Role of Csk in neural differentiation of the embryonic carcinoma cell line P19

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Received 10 October 1996; revised version received 18 February 1997

Abstract To examine the neural function of Csk (C-terminal Src kinase), a membrane-targeted form of Csk (Src/Csk) and its kinase-defective variant (DK-Src/Csk) were expressed in the embryonic carcinoma cell line P19. Expression of Src/Csk, but not DK-Src/Csk, caused reduction of the specific activities of Src and Fyn in the differentiated P19 cells. During neural differentiation, the specific activity of Src was elevated in the control P19 cells, whereas the activation was completely eliminated in the Src/Csk transfectant. In normally differentiated P19 cells, cross-linking of a cell adhesion molecule, L1, induced a short-term activation of Src and Fyn. In the Src/Csk transfectant, L1 stimulation induced delayed activation of Src and Fyn peaking at much lower levels than in the control cells. Src/Csk transfectants developed normally in the initial stages of neural differentiation, but exhibited an apparent defect in cell-to-cell interaction, i.e. neurite fasciculation and aggregation of cell bodies, in the latter stages. These findings imply that Csk is involved in the regulation of Src family kinases that play roles in cell-to-cell interaction mediated by cell adhesion molecules.

Key words: Csk; Src family kinases; Neuronal differentiation; P19 cells; L1

1. Introduction

The development of the nervous system is known to be regulated by a variety of protein tyrosine kinases. The neural functions of the receptor-type tyrosine kinases have been relatively well characterized [1], while those of the non-receptor-type tyrosine kinases including Src family kinases remain obscure. The Src family consists of 10 members which share a unique structure containing Src homology 3 (SH3) domain, SH2 domain and C-terminal regulatory domain [2]. In the lymphoid system, it has been shown that Src family kinases serve as downstream factors of receptor molecules, such as T- and B-cell receptors, to convert extracellular signals into intracellular tyrosine phosphorylation events [3]. Expression patterns of Src family kinases in the nervous system have implicated their roles in differentiation and function of neural cells [4–12]. Knockout mice for neural Src family kinases were generated to evaluate their in vivo roles, but the analyses were unsuccessful due to the gene redundancy among the family members [13–16]. In an in vitro system, however, it was observed that neurons derived from Src knockout mice exhibited a defect in neurite extension on a cell adhesion molecule, L1 [17], and that neurons from Fyn knockout mice showed impaired neurite outgrowth on N-CAM [18]. Moreover, a functional interaction between Fyn and large myelin associated glycoprotein (L-MAG) was demonstrated [19]. These findings suggest that Src family kinases play certain roles in cell-to-cell interaction mediated by neural cell adhesion molecules. However, the molecular mechanism of their neural function and their physiological roles remains to be clarified.

The kinase activity of Src family kinases is regulated negatively by the phosphorylation of their C-terminal regulatory tyrosines [20]. The phosphorylation is mainly contributed by another non-receptor tyrosine kinase, Csk (C-terminal Src kinase), which was originally protein purified as a kinase that specifically acts on the regulatory site of the Src family in vitro [21,22]. Disruption of the *csk* gene in mice leads to extraordinary activation of Src, Fyn and Lyn, resulting in embryonic lethality with impaired development of the neural tissues [23,24]. Thus Csk is thought to be an essential regulator of Src family kinases during the development of the nervous system. However, the embryonic lethality of Csk knockout mice has hampered detailed analysis of Csk function.

To study the neural function of Csk and its major targets, Src family kinases, we here employed an embryonic carcinoma cell line, P19, as an in vitro model of neural differentiation. P19 is a multipotential stem cell line that can be induced to differentiate into neural cells by treatment with retinoic acid [25]. In this study, we generated multiple P19 clones which constitutively expressed a membrane-targeted form of Csk (Src/Csk), a fusion protein having a myristilation signal of Src [26]. It is known that the membrane-localized Csk can act on Src family kinases more effectively than wild type Csk [26,27]. The effects of Src/Csk expression on the kinase activity of Src family kinases and neural differentiation of P19 cells were investigated here.

