Adenovirus-mediated Overexpression of C-terminal Src Kinase (Csk) in Type I Astrocytes Interferes with Cell Spreading and Attachment to Fibronectin

CORRELATION WITH TYROSINE PHOSPHORYLATIONS OF PAXILLIN AND FAK

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To examine the role of C-terminal Src kinase (Csk), a negative regulatory kinase of Src family tyrosine kinases, in the cell adhesion mechanism of the nervous system, wild-type Csk (Csk), and a kinase-deficient mutant of Csk (Csk-ΔK) were overexpressed in primary cultured type I astrocytes by infecting them with the recombinant adenovirus. Overexpression of Csk repressed the in vitro kinase activity of Src to as little as 10% that of control cells and interfered with cell spreading and cell attachment to fibronectin. Focal adhesion assembly and the organization of actin stress fibers were also disrupted in cells overexpressing Csk. On the other hand, overexpression of Csk-ΔK induced tyrosine phosphorylation of cellular proteins, including the paxillin and focal adhesion kinase (FAK) and enhanced to some extent the cytoskeletal organization and the rate of cell spreading on fibronectin, indicating that Src or its relatives was functionally activated in the cells. Paxillin was also tyrosine-phosphorylated in Csk-overexpressing cells, indicating that it can serve as a substrate of Csk. The phosphorylation state of paxillin in cells overexpressing Csk was indistinguishable from that in cells expressing Csk-ΔK in that both phosphorylated paxillins bound equally to SH2 domain of Csk and were co-immunoprecipitated with Csk. In contrast, tyrosine phosphorylation of FAK and its in vitro autophosphorylation activity were increased only in cells expressing Csk-ΔK. In Csk-expressing cells, the kinase activity of FAK was substantially decreased to 20–30% that of control cells, even though the expression level of FAK was rather increased. These findings suggest that Csk regulates Src family tyrosine kinases that play essential roles in the regulation of cell adhesion via a FAK-dependent mechanism and that the tyrosine phosphorylation of paxillin alone may not be sufficient for the regulation of the cell adhesion mechanism in astrocytes.

Protein-tyrosine phosphorylation is a crucial step in the signal transduction cascades triggered by a variety of extra cellular stimuli that modulate cellular functions. Receptor-type tyrosine kinases are activated by ligand binding and directly transduce the extracellular information into intracellular tyrosine phosphorylation events, whereas nonreceptor tyrosine kinases function as signal transducers in concert with receptor-like molecules that lack tyrosine kinase activities.

Src family tyrosine kinases are nonreceptor tyrosine kinases that are functionally linked to some receptor-type tyrosine kinases (1, 2), G-protein-coupled receptors (3), cytokine receptors (4), and cell adhesion molecules/receptors (5, 6). Because of their functional redundancy, the essential roles of Src family tyrosine kinases still remain to be elucidated (7). Src family tyrosine kinases are membrane-associated proteins having Src homology 2 (SH2) and SH3 domains, which are important for protein-protein interaction. The activity of Src family tyrosine kinases is regulated through intramolecular interaction between the SH2 domain and the phosphorylated tyrosine at the C-terminal regulatory site (Tyr-527) (8), and it is predicted that displacement of this interaction by another molecule that can bind to the Src SH2 or SH3 domain or dephosphorylation of Tyr-527 can activate the kinase. Although the mechanism of activation coupled with receptor stimulation is still unclear, the tyrosine kinase Csk (C-terminal Src kinase) has been identified as a negative regulator of Src family tyrosine kinases (9, 10). Csk acts specifically on the regulatory sites of the family members, and targeted disruption of the csk gene caused constitutive activation of Src family tyrosine kinases accompanied by a defect in neurulation (11, 12). On the other hand, overexpression of Csk in some cell lines induced repression of cellular signaling mediated by Src family tyrosine kinases (13, 14). From these observations, it has been suggested that a balance between Src family tyrosine kinases and Csk might define the basal function of Src family tyrosine kinases.

