Suppression of Protein-Tyrosine Kinase Activities of Src Family by A Cytoplasmic Protein-Tyrosine Kinase Csk

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Contents

General Introduction

There are many proteins that contribute to a complex intracellular signaling network, which regulates cellular functions in various aspects. A group of protein-tyrosine kinases is one of the molecules involved in cellular signaling such as cell growth and differentiation, and its signaling is initiated by phosphorylation of the downstream factors, by which some properties of the phosphorylated protein are altered. According to their structural features, protein-tyrosine kinases can be subdivided into two major groups; receptor type and non-receptor type. It is known that receptor type kinases mediate extracellular signals to change to intracellular signaling pathways. On the other hand, the function of non-receptor type kinases remains unclarified yet. Src family protein-tyrosine kinases belong to the latter type.

It is known at present that Src family consists of nine members; p60$^{c\text{-}src}$ (Takeya and Hanafusa, 1983), p59^{fyn} (Semba *et al.*, 1986; kawakami *et al.*, 1986), p62$^{c\text{-}yes}$ (Sukegawa *et al.*, 1987), p60^{yrk} (Sudol *et al.*, 1993), p55$^{c\text{-}fgr}$ (Parker *et al.*, 1985; Nishikawa *et al.*, 1986), p56^{lck} (Marth *et al.*, 1985; Voronova and Sefton, 1986), p55^{blk} (Dymecki *et al.*, 1990), p59^{hck} (Ziegler *et al.*, 1987; Quintrell *et al.*, 1987) and p53/56^{lyn} (Yamanashi *et al.*, 1987). Some of them are detected in specialized tissues, while others are distibuted ubiquitously. Certain of this family has been found to be physically and functionally associated with certain members of cell surface receptors (Table 1). For instance, p59^{fyn} is associated with CD3/TCR complex and mediates stimuli brought about by antigen recognition by the latter (Samelson *et al.*, 1990; Cooke *et al.*, 1991; Appleby *et al.*, 1992; Stein *et al.*, 1992). The functions of other Src family members such as p56^{lck}, p55^{blk}, p59^{hck} and p53/56^{lyn} are being made clear in hematopoietic tissues. On

the other hand, p60$^{c\text{-}src}$, p62$^{c\text{-}yes}$ and p59^{fyn} are highly expressed in the central nervous system, but their roles remain unsolved yet. In order to clarify their functions, anyway, it seems very important to analyze when and how the activity of Src family protein-tyrosine kinase are regulated. From this point of view, I examined the regulation mechanism of activities of Src family protein-tyrosine kinases.

All Src family members possess common structural features as shown in Figure 1a. Gly2 is conserved in all Src family members and myristylated both in p60$^{c\text{-}src}$ (Shulz *et al.*, 1985; Buss and Sefton, 1985) and p56^{lck} (Voronova *et al.*, 1984; Marchildon *et al.*, 1984). Therefore these proteins are considered to be localized in membrane periphery (Rohrschneider and Gentry, 1984). The others are also assumed to have such modification.

The invariant Gly2 is followed by a totally divergent region of 75 residues or so. Src-Homology (SH) 3 and SH2 domains, and kinase catalytic domain are followed sequentially after the variable region. The SH3 and SH2 regions are also included in some other signaling or cytoskeletal molecules than Src family. The two regions are considered to have activities to interact or be associated with other proteins. In regard to Src family, SH3 ligand has not been identified yet, but SH2 ligand was shown to be phosphotyrosyl peptide (reviewed by Pawson and Gish, 1992).

Carboxy (C)-terminal half is almost occupied by a protein-tyrosine kinase catalytic domain, in which there are two major tyrosine phosphorylation sites; tyrosine 416 (Tyr416) and tyrosine 527 (Tyr527) in chicken p60$^{c\text{-}src}$. It was shown that active p60$^{c\text{-}src}$ was autophosphorylated predominantly at Tyr416 of its own molecule *in vitro* (Casnellie *et al.*, 1982; Smart *et al.*, 1981), but that p60$^{c\text{-}src}$ labeled *in vivo* was phosphorylated at Tyr527 (Cooper *et al.*, 1986). It was

revealed that the phosphorylation state of Tyr527 was correlated with specific activity of p60$^{c\text{-}src}$; p60$^{c\text{-}src}$ lost its tyrosine kinase activity due to phosphorylation at Tyr527. Furthermore, p60$^{v\text{-}src}$, an active oncogenic variant of p60$^{c\text{-}src}$, lacks Tyr527 by mutational replacement (Takeya and Hanafusa, 1983). It has been considered that phosphorylation at Tyr527 is a key event of suppression of the activity of p60$^{c\text{-}src}$ and also observed that mutations and deletions in both SH3 and SH2 regions affected the activity of p60$^{c\text{-}src}$ (reviewed by Cooper, 1990). From these findings, a regulation model was presented as shown in Figure 1b. According to this model, C-terminal peptide of the protein phosphorylated at Tyr527 takes a unique conformation to become associated with SH3 and SH2 regions of its own molecule and thus its kinase activity is suppressed. Once Tyr527 is dephosphorylated from some reason, the protein restores original conformation and activity. Although the latter mechanism remains unknown yet, but a 50 kDa protein-tyrosine kinase purified from neonatal rat brain (Okada and Nakagawa, 1988, 1989) can be a candidates as the negative regulator that phosphorylates Tyr527 of p60$^{c\text{-}src}$ and suppresses its activity.

In this study, the primary structure of this 50 kDa protein-tyrosine kinase was deduced by means of cDNA cloning and named Csk (C-terminal Src Kinase). It was found that Csk was a new class of cytoplasmic protein-tyrosine kinase. It wa also shown that Csk was real entity that specifically phosphorylates Tyr527 of p60$^{c\text{-}src}$ *in vitro* and in yeast cells and that its phosphorylation coincidentally suppressed the activity of p60$^{c\text{-}src}$. Targeted disruption of *csk* gene in mouse embryonic stem cell revealed that Csk deficiency caused developmental lethality in mouse embryos, defects in neural tube formation with concomitant elevation of activities of Src family kinases, p60$^{c\text{-}src}$, p59^{fyn}, and p53/56^{lyn}. These findings suggest that Csk and thus Src

family play critical roles in nervous development. The details are described in this paper.

Table 1. Tissue distribution of Src family and cell surface molcules associated with it

c-src	ubiquitous, high in brain, platelet high affinity IgE receptor, PDGF receptor
fyn	ubiquitous, high in brain, thymus CD3/TCR complex, surface IgM, IgD, PDGF receptor, low affinity IgE receptor, platelet GpIV
c-yes	ubiquitous, high in brain, liver, kidney high affinity IgE receptor, PDGF receptor, platelet GpIV
yrk	cerebellum, spleen
c-fgr	macrophage, granulocyte, NK cell
lck	T cell CD4/CD8, IL-2 recepor
blk	B cell surface IgM, IgD
hck	hematopoietic cells
lyn	liver, brain, kidney, high in spleen surface IgM, IgD, high affinity IgE receptor, platelet GpIV

(Umemori and Yamamoto, 1992)