2. Materials and methods

2.1. Cell culture

P19 mouse embryonic carcinoma cells were maintained in α-modified minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Differentiation of P19 cells into neural cells was carried out essentially by the method of McCarrick and Andrews [28]. Briefly, cells were first allowed to aggregate in bacterial-grade dishes in α-MEM containing 10% FBS and 1 μM retinoic acid. After 4 days, the aggregates were dispersed into single cells by treatment with 0.01% trypsin. The cell suspension (2×10^6 cells) was then re-plated onto a 100-mm tissue culture-grade dish coated with 5 μg/ml poly-L-lysine to induce neural differentiation. After a further 2 days, 5 μg/ml cytosine arabinoside was added to the medium to kill proliferating non-neural cells.

2.2. Vector constructions and transfection

The Neo expression vector (pKJ2) driven by the promoter and polyadenylation signal of the phosphoglycerate kinase 1 (PGK) gene

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PII S0014-5793(97)00224-X

was digested with *Pst*I to cut out a Neo open reading frame (ORF), blunted and ligated to a *Mlu*I linker. The *Hin*dIII-*Sal*I fragment of Src/Csk (a gift from Dr. A. Veillete) was also blunted, ligated to a *Mlu*I linker, and inserted into the pKJ2-derived vector (PGK-Src/Csk). A kinase-deficient variant of Src/Csk (PGK-DK-Src/Csk) was generated by substituting Lys-222 for Arg by the Kunkel method. The expression vector was co-transfected with an intact pKJ2 vector into undifferentiated P19 cells by electroporation with an electric pulse of 250 V and 960 μF. Stable transfectants were selected by culturing in the presence of geneticin (150 μg/ml) for 14 days, and surviving colonies were picked up.

2.3. Western blotting

To determine subcellular localization of Src/Csk, P19 cells were rinsed twice with cold phosphate-buffered saline (PBS) and harvested in a hypotonic buffer (10 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, and 2 mM $MgCl_2$). The cells were disrupted with a Teflon pestle homogenizer, and the cytosolic and membrane-containing fractions were separated by ultracentrifugation at 70000×*g* for 90 min. For detection of neural marker proteins, cells were lysed with RIPA buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 mM sodium vanadate and 10 μg/ml aprotinin), and the supernatant obtained by ultracentrifugation was used for analysis. Proteins separated by SDS-PAGE were transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell). After blocking with 1% skim milk (or bovine serum albumin (BSA)) in Tween-TBS (Tris-buffered saline containing 0.1% Tween-20) the membrane was probed with primary antibody. The immunoreactivity was visualized with an enhanced chemiluminescence system (Dupont) using a secondary antibody conjugated with horseradish peroxidase (HRP).

2.4. Immunoprecipitation and in vitro kinase assay

Cells were rinsed twice with ice-cold PBS, then lysed with 400 μl of ice-cold RIPA buffer or TNE buffer (20 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 μM β-mercaptoethanol, 1 mM PMSF, and 10 μg/ml aprotinin). The cell lysate (200 μg of protein) was precleared with Pansorbin cells (Calbiochem), and incubated with anti-v-Src antibody (Mab327, Oncogene Science) or anti-Fyn antibody (Wako) for 1 h at 4°C. Subsequently, 0.1 μg of anti-mouse IgG was added and the mixture was further incubated for 30 min at 4°C. The immune complex was recovered by incubation with 20 μl of 10% Pansorbin cells for 30 min at 4°C, then washed once with a cushion of 500 μl RIPA buffer containing 1 M sucrose, twice with RIPA buffer, and twice with TNE buffer. The obtained immunoprecipitate was resuspended in 20 μl of TNE buffer. To estimate the protein amount of the kinase, half of the immunoprecipitate was subjected to Western blotting, and the intensity of signal was determined densitometrically.

For in vitro kinase assay, an aliquot (2 μl) of immunoprecipitated kinase was mixed with 18 μl of reaction mixture (10 mM PIPES-NaOH pH 7.0, 10 mM $MnCl_2$, 2.5 μg of rabbit muscle enolase (Sigma)) and the reaction was initiated by the addition of 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). After incubation for 10 min at 30°C, 20 μl of 2×SDS-PAGE sample buffer was added and the sample was boiled for 3 min. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. Radioactivity incorporated into enolase was semi-quantified by using a Bioimage Analyzer BAS3000 (Fuji).