It is also known that some members of the Src family are targeted to cell adhesion plaque during the process of cell adhesion (15, 16). Cells transformed by v-Src cause rearrangement of the actin cytoskeleton structure accompanied by anchorage-independent growth (17). In Src-transformed and Csk-deficient cells, tyrosine phosphorylation of some focal adhesion proteins, including FAK (focal adhesion kinase) and paxillin, were elevated, implicating the role of Src in the regulation of cell adhesion (7, 18). The cell adhesion mechanism not only allows cells to adhere to one another but also transduces signals involved in the regulation of cytoskeletal organization and gene expression. In such a signaling cascade, the nonreceptor tyrosine kinase FAK is regarded as a critical signaling mole-

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¶ The abbreviations used are: SH, Src homology; FAK, focal adhesion kinase; m.o.i., multiplicity of infection; GST, glutathione-S-transferase; poly-EE, poly-γ-glutamate-tyrosine; ECM, extracellular matrix; PBS, phosphate-buffered saline; GFAP, glial fibrillary acidic protein; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; β-gal, β-galactosidase.
cule, as deduced from observations of FAK-deficient and FAK-overexpressing cells (19, 20). FAK is enriched in focal adhesion through interaction with a cell adhesion receptor, integrin, and is activated upon cell attachment on the extracellular matrix (ECM) (21, 22). The activation is accompanied by autophosphorylation of Tyr-397, which creates an Src SH2 binding site (23). Src is subsequently activated through this interaction, thereby phosphorylating FAK at Tyr-925 to create another binding site for the signaling molecule Grb2/Sos to drive the mitogen-activated protein kinase cascade (21, 24, 25). Furthermore, a recent study has indicated that tyrosine phosphorylation of paxillin by Src may be a critical step in focal adhesion assembly (26). These observations suggest that Src is an important mediator of signals originating from cell adhesion through FAK, although the molecular mechanism leading to the regulation of cytoskeletal organization and gene expression has yet to be elucidated.

The nervous system is a highly complex structure composed of a variety of cells, including neuronal and glial cells. During the development of the nervous system, cell-cell and cell-ECM interaction is one of the key processes in the highly organized proliferation of precursor cells, differentiation, axon guidance, synapse formation, and cell death. There is a line of evidence suggesting that Src family tyrosine kinases are involved in the regulation of cell-cell and/or cell-ECM interaction in the nervous system (27–29). Previously, we have shown that suppression of Src family kinases by the expression of a membrane-targeted Csk in neurally differentiated P19 cells induced inhibition of cell-cell interaction mediated by the neural cell adhesion molecule (6). To further elucidate the involvement of Csk in the regulation of the cell adhesion mechanism mediated by the FAK/Src pathway, we here employed primary cultured type I astrocytes and an adenovirus-mediated gene transfer system to obtain the efficient overexpression of Csk in primary cultures. Overexpression of the wild-type Csk readily repressed the kinase activity of Src and inhibited cell spreading and attachment to the ECM. Biochemical links between the phenotype and the tyrosine phosphorylation of cell adhesion proteins were also investigated.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—To obtain a primary culture of type I astrocytes from mice (ICR strain), brain cortex regions were dissected from embryos at embryonic day 18 (E18) and immediately soaked in ice-cold isotonic buffer (137 mM NaCl, 0.17 mM NaHPO₄, 0.22 mM KCl, 5.5 mM glucose, 59 mM sucrose, 5.4 mM KCl). After removing the meninges, the tissues were shattered with scissors and incubated at 37 °C for 1 h in the presence of 0.1% dispase (Life Technologies, Inc.) and 0.05% DNase I (Boehringer Mannheim). The dispersed cells were further dissected by pipetting with a 10-ml disposable pipette fitted with a 0.2-ml tip for use in a nonautomated pipetter. The cell suspension was filtered through double sheets of lens cleaning paper (Fuji Film) to remove undispersed tissue fragments. After removing dispase and DNaseI by centrifugation, cells were resuspended in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% fetal bovine serum (Hyclone). Cell suspensions were plated in tissue culture-grade dishes coated with 10 μg/ml fibronectin (Sigma) and maintained at 37 °C and 5% CO₂ in a humidified atmosphere. Cells were used for experiments after passing twice to remove neuron-like cells.

