotic (lane 2) control Balb/c 3T3 cells, and unsynchronized (lane 3) and mitotic (lane 4) Csk-overexpressing cells. The cortactin-related proteins were immunoprecipitated with an anti-cortactin antibody, and then the immunoprecipitates were subjected to immunoblot analysis with the anti-cortactin antibody (C). The tyrosine phosphorylation levels of the immunoprecipitates were analyzed by immunoblot analysis with an anti-phosphotyrosine antibody (D).

confirmed by immunoprecipitation assay, followed by immunoblot analysis with an antibody against each protein (data not shown). It seemed probable that the proteins involved in cell-to-cell or cell-to-substrate attachment were disrupted concomitant with morphological change, because their protein amounts were reduced in mitotic cells (data not shown). However, phosphorylation of several proteins around 80 kDa and 90 kDa was rather enhanced in the mitotic cells (Fig. 4B). Since we found in the previous study that cortactin (p80/85) was one of the *in vivo* substrates of c-Src (manuscript submitted), and its molecular size was similar to those of tyrosine-phosphorylated proteins in the mitotic cells, we examined its phosphorylation level in the mitotic cells.

The cell lysates were subjected to immunoprecipitation with an anti-cortactin antibody, and equal amounts of immunoreactive proteins (80 kDa and 90 kDa) were recovered from all the cell types (Fig. 4C). The tyrosine phosphorylation level of these proteins was detected with an anti-phosphotyrosine antibody (Fig. 4D). The phosphorylation of the 90 kDa protein was elevated in the control mitotic cells, while it was suppressed in the mitotic cells overexpressing Csk.

DISCUSSION

In this report we have demonstrated the possible involvement of Csk in the regulation of the c-Src activity during the cell division cycle. The mitotic activation of c-Src was significantly repressed by the overexpression of Csk, while c-Src was constitutively activated in the cells lacking Csk. Our findings support the previous hypothesis that the c-Src activity is regulated through phosphorylation at its carboxy-terminal regulatory tyrosine, Tyr527, during mitosis.[21] The membrane-targeted Csk (Src/Csk) was more effective on the c-Src activity than wild-type Csk. The myristylation signal of Src/Csk was predicted to facilitate the access of Csk to the membrane structure, where c-Src is anchored, and thus enhance the efficiency of Csk function. Recently, Sabe *et al.* reported that Csk can form complexes through its SH2 domain with several tyrosine-phosphorylated proteins, including paxillin and $p125^{FAK}$, which are functionally associated with c-Src.[25] Therefore, the SH2-mediated redistribution of Csk to a membrane or cytoskeletal structure, where many substrates of c-Src and c-Src itself are present, may be involved in the regulation of Csk function, although detailed studies on its molecular mechanism will be needed.

We have shown here that a cortactin-related 90 kDa protein is a candidate target for the activated c-Src in the mitotic cells. Concomitant with the suppression of c-Src, its phosphorylation was reduced, suggesting that c-Src mediates its phosphorylation. Our previous study also revealed that cortactin is one of the major tyrosine-phosphorylated proteins in the Csk-deficient cells, in which c-Src is highly activated (manuscript submitted). Cortactin was originally isolated as a substrate of v-Src oncogene product.[8] It has an SH3 domain close to its carboxy-terminus and it is a cytoskeleton (F-actin)-associated protein. Thus, it is intriguing to speculate that phosphorylation of cortactin is related to regulation of morphological change during mitosis, although no direct evidence is yet available. We also examined the effect of Csk overexpression on the phosphorylation of other known targets for c-Src (paxillin and FAK). However, changes in their phosphorylation during mitosis were undetectable, because of extensive down-regulation of their protein levels in the mitotic cells (data not shown). Recent studies on the targets for c-Src in mitosis demonstrated that GAP-associated protein (p62)-related protein, p68, became tyrosine-phosphorylated and physically associated with c-Src during mitosis.[26,27] Because p68 can bind polyribonucleotides, it was suggested that c-Src may regulate the processing, trafficking or translation of RNA in a cell-cycle-dependent manner. Since c-Src has broad range of substrate specificity, it is plausible that c-Src shows redundancy in its cellular function. Elucidation of the actual function of c-Src must await further analysis of its substrate proteins.

In mitosis, c-Src is phosphorylated on serine and threonine residues by an essential kinase(s) in the cell cycle, such as cdc2 kinase or a related kinase, with concomitant elevation of its kinase activity.[18–20] This fact strongly implies important roles of c-Src in the cell division cycle. However, Csk-overexpressing cells, in which mitotic activation of c-Src is repressed, did not show any significant change in growth rate as compared with normal cells. Also, the Csk-deficient cells, in which Src is always fully active, had a normal growth rate (data not shown). These results raise the possibility that the change of c-Src activity during mitosis may not play essential roles, at least in the control of cell growth. In this study, however, the suppression of c-Src by Csk overexpression was not complete, and thus a slight activation, which might be sufficient for promotion of the cell cycle, was readily observed. We acquired several clones which expressed Src/Csk, but no clone expressed it over the level of endogenous Csk, suggesting that cells that expressed a higher level of Src/Csk, enough for the complete suppression of c-Src, were unable to survive. To evaluate the actual role of the c-Src activation, the complete suppression system using an inducible expression vector should be employed. Finally, a tyrosine phosphatase that acts as an activator of c-Src presumably exists. Its identification may be crucial to understand the regulation of c-Src during mitosis.