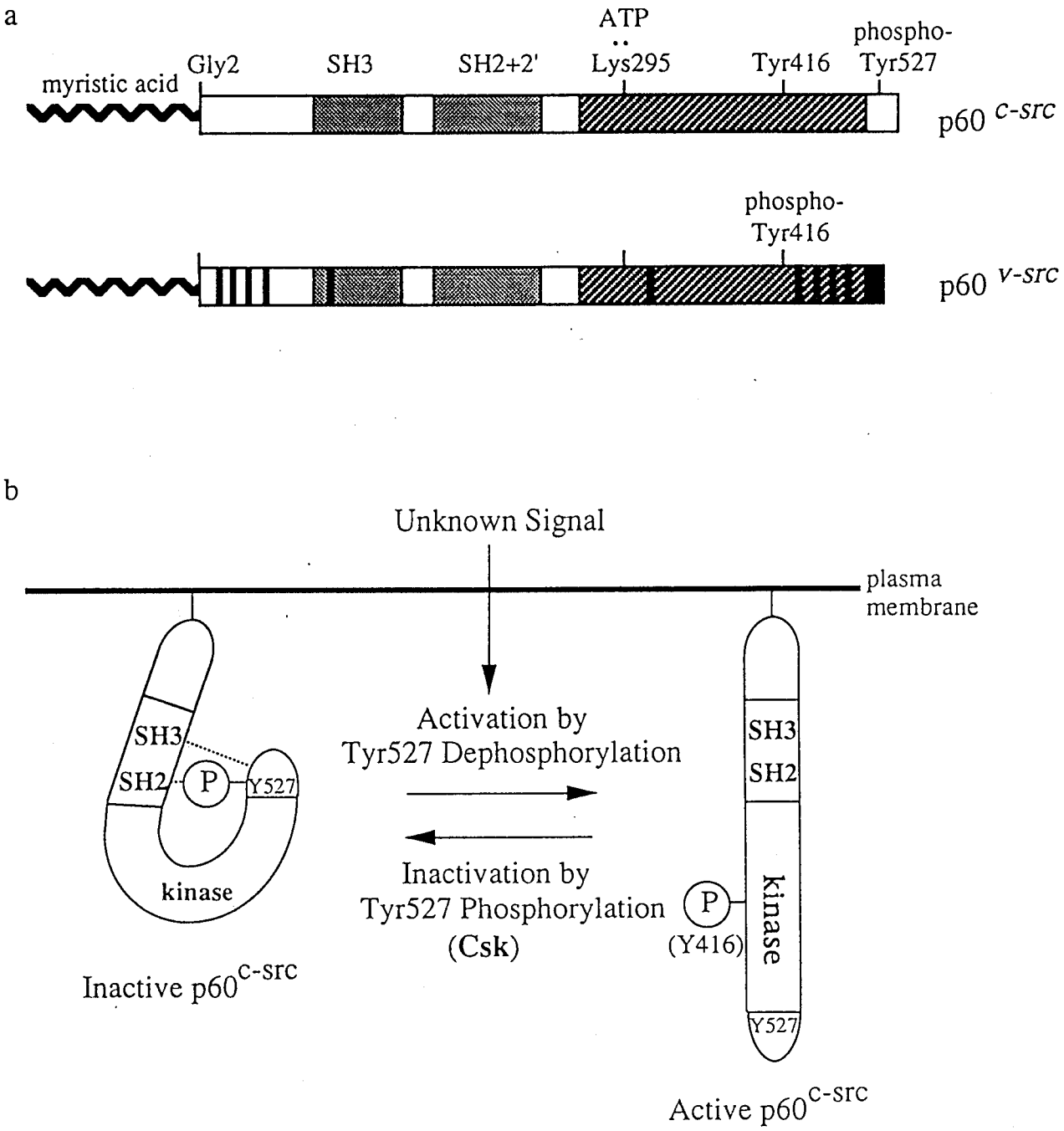

Figure 1. Conserved structure and regulation model of Src family
(a) Schematic representation of conserved features of Src family (upper) and its active variant, p60$^{v\text{-}src}$ (lower). Mutations of single amino acid change and replacement of C-terminal sequence are indicated as solid bars in p60$^{v\text{-}src}$. (b) Regulation model of p60$^{c\text{-}src}$.

Chapter I

Molecular Cloning of A Protein-Tyrosine Kinase that Specifically Phosphorylates A Negative-Regulatory Tyrosine Residue of p60$^{c\text{-}src}$

Summary

Activity of Src family protein-tyrosine kinases is suppressed by a phosphorylation of a tyrosine residue closely located at their C-terminus. In this study I cloned a cDNA that encodes a protein-tyrosine kinase purified from neonatal rat brain with an activity to phosphorylate this negative-regulatory tyrosine residue of p60$^{c\text{-}src}$. From its primary structure it was revealed that this protein-tyrosine kinase was a new class of cytoplasmic protein-tyrosine kinase, which lacks autophosphorylation and regulatory tyrosine residues and myristylation signal and that it phosphorylated specifically only the negative-regulatory tyrosine residue of p60$^{c\text{-}src}$ *in vitro*. From these characterisics, the enzyme was named Csk (C-terminal Src kinase). Csk was highly expressed in neonatal brain, spleen and thymus, where not only p60$^{c\text{-}src}$, but also other Src family members were detected abundantly. Co-expression of p60$^{c\text{-}src}$ and Csk in yeast cells showed that Csk could phosphorylate the negative-regulatory tyrosine residue and resulted in suppression p60$^{c\text{-}src}$ activity. These results indicate that Csk, a new class of cytoplasmic protein-tyrosine kinase is a negative regulator of Src family tyrosine kinases.

Introduction

As the proto-oncogene *c-src* is preferentially expressed in nervous system (Cotton and Brugge, 1983; Schartl and Barnekow, 1982; Sorge *et al.*, 1984; Fults *et al.*, 1985), its product p60$^{c\text{-}src}$ has been suggested to play an important role in development or function of nervous system. Although p60$^{c\text{-}src}$ is believed to elicit physiological functions through its protein-tyrosine kinase activity, its genuine function in nervous system remains thoroughly unknown. However, the regulation mechanism of its activity has been suggested; the activity is suppressed by phosphorylation or enhanced by dephosphorylation at a tyrosine residue located close to C-terminus, corresponding Tyr527 in chicken p60$^{c\text{-}src}$ sequence (Cooper *et al.*, 1986; Cooper and King, 1986; Courtneidge, 1985). A mutational replacement of Tyr527 to Phe was reported to cause marked stimulation of kinase activity of p60$^{c\text{-}src}$ (Kimiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Cartwright *et al.*, 1987). Since p60$^{c\text{-}src}$ itself has only little activity to phosphorylate Tyr527 (Cooper and King, 1986; Okada and Nakagawa, 1989; Kornbluth *et al.*, 1987; Cooper and MacAuley, 1988), and a mutant p60$^{c\text{-}src}$ lacking kinase activity was fully phosphorylated at Tyr527 in mammalian cells (Jove *et al.*, 1987), it has been suggested that there were some other tyrosine kinases than p60$^{c\text{-}src}$, which phosphorylate Tyr527 and regulate the function of p60$^{c\text{-}src}$. In order to clarify the function of p60$^{c\text{-}src}$, it seems important to characterize the enzyme that is involved in the regulation of the activity of p60$^{c\text{-}src}$.

Recently, a 50 kDa novel protein-tyrosine kinase that phosphorylate Tyr527 of p60$^{c\text{-}src}$ *in vitro* was found and purified from neonatal rat brain (Okada and Nakagawa, 1988, 1989). Purified enzyme is highly specific for p60$^{c\text{-}src}$; it showed no significant phosphorylating activity

for any known substrate for protein-tyrosine kinases, such as microtubule proteins, casein, enolase, lactate dehydrogenase, histone or heat-treated total protein of rat brain, except for certain synthetic peptides. This tempted me to assume tha enzyme to be a strong candidate for the specific kinase.