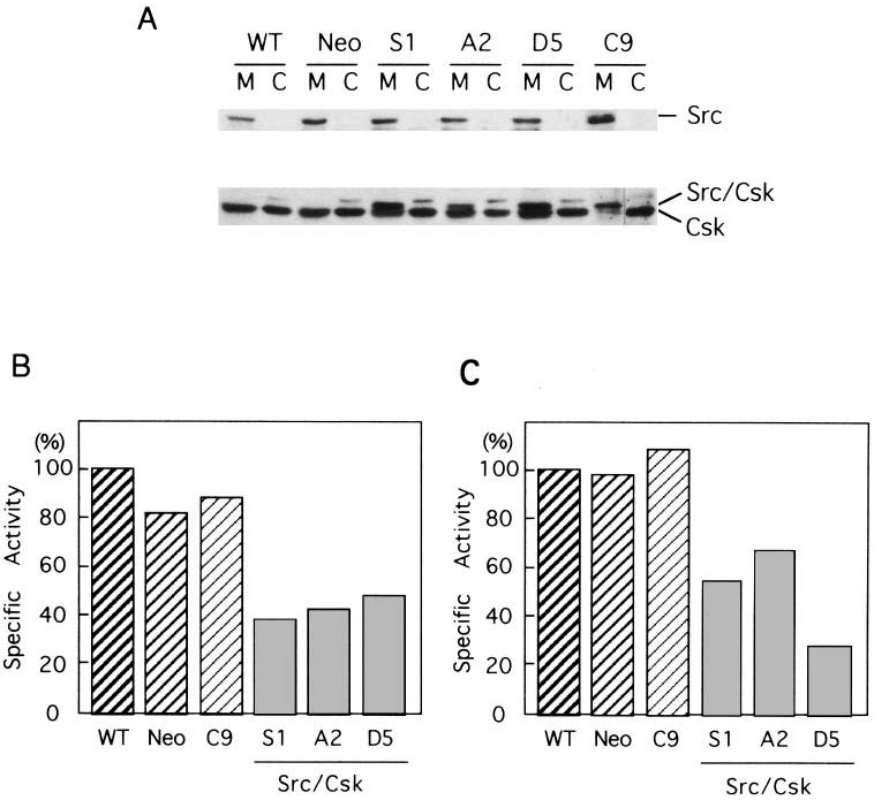

Fig. 1. A: Expression of a membrane-targeted variant of Csk (Src/Csk) in P19 cells. Wild type cells (WT), the control transfectant (Neo), three Src/Csk transfectants (S1, A2, and D5), and a DK-Src/Csk transfectant (C9) were fractionated into cytosolic (C) and membrane-containing fractions (M). Proteins in the cytosolic (10 μg) and the membrane-containing fractions (30 μg) were subjected to Western blotting with anti-Src or anti-Csk antibody. The locations of Src, Src/Csk and endogenous Csk are indicated. A faint band seen in the cytosolic fractions, which migrated more slowly than Src/Csk, was non-specific signal detected by anti-Csk antibody. B, C: Effect of Src/Csk expression on the activities of Src and Fyn. Src (B) and Fyn (C) were immunoprecipitated from differentiated (2 days after neural induction) wild type cells (WT), a control transfectant (Neo), DK-Src/Csk transfectant (C9) and the three Src/Csk transfectants (S1, A2 and D5), and subjected to in vitro kinase assay. The activity was quantified by counting the radioactivity incorporated into enolase, and the relative specific activity was obtained by dividing the activity by protein amount of the kinase in the immunoprecipitates.