**Vector Construction**—The MluI Fragment of Csk cDNA was blunted, ligated with PvuII linker, then inserted into the PacI site of the cosm id cassette of pAX1CAtwt, which carries the CAG promoter (a gift from Dr. I. Saitoh and Dr. J. Miyazaki) (30, 31). A kinase-negative mutant of Csk (Csk-ΔK) was generated by substituting Arg for Lys-222 by the Kunkel method. The construction of the mutant was confirmed by direct sequencing. Csk-ΔK was inserted into pAX1CAtwt as described for Csk. The recombinant adenovirus was obtained as described elsewhere (30). Briefly, the cassette containing the Csk expression unit was co-transfected into human kidney 293 cells together with an adenovirus genome DNA-terminal protein complex, which was digested at several sites with EcoT2231I or AvaI/EcoRI. The targeted recombinant adenovirus carrying the csk gene (Ax1CATcsk) or its kinase-deficient mutant (Ax1CATcsk-ΔK) was generated by overlapping recombination. After maintaining the 293 cells for 10–15 days, the virus clones were isolated.

**Bacterial Fusion Proteins**—Glutathione-S-transferase (GST) fusion proteins containing rat cDNA fragments of Csk domains SH2 (nucleotides 71–299), SH3 (nucleotides 300–626), or SH2/3 (nucleotides 71–626) were synthesized as described previously (32).

**Cell Adhesion Assay**—Astrocytes expressing β-galactosidase, wild-type Csk, or Csk-ΔK were detached from culture dishes by treatment with trypsin (0.25% trypsin in phosphate-buffered saline (PBS), pH 7.4) for 10 min at 37 °C. The detached cells were suspended in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% fetal bovine serum (HyClone). Cell suspensions were plated in tissue culture-grade dishes coated with 10 μg/ml fibronectin (Sigma) and maintained at 37 °C and 5% CO₂ in a humidified atmosphere. Cells were used for experiments after passing twice to remove unattached cells.

**Western Blotting**—Overexpression of the wild-type Csk readily repressed the kinase activity of Src and inhibited cell spreading and attachment to the ECM. Biochemical links between the phenotype and the tyrosine phosphorylation of cell adhesion proteins were also investigated.

**Fig. 1.** **Dose-dependent effect of Csk overexpression on specific activity of Src.** A, the lacZ gene (β-galactosidase (β-Gal), lane 2), wild-type Csk (Csk, lanes 3–6), or kinase-negative mutant of Csk (Csk-ΔK, lane 7) were introduced into primary cultured astrocytes by the recombinant adenovirus. In the case of Csk, the m.o.i. was varied in the range from 1.25 to 10. Expressions of Csk and Src were detected by Western blotting. To estimate the kinase activity of Src, the immunoprecipitate with anti-Src antibody was incubated with enolase and [γ-32P]ATP. The radioactivities incorporated into Src, and endosome were visualized by autoradiography. B, the total activity of Src was estimated by counting the radioactivity incorporated into endosome. The relative specific activity of Src was estimated by dividing the total activity by the amount of Src in the immunoprecipitates.
Mannhein) were employed. For Western blotting, horseradish peroxidase-conjugated anti-mouse IgG (Zymed Laboratories Inc.) and horse-radish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc.) were used as secondary antibodies.

**Immunofluorescence Staining**—Primary culture type I astrocytes were plated onto glass coverslips coated with collagen. The cells were allowed to grow for 5 days after infection with the adenovirus. They were then fixed in 3.7% formaldehyde in PBS for 10 min, followed by treatment with 0.1% Triton X-100 in PBS for 10 min. After this, the cells were incubated with anti-paxillin, anti-phosphotyrosine (PY20), GFAP antibodies and further incubated with fluorescein isothiocyanate- or Texas red-conjugated anti-mouse antibodies. For F-actin staining, rhodamine-conjugated phalloidin was used instead of the antibodies.

