J. Biochem. 126, 957-961 (1999)

Neuron-specific Cdk5 Kinase Is Responsible for Mitosis-Independent Phosphorylation of c-Src at Ser75 in Human Y79 Retinoblastoma Cells

Goro Kato[1] and Shuichiro Maeda

Department of Biochemistry, Yamanashi Medical University, Nakakoma, Yamanashi 409-3898

Received July 16, 1999; accepted September 6, 1999

c-Src is phosphorylated at specific serine and threonine residues during mitosis in fibroblastic and epithelial cells. These sites are phosphorylated *in vitro* by the mitotic kinase Cdk1 ($p34^{cdc2}$). In contrast, c-Src in Y79 human retinoblastoma cells, which are of neuronal origin, is phosphorylated at one of the mitotic sites, Ser75, throughout the cell cycle. The identity of the serine kinase that nonmitotically phosphorylates c-Src on Ser75 remains unknown. We now are able to show for the first time that Cdk5 kinase, which has the same consensus sequence as the Cdk1 and Cdk2 kinases, is required for the phosphorylation in asynchronous Y79 cells. The Ser75 phosphorylation was inhibited in a dose-dependent manner by butyrolactone I, a specific inhibitor of Cdk5-type kinases. Three stable subclones that have almost no kinase activity were selected by transfection of an antisense Cdk5-specific activator p35 construct into Y79 cells. The loss of the kinase activity caused an approximately 85% inhibition of the Ser75 phosphorylation. These results present compelling evidence that Cdk5/p35 kinase is responsible for the novel phosphorylation of c-Src at Ser75 in neuronal cells, raising the intriguing possibility that c-Src acts as an effector of Cdk5/p35 kinase during neuronal development.

Key words: antisense p35, Cdk5 kinase, human c-Src, Ser75 phosphorylation, Y79 retinoblastoma cells.

The cellular c-*src* gene product, c-Src, is a membrane-associated 60-kDa tyrosine kinase (*1, 2*) and is regulated by phosphorylation at various sites by multiple kinases (*1*). c-Src is phosphorylated at specific serine and threonine residues in the amino terminal 'unique' domain during mitosis in fibroblastic and epithelial cells (*3-6*). One of the mitotic phosphorylation sites in human c-Src, Ser75, is phosphorylated in a mitosis-independent manner in asynchronous Y79 human retinoblastoma cells (*6*). The published patterns of two-dimensional tryptic phosphopeptide maps indicate that the novel serine phosphorylation occurs in cultured neurons (*7, 8*), neuroblastoma (*6, 9*), retinoblastoma (*6*), and some cultured cells expressing neuronal forms of c-Src (*6, 10*). It is related to cellular morphological regulation and neuronal character (*6, 8, 11*).

In fibroblast- and epithelial-like cells, Cdk1 presumably phosphorylates the mitotic Ser75 during mitosis (*4, 5*). The protein kinase that is responsible for the mitosis-independent phosphorylation has not yet been identified. Cdk1 and its related kinases, Cdk2 and Cdk5, have the same recognition consensus sequence (*12, 13*), basic/polar-S/T-P-X-basic, which corresponds to the sequence surrounding Ser75 in human c-Src. Kinase activities of Cdk1 and Cdk2 are temporally regulated during the cell cycle (*14*). In contrast, despite the wide distribution of Cdk5 expression in tissues and cell lines, Cdk5 kinase activity is detected only in terminally differentiated neural tissues such as brain and retina (*15-17*), and it is unrelated to cell cycle regulation (*14, 18*). Thus, Cdk5 kinase is expected to be responsible for the mitosis-independent phosphorylation in human Y79 retinoblastoma cells.

To test this hypothesis, we treated Y79 cells with a Cdk5 kinase inhibitor, butyrolactone I (BL-I), and expressed an antisense Cdk5-specific activator p35 construct in Y79 cells. These inhibitions of endogenous Cdk5 kinase activity in Y79 cells abolished the Ser 75 phosphorylation. These studies provide compelling evidence that the mitosis-independent phosphorylation of c-Src at Ser 75 in Y79 cells is mediated by Cdk5/p35 kinase.

MATERIALS AND METHODS

Materials—The monoclonal anti-Src antibody 327 was obtained from Oncogene Science. The affinity-purified rabbit polyclonal anti-Cdk5 antibody (C-8) was from Santa Cruz Biotechnology. BL-I was generously provided by Dr. M. Kitagawa.