2.5. Immunocytochemistry

P19 cells differentiated in 35-mm tissue-culture dishes were rinsed with PBS, fixed in 4% paraformaldehyde in PBS, and subsequently permeabilized in PBS containing 0.2% Triton X-100. After blocking with 1% BSA in Tween-TBS, specimen was reacted with a primary antibody diluted with Tween-TBS, and washed three times with PBS. Specimen was then incubated with a secondary antibody conjugated with HRP. Immunoreactivity was detected with a staining buffer (0.05 M Tris-HCl pH 6.8, 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB), 0.5% nickel(II) ammonium sulfate hexahydrate, and 0.005% hydrogen peroxide). The primary antibodies used were anti-microtubule associated protein 2 (MAP2) and anti-syntaxin rabbit polyclonal antibodies (gifts from Dr. M. Niinobe, Osaka University), anti-neurofilament 160 kDa protein (NF) monoclonal antibody (Boehringer Mannheim), anti-N-CAM monoclonal antibody (Chemicon International Inc.), and three kinds of anti-L1 polyclonal antibodies, recognizing immunoglobulin-like, fibronectin type III-like, and intracellular domains (gifts from Dr. A. Asou, Tokyo Metropolitan Institute of Gerontology).

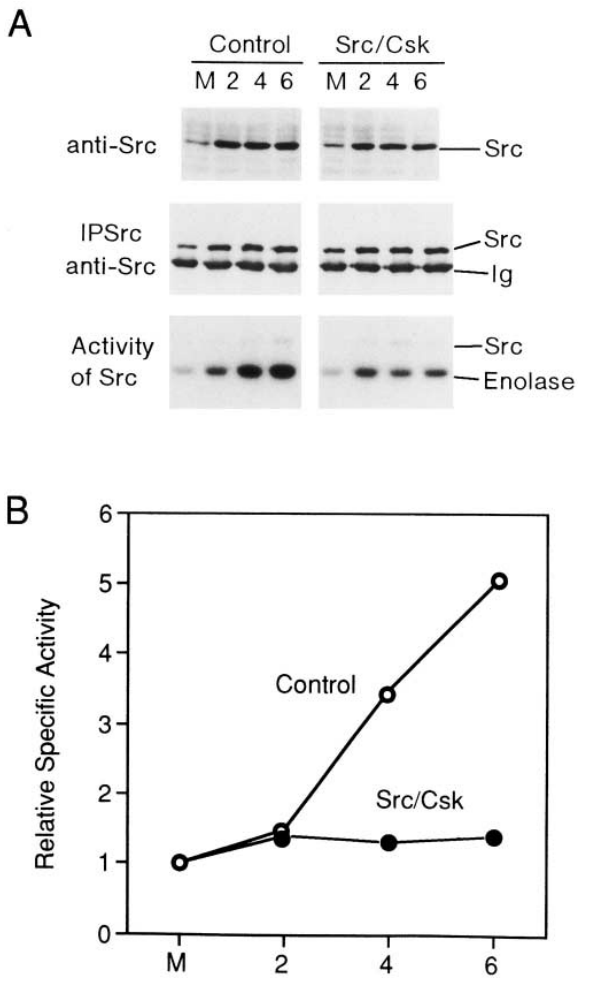

Fig. 2. Effect of Src/Csk expression on Src activity during neural differentiation of P19 cells. A: Total cell lysates were prepared from the control transfectant (Neo) and a Src/Csk transfectant (A2) at various stages of differentiation (M, undifferentiated monolayer culture; 2, 2-day cultures after neural induction; 4, 4-day cultures; 6, 6-day cultures), and analyzed for the expression of Src by Western blotting (upper panels). Src protein was immunoprecipitated from the cell lysates and the protein amounts in the immunoprecipitates were verified by Western blotting with anti-Src antibody (middle panels). The immunoprecipitates were then subjected to in vitro kinase assays with enolase as a substrate. B: The activity of Src was quantified by counting the radioactivity incorporated into enolase, and the specific activity was estimated by dividing the activity by the amount of Src protein in the immunoprecipitates. Relative specific activities against the specific activity of Src in undifferentiated culture were plotted.

3. Results and discussion

3.1. Expression of a membrane-targeted Csk in P19 cells

The expression vectors were transfected into P19 cells to generate clones constitutively expressing a membrane-targeted form of Csk (Src/Csk), and its kinase-defective variant (DK-Src/Csk). Among the multiple clones obtained, three clones expressing Src/Csk (S1, A2, and D5) and a clone expressing DK-Src/Csk (C9) were selected for analyses. To confirm the expression of Src/Csk, proteins from cytosolic and membrane-containing fractions were subjected to Western blotting with anti-Src and anti-Csk antibodies (Fig. 1). The Src/Csk protein which migrated slightly more slowly than endogenous Csk was detected only in the membrane-containing fractions where Src protein was also enriched. We previously analyzed fibroblast cell lines expressing wild type Csk and Src/Csk, and found that Src/Csk, even if its expression level was lower than that of endogenous Csk, could effectively act on Src to suppress its activation during mitosis [27]. Co-localization on a membrane structure might induce efficient and specific contact between Csk and Src family kinases.