To establish that this enzyme (hereafter this enzyme is named Csk) is real negative regulator of protein-tyrosine kinase activity of $p60^{c\text{-}src}$, I examined the substrate specificity of Csk using mutant $p60^{c\text{-}src}$ expressed in yeast cells, its cDNA cloning to deduce the primary structure of Csk, and *in vivo* functional changes of $p60^{c\text{-}src}$ when it was co-expressed with Csk in yeast cells.

Results

Substrate Specificity of Csk

The specificity of Csk against p60$^{c\text{-}src}$ was examined using mutants of p60$^{c\text{-}src}$, R295 and R295F527. R295 is a kinase-defective mutant which was generated by a mutation at Lys295 in ATP-binding site to Arg. R295F527 is also a kinase-defective mutant generated from R295 by further mutation at Tyr527 to Phe. It was shown that when synthesized in yeast cells in which Csk is not contained, wild type p60$^{c\text{-}src}$ (WT) was phosphorylated to a low degree at Tyr527 (Cooper and Runge, 1987), and the R295 was not phosphorylated at all (Cooper and MacAuley, 1988), so these molecules should be possible substrate for kinases that are specific for Tyr527. These molecules were expressed in yeast cells, and immunoprecipitated from yeast cell lysates using anti-p60$^{c\text{-}src}$ antibody (Figure 1b). Then the immunoprecipitates were incubated with or without highly purified Csk (Figure 1a) in kinase assay buffer containing [γ-^{32}P]ATP, and the phosphoproteins were resolved by SDS-PAGE and detected by autoradiography. Consequently, it was revealed that phosphorylation level of WT was increased by addition of Csk compared with that of autophosphorylation (Figure 1c, lanes 1 and 2), and that phosphorylation of R295 was dependent on the existence of Csk (lanes 3 and 4), but that of R295F527 was not detected at all (lanes 5 and 6). These results clearly indicate that Csk can specifically phosphorylate Tyr527 of p60$^{c\text{-}src}$.

cDNA Cloning and Deduced Amino Acid Sequence of Csk

Purified Csk was digested by lysyl-endopeptidase, resultant peptides were purified by HPLC and amino acid sequences of them were determined by gas phase sequencer (Figure 2, SRCK0-SRCK7). Although the sequence of SRCK2 was originally determined as "VECYRIMYCASK", the two cystein residues in this sequence were ascertained to be histidine residues after sequencing Csk cDNA. Four sequences, SRCK1, SRCK2, SRCK4 and SRCK5 were used to design both strands of degenerated oligonucleotide primers for reverse transcription and subsequent polymerase chain reaction (RT-PCR). A 260 bp product was gained from a RT-PCR using sense primer of SRCK1 and antisense primer of SRCK2 with use of cDNA reverse transcribed from neonatal rat brain mRNA using antisense primer of SRCK4 (data not shown). This 260 bp product was confirmed to contain the SRCK5 sequence (data not shown). Then it was used as a probe to screen neonatal rat brain cDNA library. From 450,000 page clones, a single positive clone was obtained, which contained a cDNA of 2125 bp in length, poly(A) tail, polyadenylation consensus sequence and a complete open reading frame encoding a protein of 450 amino acids with a molecular weight of 50,753. Since it contained the sequences of the eight peptides obtained by lysyl-endopeptidase digestion of Csk, it was indicated that the deduced amino acid sequence was identical with that of Csk (Figure 2).

Deduced amino acid sequence of Csk revealed that it was a novel protein-tyrosine kinase, because no identical protein was found in NBRF-PIR release 25.0 data base. It has similarity with all the defined subdomains noted in protein kinase and contains the 15 nearly invariant residues (Hanks *et al.*, 1988). It also contains sequences characteristic of protein-tyrosine kinase, such as "DLAARN" in subdomain VI and "KWTAPE" in subdomain VIII (Figure 3a). Upstream of the kinase domain, it has SH3, SH2 and SH2' regions. Sequence identity of the

kinase domain was 46% against p60$^{c\text{-}src}$; 46%, ABL; 41%, DER; 39%, RET; and 41%, ROS; as shown in Figure 3a (Hanks *et al.*, 1988). Total sequence identity of SH3, SH2 and SH2' was 47% against p60$^{c\text{-}src}$ (Takeya and Hanafusa, 1983); 40%, CRK (Mayer *et al.*, 1988); 30%, PLC (Stahl *et al.*, 1988); and 27%, GAP (Vogel *et al.*, 1988); as shown in Figure 3b. N-terminal sequence of Csk reveals that Csk has no myristylation signal Gly2 (Shulz *et al.*, 1985; Buss and Sefton, 1985). Csk lacks tyrosine residues at sites corresponding to Tyr416 and Tyr527 of p60$^{c\text{-}src}$.

Tissues Distribution of Csk mRNA

To determine the tissue distribution of Csk, poly(A) RNA extracted from rat tissues was subjected to Northern blot analysis using Csk cDNA as probe. As shown in Figure 4, cross-hybridizing transcripts were detected in all rat tissues examined, but markedly concentrated in neonatal brain, thymus and spleen. The size of the major transcripts differed among tissues; that in brain was 2.4 kb, while that in thymus or spleen was 2.2 kb.

Co-expression of p60$^{c\text{-}src}$ and Csk in Yeast Cells

In order to demonstrate that the protein encoded in the cloned cDNA actually possess the activity to phosphorylate Tyr527 of p60$^{c\text{-}src}$ and negatively regulate it, the cDNA was introduced in yeast cells which are expressing p60$^{c\text{-}src}$. The expression of Csk alone did not alter the intracellular tyrosine phosphorylation state in yeast cells (Figure 5a, lane 1), but the expression of WT or an active mutant, F527 caused an accumulation of tyrosine phosphorylated proteins in yeast cells (lanes 2

and 4). In the cells expressing WT, co-expression with Csk depressed the tyrosine phosphorylation of intracellular proteins except for WT, while it could not be effective in the cells expressing F527 mutant (lanes 3 and 5). WT immunoprecipitated from yeast cells co-expressing with Csk actually showed lower *in vitro* kinase activity than that of WT from cells expressing WT alone (data not shown). In the case of kinase-defective mutant, R295 or R295F527 expressors, co-expression with Csk caused tyrosine phosphorylation of R295 but R295F527 (lanes 6-9). These results clearly indicate that cloned Csk can phosphorylate only Tyr527 of p60$^{c\text{-}src}$ and negatively regulate its activity even in yeast cells.

Discussion

It was previously reported that a 50 kDa protein-tyrosine kinase purified from neonatal rat brain could phosphorylate Tyr527 of p60$^{c\text{-}src}$ *in vitro* (Okada and Nakagawa, 1988, 1989). To get more information about this enzyme termed Csk, it was purified more highly with improved method, and its substrate specificity was confirmed with kinase-defective p60$^{c\text{-}src}$ mutants. The results obtained clearly indicate that Csk is highly specific for Tyr527 and that it does not redirect the autophosphorylation activity of p60$^{c\text{-}src}$ to Tyr527. When these p60$^{c\text{-}src}$ mutants were incubated with purified insulin receptor, as a representative of tyrosine kinase having broader substrate specificity, neither R295 nor R295F527 was phosphorylated significantly (data not shown). Since specific phosphorylation of Tyr527 of p60$^{c\text{-}src}$ by any other kinase has not been reported, Csk is a strong candidate for the specific kinase involved in the regulation of p60$^{c\text{-}src}$ function.