**X-Gal Staining**—Cells fixed with 3.7% formaldehyde were washed twice in PBS and then incubated with X-gal staining solution (50 mM K4Fe(CN)6, 50 mM K3Fe(CN)6, 20 mM MgCl2, 0.01% sodium deoxycholate, and 0.2% X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside) in PBS) for 3 h at 37 °C.

**Western Blotting**—Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Schleicher & Schuell). Subsequently, the membrane was treated with blocking reagent (Tris-buffered saline containing 0.1% Tween 20 (TWEEN-TBS)) for 2 h at room temperature. The blocked membrane was probed with primary antibodies and further incubated with a secondary antibody conjugated with horseradish peroxidase. The immunoreactivity was visualized with an enhanced chemiluminescence system (DuPont).

**Immunoprecipitation**—Cells were rinsed twice with ice-cold PBS and then lysed with 400 μl of ice-cold radioummune precipitation buffer (10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM β-mercaptoethanol, 1 mM phenylmethlysulfonyl fluoride, 1 mM sodium vanadate, and 10 μg/ml apro-

![Fig. 2](image-url) Inhibition of cell spreading on collagen by overexpression of Csk. A, to determine the efficiency of infection with the recombinant adenovirus, primary culture astrocytes were infected with 10 m.o.i. of the recombinant adenovirus carrying the lacZ gene (Ax-CATlacZ). Forty-eight h after infection, the cells were fixed with form-aldehyde and incubated with X-gal-staining solution. B–E, cells were plated onto dishes coated with collagen. After 2 days, astrocytes were infected with 10 m.o.i. of the recombinant adenovirus carrying the lacZ gene (Ax-CATlacZ (C)), Csk (Ax-CATCsk (D)), or Csk-ΔK (Ax-CAT- Csk-ΔK (E)). Cells were allowed to grow for 5 days in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 5% CO2 atmosphere. The cell morphologies of adenovirus-infected cells were compared with those of the control cells (B) by means of phase-contrast microscopy.

![Fig. 3](image-url) Interference with cell attachment to fibronectin by overexpression of Csk. A, astrocytes expressing β-galactosidase (Control), Csk, or Csk-ΔK were collected from tissue culture dishes by treatment with trypsin. Portions of the suspended cells (1 × 10^6 cells) were plated onto 12-hole cell culture plates (growth area, 4 cm²) coated with 10 μg/ml fibronectin. After incubation for the indicated periods, the numbers of cells attached to the fibronectin were estimated as described under “Experimental Procedures.” The data shown were obtained from three independent experiments. The error bars represent the standard deviation (n = 4). B–D, phase contrast images of control cells (B), cells expressing Csk (C), and cells expressing Csk-ΔK (D), taken 40 min after plating.

**RESULTS**

**Overexpression of Csk Represses the Src Activity**—To analyze the role of Csk under near in vivo conditions, wild-type Csk...
(Csk) and the kinase-negative Csk mutant (Csk-ΔK) were transiently overexpressed in the primary cultured type I astrocytes. Recombinant adenovirus vectors were used to obtain an efficient introduction of ectopic genes into the primary cultures. Type I astrocytes were infected by Ax1CAT-lacZ (10 multiplicity of infection (m.o.i.)), Ax1CATcSk (1.25–10 m.o.i.), or Ax1CATcSk-ΔK (10 m.o.i.), and the expression of Csk protein was determined by Western blotting 5 days after infection. Although the expression of Csk in the control cells and cells infected by Ax1CAT-lacZ was at quite low levels, Csk protein was overexpressed in cells infected by Ax1CATcSk or Ax1CATcSk-ΔK in an m.o.i.-dependent manner (Fig. 1A). The efficiency of the infection was confirmed by X-gal staining of cells infected by Ax1CAT-lacZ, which showed that almost 100% efficiency was achieved (Fig. 2A). Depending upon the dose of Csk protein, the in vitro kinase activity of Src was repressed in cells overexpressing Csk to as little as 10% that of the control cells. However, activation of the in vitro kinase activity of Src could not be detected in cells expressing Csk-ΔK (Fig. 1B).