To verify the function of Src/Csk, wild type cells (WT), a control transfectant (Neo), Src/Csk transfectants (S1, A2, and D5) and DK-Src/Csk transfectant (C9) were induced to differentiate for 4 days, and the specific activities of Src and Fyn in these cell lines were compared. In this study, two types of Src, c-Src and a neuron-specific form of c-Src (c-Src$^+$) [5], were assayed simultaneously, thus the activity of 'Src' represents the total activity of c-Src and c-Src$^+$. Although c-Src$^+$ has a somewhat higher specific activity than c-Src [6], it has been shown that c-Src$^+$ is under the control of Csk in a similar manner to c-Src [29]. In Src/Csk transfectants (S1, A2, and D5), the specific activity of Src was decreased to about 50% of that of wild type P19 cells (WT) (Fig. 1B). The specific activity of Fyn was also repressed to about 20–60% of that of wild type cells (Fig. 1C). The control transfectant (Neo) and DK-Src/Csk transfectant (C9) did not show significant change in the specific activity of Src or Fyn. Under our conditions, dominantly negative effect of DK-Src/Csk was not observed in the C9 line. These findings demonstrate that ectopically expressed Src/Csk was capable of inhibiting the activities of Src family kinases.

3.2. Effect of Src/Csk expression on Src activity during neural differentiation

To examine the effect of Src/Csk expression on the change in Src activity during neural differentiation, the specific activities of Src in the control (Neo) and a Src/Csk transfectant (A2) at various stages of neural differentiation were estimated. In the control cells, the specific activity of Src was increased with neural differentiation, a greater than 5-fold increase being achieved in differentiated 6-day culture (Fig. 2A,B). In contrast, the specific activity of Src was almost equal level during neural differentiation of Src/Csk transfectant, although neural induction of Src protein was normally observed. It has already been reported that expression of Src, especially c-Src$^+$, is induced during neural differentiation [30] and that the specific activity of Src is also augmented in differentiated nervous system [29]. From these observations, it is suggested