The amino acid sequence reveals that Csk has all features of protein-tyrosine kinase (Hanks *et al.*, 1988), but that it has several unique characteristics for protein-tyrosine kinase never reported previously. A comparison of the kinase domain with other protein kinases discloses that Csk has higher similarity to Src family kinases. Among them, p60$^{c\text{-}src}$ (Takeya and Hanafusa, 1983) has the highest similarity, but is only 46% identical to Csk. All Src family kinases have the sequence "DLRAAN" in subdomain VI, while Csk has a sequence "DLAARN" as well as other kinases than Src family. Other protein-tyrosine kinases including receptor type kinases (Hanks *et al.*, 1988) also show similarities to Csk in the kinase domain, the sequence identity ranging from 39% to 41% (Figure 3a). This indicates that Csk might not be a member of any known family of protein-tyrosine kinase.

It is striking that Csk lacks a tyrosine residue at a site corresponding to Tyr416 of p60$^{c\text{-}src}$. Tyr416 is known to be a site for autophosphorylation in p60$^{c\text{-}src}$. The members of Src family share a highly conserved sequence around this residue, and autophosphorylate at a tyrosine residue equivalent to Tyr416. Csk, however, shows no autophosphorylation activity (Figure 1c). It is probable that the lack of autophosphorylation activity, which is a peculiar characteristic for tyrosine kinases, may be important for the determination of the substrate specificity or regulation of Csk.

The members of Src family kinase also share a highly conserved C-terminal sequence that includes a tyrosine residue equivalent to Tyr527. From this, it has been suggested that the tyrosine residue can be phosphorylated and that it can serve as a site for negative regulation of these kinases (reviewed by Hunter, 1987). In contrast, Csk has a stop codon at the site corresponding to Tyr527, indicating that the enzyme is not regulated by the phosphorylation at this site. Based on the low homology within the kinase domain with other protein-tyrosine kinases and the lack of autophosphorylation activity, Csk should be classified into a new family of protein-tyrosine kinases.

Upstream of the kinase domain, Csk contains SH3, SH2 and SH2' regions. These regions were originally found in cytoplasmic protein-tyrosine kinases (Sadowski *et al.*, 1986), where they were important for function (Hirai and Varmus, 1990). They were also found in a viral oncoprotein, p47$^{gag\text{-}crk}$ (Mayer *et al.*, 1988), phospholipase C-γ (Stahl *et al.*, 1988) and ras GTPase activating protein (Vogel *et al.*, 1988). From the findings that p47$^{gag\text{-}crk}$ affects the intracellular tyrosine phosphorylation states (Mayer and Hanafusa, 1990) and that both phospholipase C-γ (Meisenhelder *et al.*, 1989) and ras GTPase activating protein (Molloy *et al.*, 1989) can be phosphorylated on tyrosine and their

SH2 domains can directly interact with activated EGF receptor or PDGF receptor *in vitro* (Anderson *et al.*, 1990), it is believed that SH2 and SH3 are important for interaction with cellular proteins that serve as substrates or regulators for protein-tyrosine kinases. Therefore, it is possible that these regions in Csk are involved in recognition of its substrate, p60$^{c\text{-}src}$, or interaction with a regulator that modulates the kinase activity of Csk.

Csk lacks a myristylation signal at the N-terminus and has no potential membrane spanning sequence, suggesting that Csk is a cytosolic protein. Although Csk was originally purified from membrane fractions, immunoblot analysis with anti-Csk antibody indicated that Csk is also present in the soluble fraction (data not shown). This finding is consistent with the previous observation that soluble mutant of p60$^{c\text{-}src}$ was also phosphorylated effectively at Tyr527 (Schuh and Brugge, 1988). Csk recovered from membrane fractions might have been bound to p60$^{c\text{-}src}$.

Northern blot analysis of neonatal rat brain identified a single 2.4 kb transcript. Transcripts of the same size were detectable in all adult tissues examined, but markedly concentrated in neonatal brain. p60$^{c\text{-}src}$ is also known to be concentrated in developing rat brain (Cartwright *et al.*, 1988), thus Csk may play an important role in the regulation of p60$^{c\text{-}src}$ during neural differentiation. In thymus and spleen, cross hybridizing transcripts with slightly smaller size (2.2 kb) were detected at a similar level to that of neonatal transcript. Since there was no difference on the molecular weight, on the reactivity of anti-Csk antibody, and on the peptide map by lysyl-endopeptidase digestion of between Csk-like protein purified from spleen and Csk derived from neonatal brain (data not shown), the difference of transcripts in length may be due to an alternative splicing at a site in the untranslated region of Csk mRNA. These tissues are known to contain different type of Src

family kinases, such as p56*lck*, p59*fyn* and p53/56*lyn*. These kinases are thought to be involved in the regulation of function or differentiation of lymphoid cells. Therefore, Csk may regulate not only p60*c-src* but also the other Src family members in some non-neuronal cell types.

Co-expression experiments of p60*c-src* and Csk in yeast cells demonstrates that Csk can specifically phosphorylate Tyr527 of p60*c-src* *in vivo*. This finding also indicates that Csk acts as a negative regulator for p60*c-src* under physiological conditions. When p60*c-src* is expressed in yeast cells, where Csk is absent, it was reported that p60*c-src* was only phosphorylated at a low degree at Tyr527, but that it was phosphorylated at Tyr416 stoichiometrically (Kornbluth *et al.*, 1987; Cooper and Runge, 1987). Under that condition, p60*c-src* is known to be fully active (Cooper and Runge, 1987). Thus, high level of phosphotyrosine was observed in yeast cells (Figure 5a, lane 2). However, when p60*c-src* was co-expressed with Csk, the level of phosphotyrosine was substantially lowered, and phosphotyrosine was detected on p60*c-src* alone (Figure 5a, lane 3). This fact suggests that Tyr527 of p60*c-src* is fully phosphorylated by Csk, so that the kinase activity of p60*c-src* is markedly decreased. Since the level of phosphotyrosine on p60*c-src* in such case was almost equivalent to that on R295 (Figure 5a, lane 7), it is likely that Tyr527 phosphorylation by Csk occurs stoichiometric. These observations further support the possibility that Csk is the negative regulator for p60*c-src*.

Experimental Procedures

Purification of Csk

Csk was purified from the membrane fraction of neonatal brain by sequential column chromatography. The method was essentially the same as described previously (Okada and Nakagawa, 1989), but it was slightly modified to improve the yield and purity. All operations were carried out at 0-4 °C. At each purification step protein-tyrosine kinase activity was monitored by phosphorylation of poly(Glu, Tyr) (Okada and Nakagawa, 1989). Rat brain of postnatal 2-4 days was homogenized in the buffer containing 50 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 10,000xg for 15 min. The supernatant was recentrifuged at 100,000xg for 60 min and resulting precipitate was extracted with the buffer containing 50 mM Tris-HCl pH 9.0, 1 mM EDTA, 5 mM β-mercaptoethanol, 10% (v/v) glycerol and 5% (v/v) NP40 with stirring for 2 h. The suspension was sequentially subjected to column chromatography of DE52, poly(Glu, Tyr) Sepharose CL4B, MonoQ, Sephacryl S200HR and MonoS, and proteins were eluted with 0.1 M KCl, 0.1 M NaCl, linear gradient of 0-0.35 M NaCl (collected 0.25 M NaCl fraction), 0.2 M NaCl (only in this step the buffer supplemented with 0.2 M NaCl was used) and linear gradient of 0-0.2 M NaCl, respectively. At each purification step of column chromatography, the universal buffer used for equilibration of column, elution of protein with addition of salt and dialysis of eluate was buffer A containing 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 5 mM β-mercaptoethanol, 10% (v/v) glycerol and 0.1% (v/v) NP40. Purified Csk was stored at -20 °C.