Cell Culture—Human Y79 retinoblastoma cells were obtained from the RIKEN Cell Bank and cultured in suspension in RPMI 1640 plus 10% fetal bovine serum.

Radiolabeling of Cells—Unsynchronized Y79 cells in log-phase growth were cultured with or without BL-I (12-96 μM) for 18 h, then labeled with 1 mCi/ml [^{32}P]orthophosphate (ICN) as previously described (*6*) except that BL-I was included in the phosphate-free medium for the BL-I-treated cells.

Immunoprecipitation—To immunoprecipitate Cdk5 ki-

[1] To whom correspondence should be addressed. E-mail: gkato@res.yamanashi-med.ac.jp

Abbreviations: Cdk, cyclin-dependent kinase; BL-I, butyrolactone I.

nase, the unsynchronized cells were washed with PBS and lysed with RIPA buffer plus inhibitors (10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 μg/ml leupeptin, 20 μg/ml aprotinin, 50 mM β-glycerophosphate, 25 mM NaF, and 0.15 mM sodium orthovanadate). Lysates (150-300 μg protein) were precleared with protein A/G plus agarose and immunoprecipitated with C-8 polyclonal antibody. The precipitates were washed with RIPA buffer and separated on SDS-12.5% PAGE. c-Src was immunoprecipitated with monoclonal anti-Src antibody 327 from cell lysates and separated on SDS-10% PAGE as previously described (*6*).

Immunoblot Analysis of Cdk5 Kinase—The Cdk5 immunoprecipitates were separated on SDS-PAGE, transferred to nitrocellulose, and probed with a 1:500 dilution of C-8 antibody. The filters were treated with a 1:1,000 dilution of biotynylated goat anti-rabbit IgG (Life Technologies), then with a 1:1,000 dilution of tangerine-labeled streptoavidin (Molecular Probes). Proteins were detected with an FMBIO-100 Fluorescent Image Analyzer (TAKARA).

Kinase Activity of Cdk5 Kinase—The Cdk5 immunoprecipitates were washed three times with RIPA buffer and once with kinase buffer (*16*), then examined for their activity to phosphorylate histone H1 with or without 10 μM BL-I as previously described (*16*).

Generation of Antisense p35 Construct—A human p35 cDNA, derived from a plasmid pCMV-p35 (a gift from Dr. L.-H. Tsai), was subcloned into the pCMV-NEO-BAM expression vector in the antisense orientation as previously described (*19*).

Transfection—Y79 cells (8×10^6) were incubated on ice for 10 min, mixed with 20 μg of the p35 antisense construct or 20 μg of the sense construct, then electroporated with a Bio-Rad Gene Pulser at a setting of 0.8 kV and 25 μF. After 43 h, cells were cultured in selective medium containing G418 (500 μg/ml), and G418 resistant clones were selected.

Phosphopeptide Analyses—The phosphorylated proteins were excised from unfixed gels and subjected to V8 protease mapping (*6*). The tryptic digests of the N-terminal 16-kDa V8 fragments were subjected to two-dimensional tryptic phosphopeptide analysis (*6*) or to one-dimensional analysis on Tricine-SDS-PAGE (16.5% T/3% C) as previously described (*6*).

Detection and Quantitative Analysis of Radioactivity—A BAS2000 (Fuji) equipped with imaging plates was used as previously described (*6*).

RESULTS AND DISCUSSION

We first examined whether Cdk5 kinase activity is detected in asynchronous Y79 cells. Immunoblot analysis with the Cdk5 antibody C-8 revealed that the antibody specifically reacted with a 32-kDa protein immunoprecipitated from Y79 cell lysates (Fig. 1a). *In vitro* kinase assays of the immunoprecipitated Cdk5 were performed with histone H1 as an exogenous substrate. Cdk5 kinase from asynchronous Y79 cells exhibited an approximately 5-fold higher level of kinase activity than Cdk5 from asynchronous Hela and HepG2 cells (Fig. 1, b and c). The levels of phosphorylation at Ser75 in c-Src from these epithelial-like cells were reduced to less than 6% of that in Y79 cells (*6*). These data suggest that the difference in Cdk5 activity levels may reflect the Ser75 phosphorylation status in c-Src from these cells.