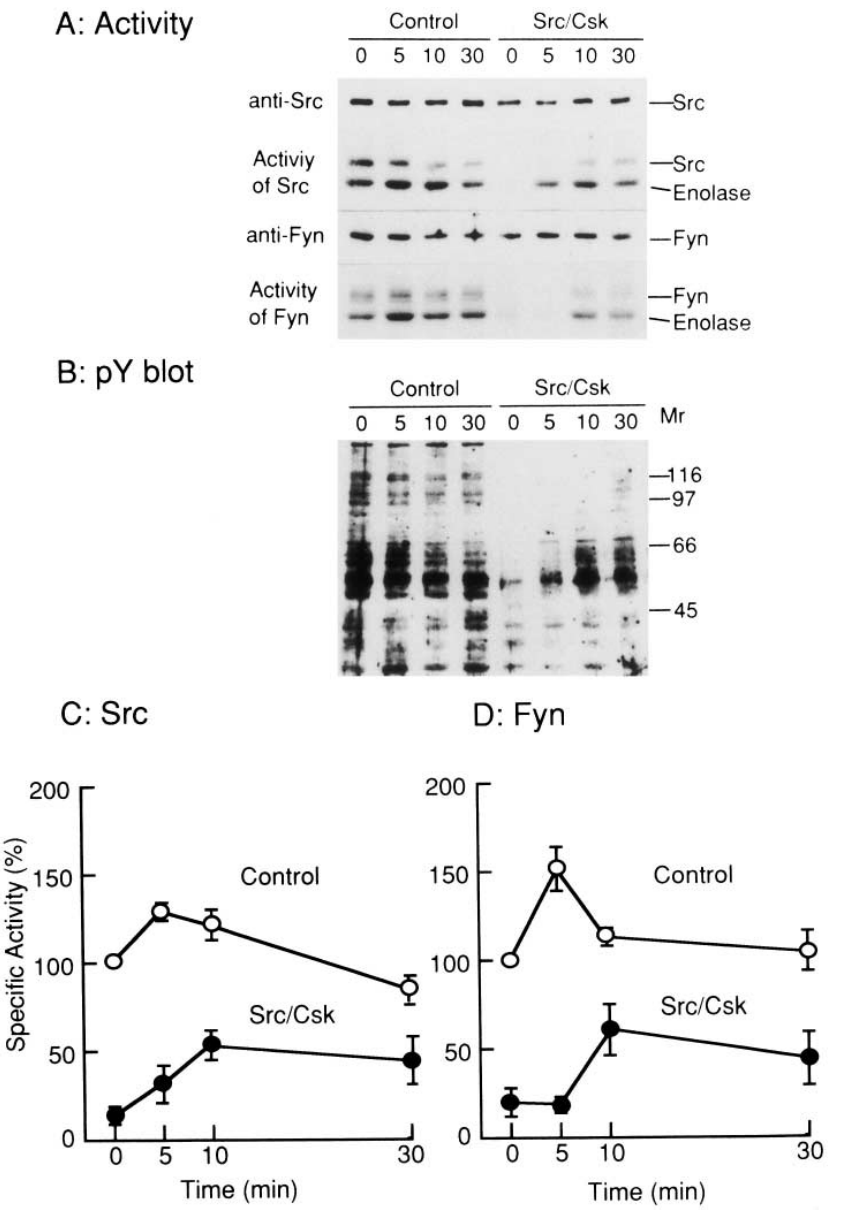

Fig. 3. Effect of Src/Csk expression on the activation of Src and Fyn induced by cross-linking of a cell adhesion molecule L1. A: The control transfectant (Neo) and Src/Csk transfectant (A2) were allowed to differentiate for 5 days. After incubation with an anti-L1 antibody (50 μg/ml) recognizing an extracellular domain for the indicated time (min), the cells were lysed in TNE buffer. The expression levels of Src and Fyn were confirmed by Western blotting, and Src and Fyn proteins were immunoprecipitated from the cell lysate (50 μg protein). The kinase activities of Src and Fyn were determined with enolase as a substrate. B: Whole cell lysates were prepared from the control cells and Src/Csk transfectant following L1 stimulation for the indicated time (min), and aliquots (10 μg protein) were subjected to Western blotting with anti-phosphotyrosine antibody (4G10). The molecular sizes of the marker proteins are indicated in kDa. C, D: The specific activities of Src (C) and Fyn (D) were calculated from 4 (Src) and 3 (Fyn) independent experiments (a representative result is shown in panel A). Relative values against the activity in unstimulated control cells are expressed as means ± S.E.M.

that constitutively high activity of Src is required for progression or maintenance of neural differentiation. In Src/Csk transfectants, the constitutive activation of Src was almost completely eliminated. Therefore, Src/Csk transfectants might provide a good system for studying the mechanism and physiological role of Src activation during neural differentiation.

3.3. *Effect of Src/Csk expression on the activation of Src family kinases induced by cross-linking of a cell adhesion molecule, L1*

To examine the effect of Src/Csk expression on the potentially Src-mediated signaling pathway evoked by cell-to-cell interaction, we here investigated the activity change in Src family kinases after cross-linking of L1 using anti-L1 antibody. L1 is a neural cell adhesion molecule consisting of immunoglobulin-like and fibronectin type III-like extracellular domains and a short cytoplasmic tail, and is known to be a key molecule involved in neurite fasciculation [31–34]. In differentiated control cells, the basal activities of Src and Fyn were substantially higher than those in Src/Csk transfectant. Nevertheless, rapid (peaking within 5 min after stimulation) and significant activation of Src and Fyn was observed (Fig.