Yeast Cell Culture

The *Saccharomyces cerevisiae* strain BJ2168 (*a, ura3-52, leu2, trp1, prb1-1122, pep4-3, prc1-407, gal2*)(Cooper and MacAuley, 1988) was used throughout. p60$^{c\text{-}src}$ and its mutants expressor were given by Jonathan A. Cooper. The synthetic medium (HD medium) used to grow yeast cells consisted of 6.6 g yeast nitrogen base w/o amino acid (Difco), 20 g glucose, 20 mg uracil, 20 mg tryptophan, 20 mg histidine, 20 mg arginine, 20 mg methionine, 30 mg tyrosine, 30 mg leucine, 30 mg isoleucine, 30 mg lysine, 60 mg phenylalanine and 150 mg valine in 1 litter of water.

Immunoprecipitation of p60$^{c\text{-}src}$

Immunoprecipitation of p60$^{c\text{-}src}$ and its mutants from yeast cells was carried out by the method described previously (Cooper and Runge, 1987). In brief, yeast cells were disrupted by vortexing with glass beads in LIPA buffer (50 mM Tris-HCl pH 8.2, 0.15 M NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 100 KIU/ml aprotinin, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate and 0.1% SDS). After centrifugation at 15,000 rpm for 20 min at 4 °C, mouse monoclonal anti-p60$^{c\text{-}src}$ antibody (MAb327; Oncogene Science Inc.)(Lipsich *et al.*, 1983) was added to the supernatant and incubated for 1 h on ice, and rabbit anti-mouse IgG (Zymed) was added and incubated for 15 min on ice, and then Pansorbin (Carbiochem) was added and incubated for further 30 min on ice. After centrifugation at 5,000 rpm for 2 min at 4 °C, the precipitate was washed for three times with LIPA buffer and further 3 times with buffer A.

In vitro Kinase Assay

Immunoprecipitated p60$^{c\text{-}src}$ derived from 10^9 yeast cells and 50 ng of purified Csk was included in a assay mixture (10 μl of 50 mM Tris-HCl

pH 7.4, 3 mM $MnCl_2$, 0.1 mM Na3VO4, 1 mM [γ-^{32}P]ATP (74 kBq)). Phosphorylation was allowed to proceed for 10 min at 30 °C, phosphoproteins were resolved by 10% SDS-PAGE, and phosphorylation signal was detected by autoradiography.

Lysyl-endopeptidase Treatment of Csk and Isolation of Peptides

Fifty μg of purified Csk were digested by 0.4 μg of lysyl-endopeptidase (Wako) in 1 ml of 10 mM Tris-HCl pH 8.0 at room temperature overnight. The reaction mixture was freeze-dried and redissolved in 200 μl of 0.1% trifluoroacetic acid. This peptide mixture was subjected to HPLC using C18 reverse phase column. The resultant peptides were then subjected to gas-phase sequencer (Applied Biosystems 470-A sequencer).

RT-PCR

One μg of poly(A) RNA extracted from neonatal rat brain was reverse-transcribed in 10 μl of 50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dNTP, 20 units of RNasin (Toyobo), 20 pmole of degenerate oligonucleotide primer and 200 units M-MLV reverse transcriptase (BRL) for 60 min at 37 °C. The reaction mixture was heat-treated at 95 °C for 5 min prior to use for PCR template. PCR mixture (50 μl) contained the cDNA of 5 μl of reverse-transcription reaction mixture, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1 mM DTT, 0.2 mM each of dNTP, 0.01% gelatin, 50 pmole each of upstream and downstream oligonucleotide primers and 2 units of Taq DNA polymerase (BRL) with an overlay of liquid paraffin. PCR was executed as thirty cycles of three incubation steps; denaturing at 92 °C for 20 sec, annealing at 42 °C for 1 min and incubating at 72 °C for 2.5 min. The product was analyzed by non-denaturing 5% PAGE.

DNA Sequencing

The DNA sequence was determined using Sequenase system (Sequenase Version 2.0 7-deaza-dGTP Edition; USB) with double stranded plasmid DNA as template.

RNA Extraction and Northern Blot

Total RNA was extracted from rat tissues by the method described previously (Glisin *et al.* 1974). Crude RNA preparation was applied to oligo(dT)-cellulose column chromatography or affinity purification of oligotex-dT30 (JSR) to select poly(A) RNA fraction. For Northern analysis, poly(A) RNA (1.5 μg in each lane) was applied to denaturing agarose gel containing formaldehyde (Lehrach *et al.*, 1977). After electrophoresis, RNAs were capillary-transferred onto positive charged nylon membrane (Hybond N+; Amersham) and probed by radio-labeled Csk cDNA.

Expression of Csk in Yeast Cells

The open reading frame of Csk cDNA was cloned into yeast expression vector, pHM209, which was derived from pHM153 (Matsuzaki *et al.*, 1990). All p60$^{c\text{-}src}$ mutants and Csk were induced to be expressed by exchange the component of yeast cell culture medium from 2% glucose to 2% galactose. Induction was allowed for 1 day of culture period.

Immunoblot Analysis

Samples were subjected to SDS-PAGE and transferred to nitrocellulose filter. For detection of phosphotyrosine, PY20 (1/300 dilution in 3% BSA in PBS; ICN)(Glenny *et al.*, 1988), for p60$^{c\text{-}src}$, MAb327 (1/1,000 dilution), and for Csk, rabbit anti-Csk antiserum (raised against

bacterially expressed Csk; 1/2,000 dilution) were used as first antibody. Rabbit anti-mouse IgG (1/500 dilution; Zymed) or goat anti-rabbit IgG (1/2,000 dilution; Zymed) were used as the second antibody. Soluble complex of horseradish peroxidase and anti-peroxidase complex of derived from mouse or rabbit (Zymed) was used for visualization with staining in 0.1 M Tris-HCl pH 7.4, 0.25 mg/ml 3,3'-diaminobenzidine, 40 mg/ml nickel(II) ammonium sulfate and 0.012% H_2O_2.