Overexpression of Csk Interferes with Cell Spreading—The effect of Csk overexpression on the morphology of astrocytes was observed under phase-contrast microscopy. The control astrocytes exhibited widely spread, polygonal shapes with obvious cytoplasmic edges, although some developing cells were rounded (Fig. 2B). Cells expressing β-galactosidase did not show any morphological change (Fig. 2C). In contrast, overexpression of Csk induced a dramatic change in the cell morphology; the majority of the cells had spread very poorly and remained rounded with only a few attachment sites (Fig. 2D). The morphology of cells expressing Csk-ΔK appeared to be almost the same as that of the control cells, although some had spread more vigorously than the control cells (Fig. 2E).

Overexpression of Csk Interferes with Cell Attachment to Fibronectin—To compare the abilities of cells to attach to the ECM, cell adhesion assays were performed. The control cells and those expressing Csk-ΔK began to attach to fibronectin-coated dishes within 20 min after plating, and about 60% of them were attached after around 60 min. In contrast, the Csk-expressing cells did not begin to attach to the dishes until 60 min after plating (Fig. 3A), and the majority still remained unattached even after 6 h, when attachment of the control cells and those expressing Csk-ΔK were almost completed (data not shown). The morphologies of cells 40 min after plating are compared in Fig. 3, B–D. It can be seen that cells expressing Csk-ΔK spread more rapidly than control cells (Fig. 3D), whereas none of cells expressing Csk had become attached at this point (Fig. 3C).

Focal Adhesion Structure and Organization of F-actin Cytoskeleton in Csk-overexpressing Astrocytes—To examine the molecular basis of the phenomena observed in Csk-expressing astrocytes, the organization of the F-actin cytoskeleton and the distribution of the focal adhesion proteins were analyzed. Cells expressing β-galactosidase, Csk, or Csk-ΔK were all stained by an antibody against GFAP, confirming that these cells maintained their astrocyte characteristics (Fig. 4, A–C). Staining with rhodamine-conjugated phalloidin indicated that the total amount of F-actin in the Csk-expressing cells was comparable with that in the other cell types. However, the F-actin in the Csk-expressing cells displayed a disordered structure and was condensed at the peripheral regions of the cells (Fig. 4E). In some cells expressing Csk-ΔK, the organization of F-actin was more evident than in the control cells (Fig. 4, G–L). Patchy staining around the cell peripheries, which is characteristic of focal adhesion, was observed in the control cells and in those expressing Csk-ΔK, whereas the focal adhesion staining was dramatically reduced in the Csk-expressing cells, indicating that the formation or maintenance of a focal adhesion structure was disturbed by Csk expression.

Tyrosine Phosphorylation of Paxillin and FAK in Astrocytes Expressing Csk and Csk-ΔK—The effects of Csk and Csk-ΔK overexpression on assembly of focal adhesion and organization of the actin cytoskeleton Five days after infection with the recombinant adenovirus, primary cultured type I astrocytes expressing β-galactosidase (A, D, G, and J), Csk (B, E, H, and K), or Csk-ΔK (C, F, I, and L) were stained with anti-GFAP antibody (A–C), rhodamine-conjugated phalloidin (D–F), anti-paxillin monoclonal antibody (G–I), or anti-phosphotyrosine antibody (J–L).
expression on the tyrosine phosphorylation of cellular proteins were investigated by Western blotting with anti-phosphotyrosine antibody (Fig. 5A). In the control and β-galactosidase-expressing cells, the tyrosine phosphorylation levels were relatively low (lanes 1 and 2). Overexpression of Csk induced the tyrosine phosphorylation of several proteins in a dose-dependent manner, with prominent phosphorylation being obtained for 70- and 100-kDa proteins (lanes 3–6). Overexpression of Csk-ΔK also induced the tyrosine phosphorylation of some proteins, including a 70-kDa protein, although the phosphorylation of a 125-kDa protein was induced in place of a 100-kDa protein (lane 7).