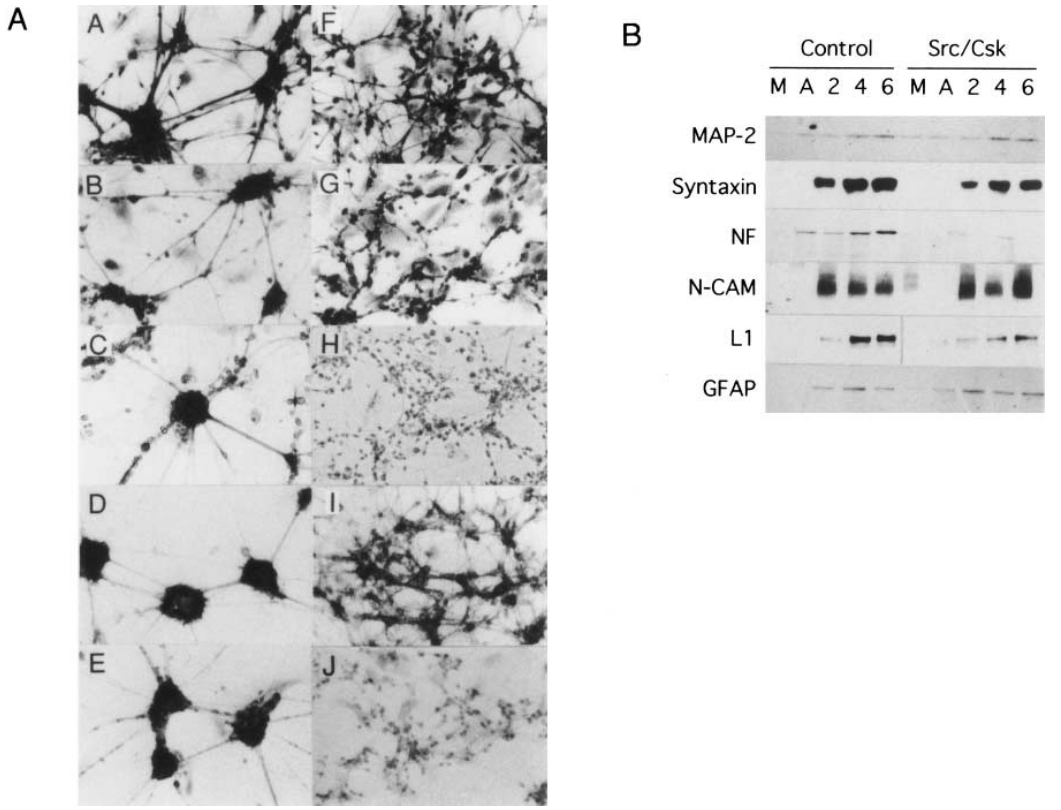

Fig. 4. A: Immunocytochemical analyses of the expressions of neural marker proteins and cell adhesion molecules in the control transfectant (Neo) and Src/Csk transfectant (A2). The control (A–E) and Src/Csk (F–J) transfectants were allowed to differentiate for 6 days, and the cultures were immunostained with anti-MAP2 (A, F), anti-syntaxin (B, G), anti-NF160 (C, H), anti-N-CAM (D, I), and anti-L1 (E, J) antibodies. B: Western blot analyses of the expressions of marker proteins. Whole cell lysates (20 μg protein) of control (Neo) and Src/Csk transfectant (A2) at various differentiation stages were subjected to Western blotting with antibodies against the indicated marker proteins. M, undifferentiated monolayer culture; A, cell aggregates in suspension culture; 2, 2-day cultures after neural induction; 4, 4-day cultures; 6, 6-day cultures.

3A,C,D). Surprisingly, it was found in this study that Fyn was also functionally coupled with L1 stimulation. In Src/Csk transfectants, in which Src family kinases were present as suppressed forms, relative specific activities of Src and Fyn were apparently increased by L1 stimulation. However, the peaking times of the activation were significantly delayed (beyond 10 min after stimulation), and the absolute values of their specific activities were much less than those in the control cells. These findings suggest that Src as well as Fyn could act as intracellular transducers of signals evoked by cell-to-cell interaction mediated by L1, and that Csk is involved in the regulation of this signaling pathway.