Figures and Legends

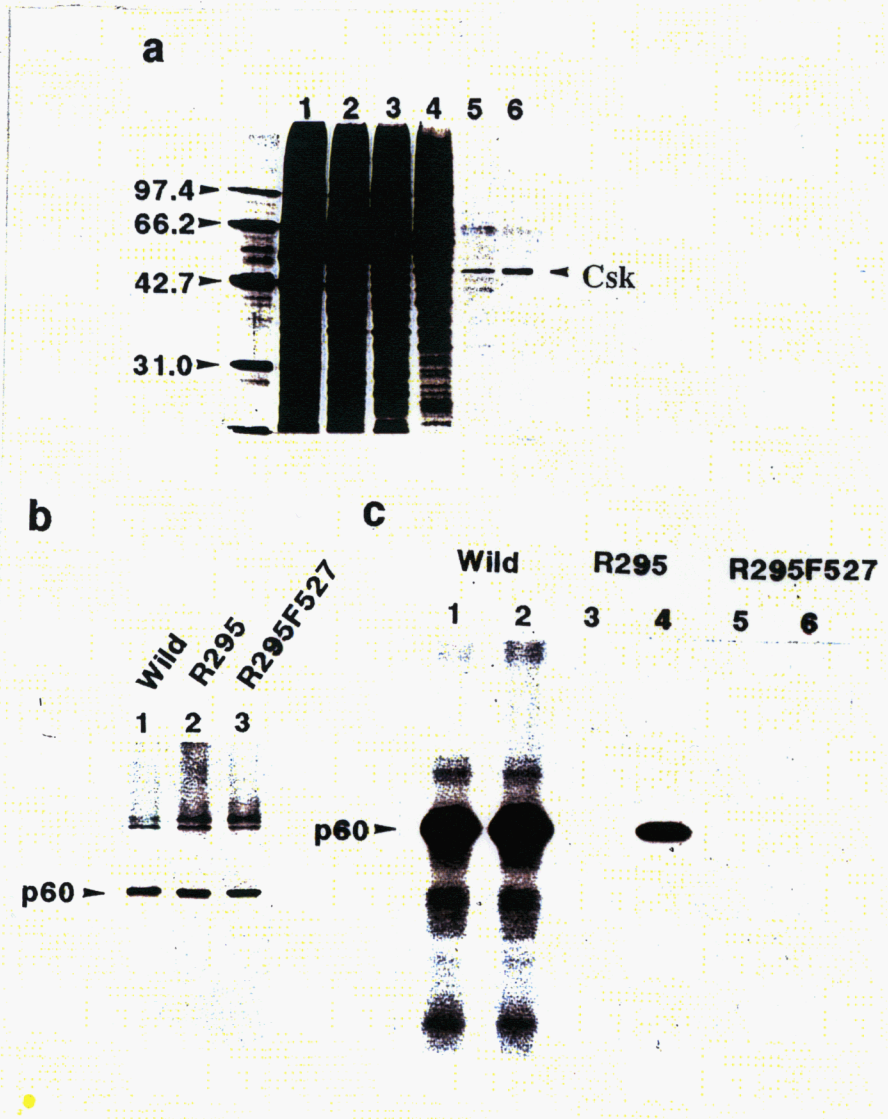

Figure 1. Phosphorylation of mutant p60$^{c\text{-}src}$ by Csk
(a) Sample of each purification step of Csk was analyzed by SDS-PAGE and stained with silver. Lane 1, Nonidet P40 extract; lane 2, DEAE-cellulose column chromatography; lane 3, poly(Glu, Tyr) Sepharose CL4B; lane 4, MonoQ; lane 5, Sephacryl S200HR; lane 6, MonoS. Positions of relative molecular mass (*Mr*) markers (in *Mr* x10^{-3}) are indicated. (b) Expression of wild-type p60$^{c\text{-}src}$ (WT; lane 1) and its mutants, R295 (lane 2) and R295F527 (lane 3) in yeast cells were detected by immunoblotting of the immunoprecipitates. The bands above p60 are immunoglobulin. (c) Phosphorylation of p60$^{c\text{-}src}$ by Csk was determined by incubating immunoprecipitates of WT (lanes 1 and 2), R295 (lanes 3 and 4) and R295F527 (lanes 5 and 6) with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) Csk. The reaction products were analyzed by SDS-PAGE and autoradiography.

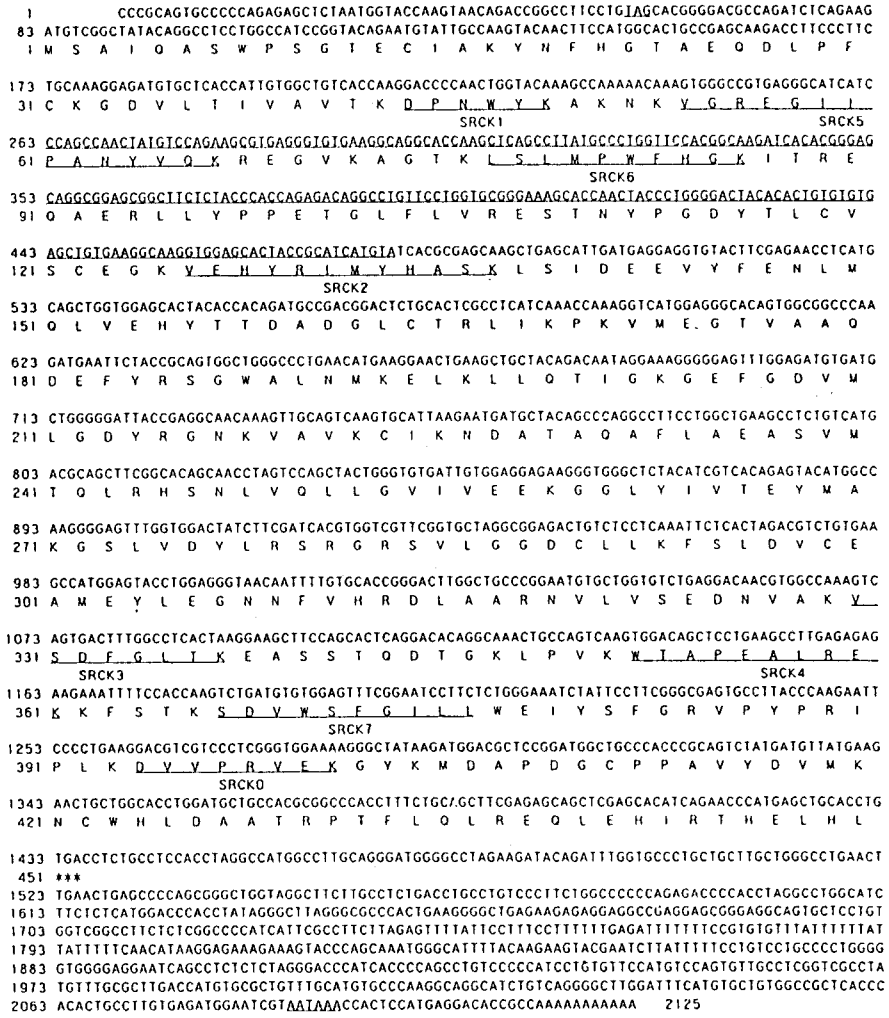

Figure 2. Nucleotide sequence and deduced amino acid sequence of Csk The 2,125-nucleotide cDNA insert contains a single open reading frame that encodes a protein of 450 amino acids. An in-frame stop codon in the 5'-untranslated region and a polyadenylation consensus sequence in the 3'-untranslated region are underlined. Amino acid sequences of eight peptides obtained by lysyl-endopeptidase digestion are indicated by underlines designated as SRCK0-7. A DNA fragment amplified by RT-PCR and used as a probe to screen a cDNA library is indicated by an underline.