We next tested whether BL-I, a specific inhibitor of Cdk5 kinase (*20, 21*), inhibits the Ser75 phosphorylation in Y79 cells. The inhibitory activity of BL-I was confirmed by the *in vitro* Cdk5 kinase assay of the Y79 immunoprecipitate. BL-I (10 μM) decreased the kinase activity to the control level (Fig. 1c). Figure 2a shows the one-dimensional separation (top) of the tryptic phosphopeptides, phosphoserine 75-containing peptide 7 and phosphoserine 17-containing peptides 6 and 9 eluted from the conventional two-dimensional phosphopeptide maps (bottom) of c-Src from asynchronous Y79 cells. This one-dimensional analysis gives more reproducible solubilization of each peptide than the two-dimensional analysis and is suitable for the determination of the stoichiometry of phosphorylation at each site (*7*). Tryptic phosphopeptides from c-Src in Y79 cells cultured in the presence or absence of BL-I were separated on one-dimensional Tricine-SDS-PAGE (Fig. 2b). The relative intensity (*6*) of Ser75 phosphorylation in the absence of BL-I, calculated as the ratio of ^{32}P incorporation

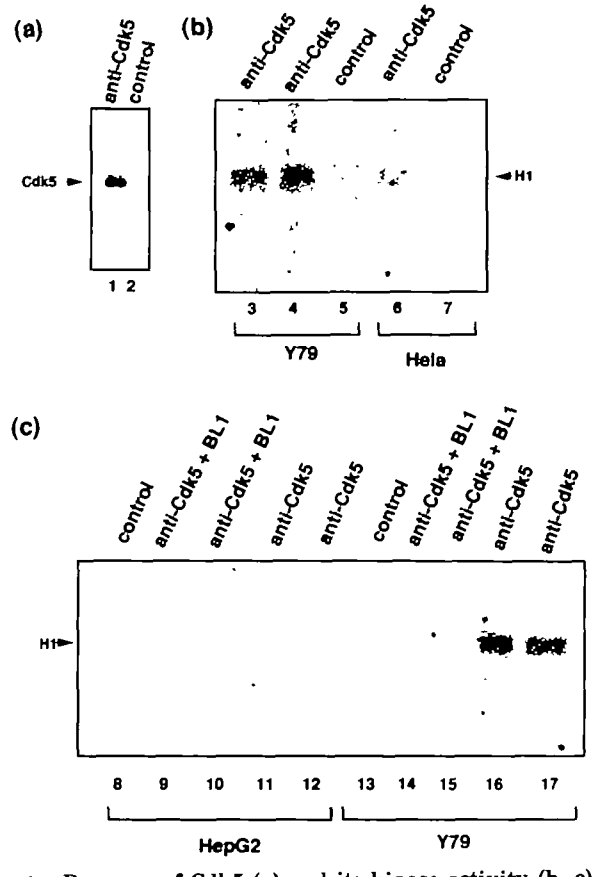

Fig. 1. **Presence of Cdk5 (a) and its kinase activity (b, c) in cultured Y79 cells.** Unsynchronized exponentially growing Y79 (lanes 1-5 and 13-17), HeLa (lanes 6 and 7), and HepG2 (lanes 8-12) cells, were lysed, and Cdk5 kinase was immunoprecipitated from each cell lysate (200 μg protein) with anti-Cdk5 antibody (lanes 1, 3, 4, 6, 9-12, and 14-17) or normal rabbit IgG (control antibody, lanes 2, 5, 7, 8, and 13). The immunoprecipitates were subjected to anti-Cdk5 immunoblot analysis (a) and *in vitro* histone H1 (H1) kinase assay (b, c) with (lanes 9, 10, 14, and 15) or without (lanes 3-8, 11-13, 16, and 17) 10 μM BL-I.

of peptide 7 to the total incorporation of peptides 6 and 9 (lane 5), was 19%. As shown in lanes 5-9, BL-I specifically inhibited the Ser75 phosphorylation in a dose-dependent manner and the relative intensity decreased to 1.1% at 96 μM. Thus, BL-I inhibited the activity of the protein kinase responsible for Ser75 phosphorylation in Y79 c-Src.