Consistent with the activities of Src family kinases, the basal levels of tyrosine phosphorylation of cellular proteins were already high in differentiated control cells (Fig. 3B). Probably because the phosphorylation was nearly saturated, there was no significant change in tyrosine phosphorylation even after L1 stimulation. In contrast, the basal levels of tyrosine phosphorylation were very low in Src/Csk transfectants, indicating that the activities of Src family kinases were efficiently suppressed by Src/Csk in these cells. Although several reports proposed that Csk has some targets other than Src family kinases [35,36], there was no induction of any tyrosine phosphorylated protein in Src/Csk transfectant. This suggests that Src/Csk mainly acts on Src family kinases to suppress their functions under our conditions. In Src/Csk transfectants, however, induction of tyrosine phosphorylation by L1 stimulation was detected in parallel with the activation profiles of Src and Fyn, supporting that Src family kinases are important mediators of L1 signaling. Thus it is likely that the constitutive activation of Src in differentiated normal cells is induced by an active cell-to-cell interaction potentially mediated by L1, although the mechanism remains to be studied.

3.4. Phenotype of Src/Csk transfectants

When the three Src/Csk transfectants and a DK-Src/Csk transfectant were cultured in an undifferentiated state, no significant change was observed in rates of cell growth or cell morphology as compared with those of wild type cells. Furthermore, the initial events of differentiation (until 4 days after neural induction) appeared to be indistinguishable from those of wild type cells, in that they generated small cell clusters and extended neurites to form a fine network (data not shown). These observations demonstrate that the expression of Src/Csk does not affect the determination of neural cell fate. Since Src was present as a suppressed form in undifferentiated P19 cells, the effect of Src/Csk on Src was moderate in the early stages of differentiation. Previously it was reported that overexpression of c-Src and c-Src^{+} in P19 cells rather inhibited neural differentiation, suggesting that the activity of Src is not essential for commitment of neural differentiation [37]. This implies that Src should be down-regulated in undifferentiated cells until neural differentiation is committed by certain mechanism. Indeed, the level of Csk expression was greater in developing neural cells than in mature ones [29].

In the control cells, thereafter, the dispersed small cell clusters became integrated into larger cell aggregates and the fine networks of neurites were converted into large meshed networks consisting of thick neurite bundles (neurite fasciculation) within 6 days after neural induction (Fig. 4A (A–E)). In contrast, Src/Csk transfectants were still dispersed into small cell clusters and their neurites were no longer integrated into thick bundles (Fig. 4A (F–J)). DK-Src/Csk transfectant exhibited a morphology indistinguishable from that of wild type cells (data not shown).

To further characterize the phenotype of Src/Csk transfectant, expressions of some neuronal marker proteins and cell adhesion-related proteins were analyzed by immunocytochemistry. In the differentiated control cells, MAP2, syntaxin, NF, L1 and N-CAM immunoreactivities were detected in the aggregates of both neuronal cell bodies and neurite bundles (Fig. 4A (A–E)). Src/Csk transfectant also showed high immunoreactivity against anti-MAP2, anti-syntaxin, and anti-N-CAM antibodies (Fig. 4A (F,G,I)). However, the immunoreactivities for NF and L1 were extremely low in Src/Csk transfectant (Fig. 4A (H,J)). The expression of these proteins was further confirmed by Western blotting (Fig. 4B). Consistent with the results of immunocytochemistry, induction of NF and L1 during neural differentiation was suppressed in Src/Csk transfectants. Induction of other neural proteins including neuron-specific enolase, focal adhesion kinase (FAK), Fyn, Src and Csk was not significantly affected by the expression of Src/Csk (data not shown).

Taken these phenotypes together with the finding that Src-mediated signaling through L1 was attenuated in Src/Csk transfectants, it is plausible that abrogated cell-to-cell interaction mediated by L1 is one of the major causes of the phenotype. Indeed, interference with L1-L1 interaction of normal P19 cells by the addition of antibody against extracellular domain of L1 caused a phenotype very similar to that of Src/Csk transfectant (unpublished observation). Thus Src family kinases and Csk may play important roles in the regulation of cell-to-cell interaction which is an essential element for the progression of neural differentiation. However, expression of neurofilament protein was also downregulated in Src/Csk transfectant. The possibility that Src family kinases/Csk is involved in the regulation of multiple gene expressions required for neural differentiation should be examined in a future study.

Acknowledgements: This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, the Yamanouchi Foundation for Research on Metabolic Disorders, The Naitou Foundation and The Sankyo Foundation.

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