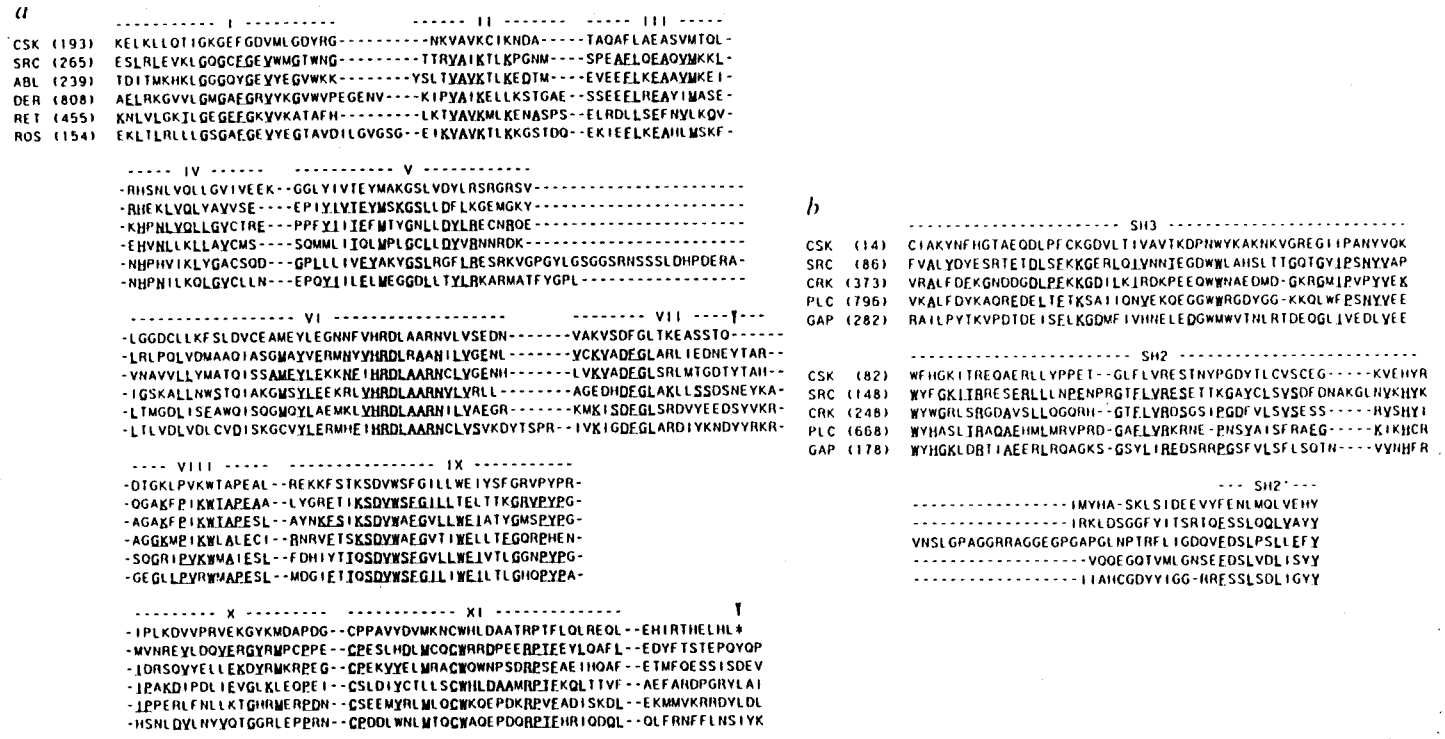

Figure 3. Amino acid sequence comparisons of catalytic domains, SH3 and SH2 regions

(a) Amino acid alignment of the conserved kinase domain (Hanks *et al.*, 1988) in Csk; *c-src* gene product (SRC; a representative of Src family); *c-abl* gene product (ABL; Abl subfamily); *Drosophila* gene product related to the EGF receptor (DER; EGF receptor subfamily); *ret* gene product (RET; PDGF receptor subfamily); and *c-ros* gene product (ROS; insulin receptor subfamily). Underlined bold type indicates residues identical to the kinase domain sequence in Csk. Roman numbers designate subdomains conserved in protein kinases (Hanks *et al.*, 1988). The number in parenthesis is the first amino acid of sequence shown. Sequence identities are 46% (SRC), 46% (ABL), 41% (DER), 39% (RET) and 41% (ROS). Sequence identities to other Src family kinases are 45% (*c-fgr*), 44% (*lyn*), 43% (*c-yes*), 43% (*hck*), 42% (*fyn*) and 42% (*lck*)(Hanks *et al.*, 1988). The percentage identities were determined over the length of the kinase domain of Csk; residues in insertions were ignored for these calculations. Arrows denote the residues corresponding to Tyr416 and Tyr527 of p60$^{c\text{-}src}$. (b) Amino acid alignment of SH3, SH2 and SH2' regions in Csk, *c-src* gene product (SRC)(Takeya and Hanafusa, 1983), *gag-crk* gene product (CRK)(Mayer *et al.*, 1988), phospholipase C-γ (PLC)(Stahl *et al.*, 1988), and GTPase activating protein (GAP)(Vogel *et al.*, 1988). Total sequence identities in these regions are 47% (SRC), 40% (CRK), 30% (PLC) and 27% (GAP). The percentage identities were determined as described above.

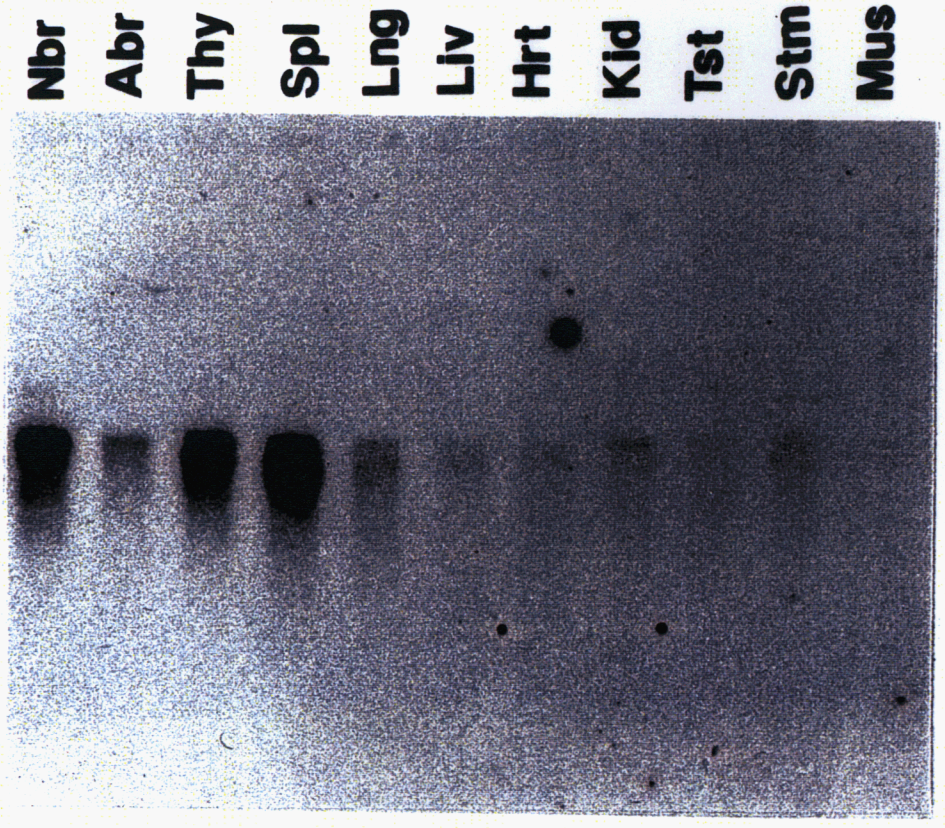

Figure 4. Distribution of Csk mRNA in rat tissues
Northern blot analysis of poly(A) RNA prepared from rat tissues demonstrates the distribution Csk expression. Lanes, NBr, neonatal brain; ABr, adult brain; Thy, thymus; Spl, spleen; Lng, lung; Liv, liver; Hrt, heart; Kid, kidney; Tst, testis; Stm, stomach; Mus, muscle.