BL-I inhibits Cdk1 and Cdk2 kinase activity *in vitro* in addition to Cdk5 kinase activity. Cdk5 kinase is stimulated by either one of two proteins unrelated to cycline, called p35 and p39 (*22-24*). Recently, their expression was found in the rat retina (*25*). The expression of the antisense p35 construct is an effective measure to specifically suppress only endogenous Cdk5/p35 kinase activity in neuronal cells (*19*). Therefore, we sought to determine whether the specific loss of Cdk5 kinase activity by expression of the antisense p35 vector causes inhibition of Ser75 phosphorylation in Y79 cells. We transfected an antisense p35 vector or a sense vector into Y79 cells. Three stable antisense-transfected clones and two stable sense-transfected clones were isolated. None of these clones showed any changes in morphology or growth in suspension cultures. Figure 3 shows that all three antisense p35-transfected clones displayed almost no Cdk5 kinase activity. The kinase activity of the two sense p35-transfected clones was slightly (1.1-1.4-fold) higher than that of the parent Y79 cells (data not shown). We then compared the tryptic phosphopeptide maps of c-Src prepared from these antisense and sense p35 transfectants. As shown in Fig. 4, the phosphoserine 75-containing peptide 7 diminished in the antisense p35-transfected clones. This loss of Cdk5 kinase activity caused a 70-96% decrease in the relative intensity of phosphoserine 75. A low level (approximately 10-20%) of ^{32}P incorporation into the Ser75-containing peptide 7 was detected in the asynchronous antisense-transfected clones (Fig. 4). The greater part of this residual Ser75-phosphorylating activity is presumably attributable to the M-phase-specific Cdk1 kinase activity in mitotic cells, for the following reasons. (i) BL-I almost completely inhibited ^{32}P incorporation into Ser75-containing peptide 7 (Fig. 2b). (ii) The antisense p35 construct expression almost completely inhibited the Cdk5 kinase activity in the asynchronus clones (Fig. 3). (iii) Unsynchronized logarithmically growing Y79 cells contain a G2/M phase cell population of about 20% (*6*). (iv) Y79 cells in the G2/M phase exhibit an increase of about 50-60% in ^{32}P incorporation into the Ser 75-containing peptide 7 compared to the asynchronous Y79 cells (*6*).

These data present compelling evidence that Cdk5/p35 kinase is responsible for the novel mitosis-independent phosphorylation at a mitotic site, Ser75, in c-Src from Y79 cells, raising the intriguing possibility that c-Src is an effector of Cdk5/p35 kinase. Gross inhibition of Ser75 phosphorylation by loss of Cdk5 kinase activity provides a strong indication that Cdk5 kinase directly phosphorylates c-Src on Ser75 in asynchronous Y79 cells, because Cdk1 and its related kinase, which have the same substrate specificity as Cdk5 kinase, phosphorylate c-Src at the

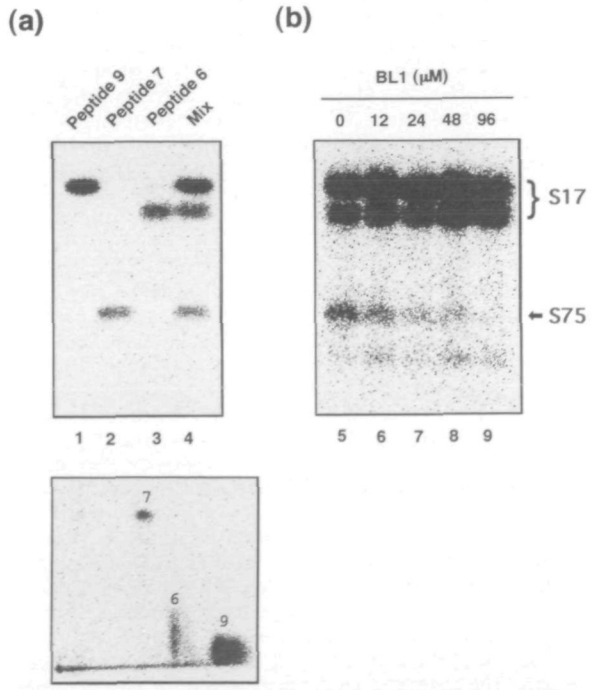

Fig. 2. **Inhibition of Ser75 phosphorylation in Y79 c-Src by BL-I.** (a) One-dimensional separation of the tryptic phosphopeptides eluted from the two-dimensional maps of Y79 c-Src. Y79 cells were labeled with [^{32}P]orthophosphate, and the immunoprecipitated c-Src was subjected to two-dimensional tryptic phosphopeptide analysis (bottom). Spots of peptides 6, 7, and 9 on the map were eluted, and each eluate (lanes 1-3) and their mixture (lane 4) were electrophoresed on a Tricine-SDS polyacrylamide gel (top). (b) Effect of BL-I on Ser75 phosphorylation in Y79 c-Src. Y79 cells treated with 0-96 μM BL-I (lanes 5-9) for 18 h were labeled with [^{32}P]orthophosphate. The immunoprecipitated c-Src was subjected to V8 protease mapping. The N-terminal 16-kDa V8 fragments were subjected to one-dimensional tryptic phosphopeptide analysis. S17, phosphoserine 17; S75, phosphoserine 75.