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REFERENCES

1) Sudol, M. Nonreceptor protein tyrosien kinases. *In* "The Molecular Basis of Human Cancer," ed. B. Neel and R. Kumar, pp. 203–233 (1993). Futura Publishing Co., Inc., Mount Kisco, New York.
2) Clark, E. A. and Brugge, J. S. Redistribution of activated pp60$^{c\text{-}src}$ to integrin-dependent cytoskeletal complexes in thrombin-stimulated platelets. *Mol. Cell. Biol.*, **13,** 1863–1871 (1993).
3) Hausdorff, W. P., Pitcher, J. A., Luttrell, D. K., Kurose, H., Parsons, S. J., Caron, M. G. and Refkowttz, R. J. Tyrosine phosphorylation of G protein a subunit by pp60$^{c\text{-}src}$. *Proc. Natl. Acad. Sci. USA,* **89,** 5720–5724 (1992).
4) Chen, J., Martin, B. L. and Brautigan, D. L. Regulation of protein serine-threonine phosphatase type 2A by tyrosine phosphorylation. *Science,* **257,** 1261–1264 (1992).
5) McGiade, J., Cheng, A., Pelicci, G. and Pawson, T. Shc proteins are phosphorylated and regulated by the v-src and v-fps protein tyrosine kinases. *Proc. Natl. Acad. Sci. USA,* **89,** 8869–8873 (1992).
6) Wu, H., Reynolds, A. B., Kanner, S. B., Vines, R. R. and Parsons, J. T. Identification and characterization of a novel cytoskeleton-associated pp60^{src} substrate. *Mol. Cell. Biol.,* **11,** 5113–5124 (1991).
7) Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA,* **89,** 5192–5196 (1992).
8) Kaplan, K. B., Swedlow, J. R., Vermus, H. E. and Morgan, D. O. Association of p60$^{c\text{-}src}$ with endosomal membranes in mammalian fibroblasts. *J. Cell. Biol.,* **119,** 893–903 (1992).
9) David-Pfeuty, T. and Nouvian-Dooghe, N. Immunolocalization of the cellular src protein in interphase and mitotic NIH c-src overexpressor cells. *J. Cell Biol.,* **111,** 3097–3116 (1990).
10) Cooper, J. A. and Howell, B. The when and how of Src regulation. *Cell,* **73,** 1051–1054 (1993).
11) Zheng, X. M., Wang, Y. and Pallen, C. J. Cell transformation and activation of pp60$^{c\text{-}src}$ by overexpression of a protein tyrosine phosphatase. *Nature,* **359,** 336–339 (1992).
12) Okada, M. and Nakagawa, H. A protein tyrosine kinase involved in regulation of pp60$^{c\text{-}src}$. *J. Biol. Chem.,* **264,** 20886–20893 (1989).
13) Nada, S., Okada, M., MacAuley, A., Cooper, J. A. and Nakagawa, H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60$^{c\text{-}src}$. *Nature,* **351,** 69–72 (1991).
14) Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T. and Nakagawa, H. CSK: a protein tyrosine kinase involved in regulation of src family kinases. *J. Biol. Chem.,* **256,** 24249–24252 (1991).
15) Sabe, H., Okada, M., Nakagawa, H. and Hanafusa, H. Activation of c-Src in cells bearing v-Crk and its suppression by Csk. *Mol. Cell. Biol.,* **12,** 4706–4713 (1992).
16) Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M. and Aizawa, S. Constitutive activation of src family kinases in mouse embryos that lack Csk. *Cell,* **73,** 1125–1135 (1993).
17) Imamoto, A. and Soriano, P. Disruption of the csk gene, encoding a negative regulator of src family kinases, leads to neural tube defects and embryonic lethality in mice. *Cell,* **73,** 1117–1124 (1993).
18) Chackalaparampil, I. and Shalloway, D. Altered phosphorylation and activation of pp60$^{c\text{-}src}$ during fibroblast mitosis. *Cell,* **52,** 801–810 (1988).
19) Morgan, D. O., Kaplan, J. M., Bishop, J. M. and Varmus, J. E. Mitosis-specific phosphorylation of p60$^{c\text{-}src}$ by p34^{cdc2}-associated protein kinase. *Cell,* **57,** 775–786 (1989).
20) Shenoy, S., Choi, J. K., Bagrodia, S., Copeland, T. D., Maller, J. L. and Shalloway, D. Purified maturation promoting factor phosphorylates p60$^{c\text{-}src}$ at the sites phosphorylated during fibroblast mitosis. *Cell,* **57,** 763–774 (1989).
21) Bagrodia, S., Chackalaparampil, I., Kmiecik, T. E. and Shalloway, D. Altered tyrosine 527 phosphorylation and mitotic activation of p60$^{c\text{-}src}$. *Nature,* **349,** 172–175 (1991).
22) Taylor, S. J. and Shalloway, D. The cell cycle and c-Src. *Curr. Opin. Gen. Dev.,* **3,** 26–34 (1993).
23) Chow, L. M. L., Fournel, M., Davidson, D. and Veillette, A. Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50^{csk}. *Nature,* **365,** 156–160 (1993).
24) Chen, C. and Okayama, H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.,* **7,** 2745 (1987).
25) Sabe, H., Hata, A., Okada, M., Nakagawa, H. and Hanafusa, H. Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. *Proc. Natl. Acad. Sci. USA,* **91,** 3984–3988 (1994).
26) Tayler, S. J. and Shalloway, D. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. *Nature,* **368,** 867–871 (1994).
27) Fumagalli, S., Totty, N. F., Hsuan, J. J. and Courtneige, S. A. A target for Src in mitosis. *Nature,* **368,** 871–874 (1994).