To identify the tyrosine-phosphorylated proteins, several candidate proteins involved in cell adhesion were examined with regard to their expression and tyrosine phosphorylation levels. The expressions of the focal adhesion proteins vinculin, FAK, and paxillin was first confirmed by Western blotting (Fig. 5B). The expression of vinculin was not affected by the expression of Csk or Csk-ΔK. The total amount of paxillin was not affected, but mobility shifts on SDS-PAGE, probably because of the phosphorylation, were observed with cells expressing Csk and Csk-ΔK. A slight increase in the expression of FAK was observed only in the case of Csk-expressing cells. Overall, however, there were no obvious changes in the expression of focal adhesion proteins in either cell type.

Tyrosine phosphorylation of the proteins was detected by immunoprecipitation followed by Western blotting. As shown in Fig. 5C, tyrosine phosphorylation of paxillin (70 kDa) was dramatically elevated in cells expressing Csk or Csk-ΔK. FAK (125 kDa) was found to be heavily tyrosine-phosphorylated only in cells expressing Csk-ΔK (Fig. 5D). The tyrosine phosphorylation of other proteins having a similar M₀, to that of FAK, such as p130cas or PYK2 (CAKβ), was not detected (data not shown). Thus, it is suggested that the 70-kDa protein phosphorylated in both Csk- and Csk-ΔK-expressing cells was paxillin, whereas the 125-kDa protein in cells expressing Csk-ΔK was FAK. The phosphorylated 100-kDa protein found in Csk-expressing cells was not identified.

Phosphorylation of Paxillin by Src and Csk in Vitro—Paxillin is known to be a good substrate for Src, Csk, and FAK (33). To further confirm the identification of the 70-kDa protein as paxillin and determine the kinase responsible for paxillin phosphorylation in the cells, Src, Csk, and Csk-ΔK were co-immunoprecipitated with paxillin, and the immunocomplexes were subjected to in vitro kinase assay (Fig. 6). Paxillin was phosphorylated by Src or Csk but not by Csk-ΔK, suggesting that paxillin phosphorylation was mediated by Csk itself in Csk-expressing cells. In cells expressing Csk-ΔK, the paxillin was phosphorylated by a kinase(s) activated by the expression of Csk-ΔK, possibly Src and/or FAK.

It has been reported that Src and Csk phosphorylate the identical paxillin residues in vitro (33) To compare the properties of the tyrosine-phosphorylated paxillins in cells expressing Csk and Csk-ΔK, the abilities of the two species of paxillin to interact with Csk in vitro were investigated. As shown in Fig. 7A, the tyrosine-phosphorylated paxillin in cells expressing Csk and Csk-ΔK were both co-precipitated with GST fusion proteins containing the SH2 domain of Csk. There were no differences in the amount of paxillin precipitated, the migration pattern on SDS-PAGE, or the tyrosine phosphorylation level between the two species of paxillin. In addition, they were equally co-immunoprecipitated with Csk from the cell lysates of cells expressing Csk or Csk-ΔK (Fig. 7B). These observations suggest that the phosphorylation states of the two species of paxillin were functionally identical.

**Kinase Activity of FAK in Astrocytes Expressing Csk or Csk-ΔK**—Tyrosine phosphorylation and the kinase activity of FAK have been reported to increase upon cell attachment to fibronectin (22). In addition, FAK-deficient cells displayed poor spreading and a rounded cell morphology, indicative of the critical role of FAK in the regulation of cell adhesion (19). As Csk-overexpressing cells displayed the phenotype similar to that of FAK-deficient cells, we estimated the kinase activity of FAK in cells expressing Csk or Csk-ΔK.