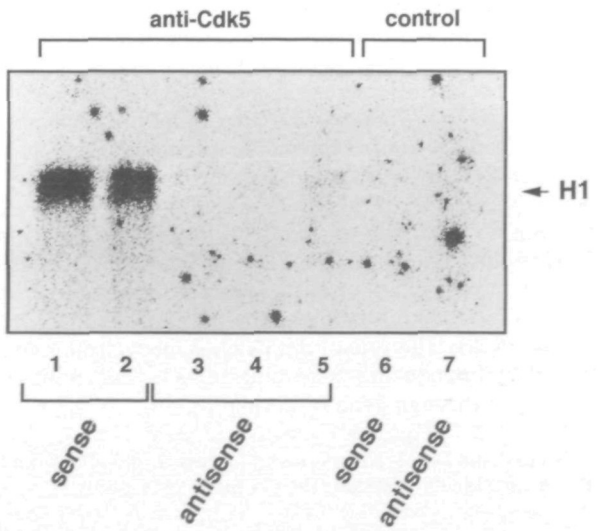

Fig. 3. **Endogenous Cdk5 kinase activity of antisense p35-transfected clones.** Cells were transfected with pCMV-antisense p35 vector or pCMV-sense p35 vector, and clones resistant to G418 were isolated and expanded. Sense p35-transfected clones S2 (lanes 1 and 6) and S22 (lane 2), and antisense p35-transfected clones AS21 (lanes 3 and 7), AS303 (lane 4), and AS333 (lane 5) were lysed, and the Cdk5 kinase immunoprecipitated from each cell lysate (300 μg protein) with anti-Cdk5 antibody (lanes 1-5) or normal rabbit antibody (lanes 6 and 7) was subjected to *in vitro* histone H1 (H1) kinase assay. Almost the same amounts of Cdk5 protein were immunoprecipitated from all the antisense and sense clones.

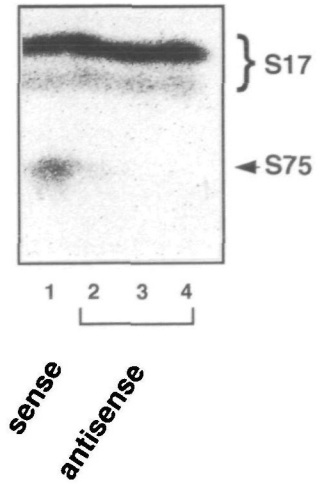

Fig. 4. **Inhibition of Ser75 phosphorylation in antisense p35-transfected clones.** ^{32}P-metabolically labeled c-Src immunoprecipitated from a sense-transfected clone S2 (lane 1), and antisense-transfected clones AS21 (lane 2), AS303 (lane 3), and AS333 (lane 4) was subjected to one-dimensional tryptic phosphopeptide analysis as described in the Fig. 2b legend. Another independent experiment gave similar results.

mitotic site, Ser75, *in vitro* (*4, 5*).

Many lines of evidence suggest that c-Src participates in neuronal development and function (*1, 2, 26*). Human Y79 retinoblastoma cells, which are of neuronal origin and retain many neuronal characteristics, have been used to investigate the neuronal phenotype (*27*). Mitosis-independent Ser75 phosphorylation by Cdk5 kinase in asynchronous Y79 cells may be related to neuronal functions. Cdk5 kinase, p35, and c-Src are present in the growth cones of developing neurons and play important roles in neurite outgrowth during neuronal differentiation (*19, 26*). A Src-binding protein, mDab1, is expressed in certain neurons (*28*). The *cdk5, p35*, and *mDab1* knockout mice have been shown to display similar cortical lamination defects in the brain (*29-31*). Our proposal that c-Src is an effector of Cdk5/p35 kinase raises the possibility that c-Src is a newcomer in signaling cascades requiring Cdk5/p35 and mDab1 in the CNS neurons. c-Src knockout mice display no obvious neurological defects, which may reflect functional compensation by other nonreceptor-type kinases or related molecules (*32*). The role of Ser75 phosphorylation may be assessed by introducing point mutations at Ser75 into the mouse c-*src* through gene targeting.

We wish to thank Dr. M. Kitagawa for kindly providing BL-I and Dr. L.-H. Tsai for kindly providing the plasmid pCMV-p35.

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