**DISCUSSION**

We have shown that adenovirus-mediated overexpression of Csk in type I astrocytes inhibited cell spreading and attach-
immunoprecipitate with anti-Csk antibody. The proteins were then collected by incubation with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h. Immunoreactivities in the immunocomplex were detected as described in "Experimental Procedures," except that 5 μg of polyglutamate-tyrosine (poly-EY) was added to the reaction mixture as a substrate. The radioactivity incorporated into poly-EY was quantified by a BAS2000 image analyzer (Fuji Film). To estimate the autophosphorylation activity of FAK, the immunoprecipitates were subject to in vitro kinase assay in the absence of ectopic substrate. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

Fig. 6. In vitro phosphorylation of paxillin by Src and Csk. In vitro phosphorylation of immunoprecipitated paxillin (lane 1), Src (lane 2), Src + paxillin (lane 3), Csk (lane 4), Csk + paxillin (lane 5), Csk-ΔK (lane 6), and Csk-ΔK + paxillin (lane 7). Src and paxillin were immunoprecipitated from cell lysates (100 and 500 μg of proteins, respectively) prepared with radiomune precipitation buffer from control astrocytes. Csk and Csk-ΔK were immunoprecipitated from cell lysates (200 μg of protein) prepared with TNE buffer from Csk- and Csk-ΔK-expressing cells, respectively. In the cases of lanes 3, 5, and 7, the two proteins were simultaneously immunoprecipitated from the same cell lysates. The immunocomplexes obtained were then subjected to in vitro kinase assay in the absence of ectopic substrate. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography.

Fig. 7. Interaction of tyrosine-phosphorylated paxillin with SH2 domain of Csk. A, 10 μg of GST fusion proteins (GST lanes 1 and 5), GST-CskSH2 lanes 2 and 6), GST-CskSH3 lanes 3 and 7), GST-CskSH2SH3 lanes 4 and 8) were incubated with cell lysates (500 μg of protein) prepared from cell lysates (100 μg of protein) prepared with TNE buffer from Csk-expressing (lanes 1–4) or Csk-ΔK-expressing (lanes 5–8) cells for 12 h at 4 °C. The GST fusion proteins were then collected by incubation with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 1 h. Immunoreactivities against anti-phosphotyrosine antibody (α-PY, upper panel) and anti-paxillin antibody (lower panel) in the collected beads were detected by Western blotting. B, TNE cell lysates (500 μg of protein) prepared from control astrocytes (lanes 1), β-galactosidase (β-Gal)-expressing cells (lanes 2), Csk-expressing cells (lanes 3), or Csk-ΔK-expressing cells (lanes 4) were subjected to immunoprecipitation with anti-Csk antibody. The immunoreactivities in the immunocomplex were detected as described in A. The arrows indicate the position of paxillin on SDS-PAGE.

amount of Csk protein expressed, showing that the function of the Src was successfully attenuated by Csk. Unexpectedly, overexpression of Csk-ΔK did not have a dominant-negative effect on in vitro Src activity. However, the tyrosine phosphorylation of some cellular proteins, including the Src substrates paxillin and FAK, was increased in cells expressing Csk-ΔK. It has been reported that overexpression of Csk in CHO cells enhanced insulin-stimulated dephosphorylation of FAK, whereas overexpression of Csk-ΔK inhibited the dephosphorylation of FAK and paxillin but somewhat enhanced the basal tyrosine phosphorylation of FAK (54). These findings together with our observations suggest that Src or its relatives are functionally activated in cells overexpressing Csk-ΔK. Although we do not have direct evidence, it is likely that the turnover rate of phosphorylation at the C-terminal regulatory site of Src is accelerated through inhibition of Csk by the overexpression of Csk-ΔK and that the transiently activated Src may be involved in the accumulation of tyrosine-phosphorylated proteins. In cells overexpressing Csk, tyrosine phosphorylation of several proteins, including paxillin and an as-yet unidentified 100-kDa protein, was increased, suggesting that Csk, when substantially overexpressed, can act on proteins other than Src family tyrosine kinases, although the physiolog-
ical meanings of this has yet to be determined.

There is accumulating evidence that FAK and Src are essential signaling molecules in the regulation of the cell adhesion mechanism (19–23). We have demonstrated here that the tyrosine kinase activity of FAK was decreased in cells overexpressing Csk and that the expression of Csk-ΔK induced elevation of the autophosphorylation activity and tyrosine phosphorylation level of FAK. The phenotype of Csk-expressing cells was also consistent with that of cells obtained from FAK-deficient mice (19), suggesting that loss of FAK function may account for some aspects of the phenotype of Csk-expressing cells. It is well known that FAK activation induced by cell attachment recruits Src to activate it, thereby inducing the phosphorylation of Src substrates, including FAK itself and paxillin (23). This phosphorylation recruits Grb2-Sos and Paxillin-Crk-C3G adapter protein complexes into the focal adhesion and ultimately activates the Ras and mitogen-activated protein kinase signaling pathway (21). The Ras-mitogen-activated protein kinase pathway is involved in the regulation of cell adhesion by modulating the organization of the cytoskeletal structure. Since Src was targeted in Csk-expressing cells, the pathway downstream of Src might be shut down in the cells, resulting in defects in cytoskeletal organization. Thus, it is more likely that loss of FAK activation in Csk-expressing cells is a secondary result of loss of cell adhesion elicited by defects in the cytoskeletal organization mediated by Src.

We observed that the expression level of FAK was slightly increased in cells expressing Csk, in which the kinase activity of FAK was repressed. This suggests that there is an up-regulation system in FAK expression regulated by the total activity of FAK itself. A similar phenomenon was observed in the cells obtained from csk knockout mice, in which the expression of Src was down-regulated by the constitutive activation of Src because of the lack of Csk (18). Further studies on the molecular mechanism leading to the feedback regulation of gene expression might provide a new clue to understanding the roles of tyrosine kinases.

Recently, it has been shown that overexpression of the C-terminal portion of FAK inhibits tyrosine phosphorylation of FAK and paxillin and, in addition, delays cell spreading and focal adhesion assembly (26) and that the inhibitory effects of the C-terminal FAK can be rescued by coexpression of FAK or Src but not by a FAK mutant that fails to bind Src and paxillin (26). Because tyrosine phosphorylation of paxillin is always observed when overcoming the inhibition of cell spreading by the C-terminal FAK, it is suggested that tyrosine phosphorylation of paxillin mediated by Src is a critical step in focal adhesion assembly. In this study, the tyrosine phosphorylation of paxillin was induced by overexpressing active Csk in a manner independent of FAK and/or Src. Paxillin in Csk-expressing cells was tyrosine-phosphorylated at an equivalent level to that in cells expressing Csk-ΔK, and the two species of phosphorylated paxillin were functionally identical in terms of specific interaction with the SH2 domain of Csk. In addition, it has been shown that Src, Csk, and FAK phosphorylate identical sites of paxillin in vitro (33). However, cell morphology and the ability of cells to spread and attach to the ECM were selectively abrogated in cells expressing Csk. Thus it is suggested from the findings of this study that although tyrosine phosphorylation of paxillin may be required, it is not sufficient to regulate the cell adhesion mechanism of astrocytes. Identification of another molecule(s) downstream of Src may be necessary to elucidate the pathway leading to the regulation of cytoskeletal organization.

This work has further highlighted the importance of Src in the regulation of the cell adhesion mechanism by using Csk, a regulator kinase of Src. A crystallographic analysis of the repressed form of c-Src indicated that displacement of the intracellular binding between the SH2 domain and the C-terminal phosphotyrosine (Tyr-527) by another protein can activate Src without dephosphorylation of Tyr-527 (35). Indeed, it is shown that autophosphorylated FAK can act as an activator through such a mechanism. However, overexpression of Csk readily modulated the kinase activity of Src in the cells, suggesting that a tyrosine phosphate(s) directed to Tyr-527 is also important during the process of cell adhesion. To identify such a phosphatase, our system for obtaining efficient overexpression of Csk may be useful.

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