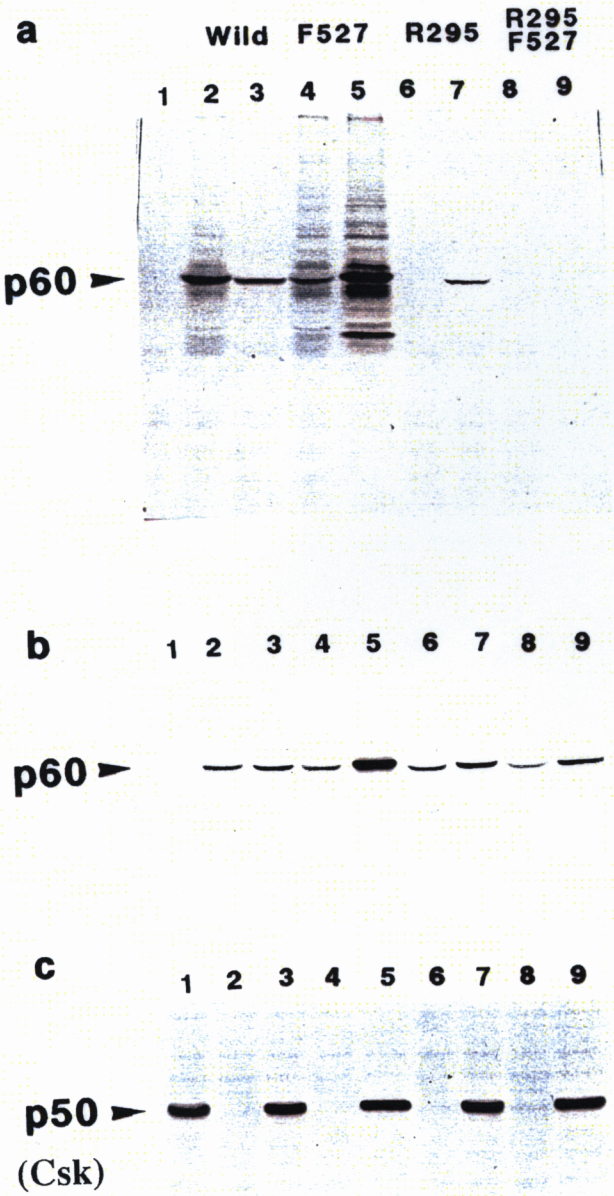

Figure 5. Co-expression of p60$^{c\text{-}src}$ and Csk in yeast cells
(a) Csk expression vector was introduced to yeast carrying p60$^{c\text{-}src}$ or its mutants, and tyrosine phosphorylation of p60$^{c\text{-}src}$s and yeast cellular proteins were estimated by immunoblot analyses using anti-phosphotyrosine antibody. Yeast cell lines used were wild-type p60$^{c\text{-}src}$ (lanes 2 and 3), F527 mutant (lanes 4 and 5), R295 mutant (lanes 6 and 7), and R295F527 mutant expressor (lanes 8 and 9) with (lanes 1, 3, ,5 ,7 and 9) or without (lanes 2, 4, 6 and 8) expressing Csk. The expressions of p60$^{c\text{-}src}$s and Csk were confirmed in panels (b) and (c), respectively, by immunoblotting.

Chapter II

Constitutive Activation of Src Family Protein-Tyrosine Kinases Caused Developmental Defects in Mouse Embryos Lacking Csk

Summary

Csk is a novel cytoplasmic protein-tyrosine kinase that has been shown to inactivate members of the Src family protein-tyrosine kinases *in vitro*. To examine the function of Csk *in vivo*, Csk-deficient mouse embryos were generated by gene targeting in embryonic stem cells. These embryos were developmentally arrested at the 10 to 12 somite stage and exhibited growth retardation and necrosis in the neural tissues. The kinase activity of p60$^{c\text{-}src}$, p59^{fyn} and p53/56^{lyn} in these embryos was greatly enhanced as an apparent consequence of enhanced specific activity. The increase in kinase activity was associated with an increase in tyrosine-phosphorylation of several proteins, especially those at around 85 and 120 kDa. Thus, these results suggest that Csk indeed acts as an indispensable negative regulator of Src family kinases *in vivo*.

Introduction

Increasing evidence has shown that protein-tyrosine kinases play essential roles in those signal transduction pathways which regulate cell proliferation, differentiation and function. The non-receptor protein-tyrosine kinases of the Src family are anchored at the inner surface of the plasma membrane through their myristilated N-terminus and have been proposed to be associated with cell surface receptors; the binding of ligand to receptor causes rapid activation of Src family kinases and activation of intracellular signal transduction pathways (Eiseman and Bolen, 1990). Nine Src family kinases have been now identified and several show tissue specific expression. In particular, the expression of p56^{lck} is confined to cells of the lymphoid lineage and p56^{lck} has been shown to be associated with CD4 and CD8 T cell surface antigens, thereby transducing independent signals during the process of T cell activation (Veillette *et al.*, 1988). The association of other Src family members (i.e. *c-src*, *c-yes*, *fyn*, *lyn* and *blk* gene products) with hematopoietic cell surface receptors has also been reported (Burkhardt *et al.*, 1991; Sugie *et al.*, 1991; Yamanashi *et al.*, 1991; Eiseman and Bolen, 1992; Campbell and Sefton, 1992; Bell *et al.*, 1992). The roles of Src family kinases *in vivo* have been addressed by generating mice lacking a particular member of the Src family. The p56^{lck} is shown to play crucial role in the thymocyte development (Molina *et al.*, 1992), and the role of p59^{fyn} in signal transduction through T cell receptor is demonstrated (Appleby *et al.*, 1992; Stein *et al.*, 1992).

The kinase activity of the Src family members has been reported to be regulated by the phosphorylation and dephosphorylation of a tyrosine residue located close to the C-terminus, corresponding to Tyr527 in chicken *c-src* (reviewed in Cantley *et al.*, 1991). The Tyr527 of p60$^{c\text{-}src}$

is known as the major tyrosine phosphorylation site *in vivo* (Cooper *et al.*, 1986). Dephosphorylation of this site causes a 10 to 20-fold increase in the kinase activity of p60$^{c\text{-}src}$ *in vitro* (Courtneidge *et al.*, 1985; Cooper and King, 1986). The p60$^{v\text{-}src}$, or other mutationally activated variants of p60$^{c\text{-}src}$ that lack Tyr527 are capable of transforming fibroblast cell lines *in vitro* (Parker *et al.*, 1984; Piwnica-Wormset *et al.*, 1987; Kmiecik and Shalloway, 1987; Cartwright *et al.*, 1987; Reynolds *et al.*, 1987). Furthermore, the constitutive reduction of phosphate on Tyr527 of p60$^{c\text{-}src}$ by the overexpression of a PTPase, PTPα, causes the activation of p60$^{c\text{-}src}$ as well as transformation of rat embryonic fibroblasts (Zheng *et al.*, 1992). Thus, it is suggested that transforming potential of p60$^{c\text{-}src}$ is suppressed by the high level of phosphorylation at Tyr527 *in vivo*.

Phosphorylation of Tyr527 has been shown to be catalyzed by a protein-tyrosine kinase other than p60$^{c\text{-}src}$ (Jove *et al.*, 1987; Schuh and Brugge, 1988; Thomas *et al.*, 1992). A novel cytoplasmic protein-tyrosine kinase, Csk, was previously isolated from neonatal rat brain (Okada *et al.*, 1989; Nada *et al.*, 1991). Csk contains SH3 and SH2 regions upstream of the kinase domain and the primary structure has strong similarity to Src family kinases. However, Csk has several unique characteristics; it does not have a myristilation signal, and lacks an autophosphorylation site (Tyr416 in chicken *c-src*) and a negative-regulatory tyrosine residue (equivalent to Tyr527). In *in vitro* experiments, we found that Csk can phosphorylate p60$^{c\text{-}src}$ specifically at Tyr527, and thereby inhibits its kinase activity (Okada *et al.*, 1989; Nada *et al.*, 1991). The pattern of Csk expression corresponded to that of Src family kinases in general; it was ubiquitous, but expressed at the highest levels in thymus, spleen and neonatal brain (Okada *et al.*, 1991), and no homologous gene has been found in the avian genome (Sabe *et al.*, 1992).

These findings raised the possibility that Csk acts as a unique negative-regulator for all Src family kinases *in vivo*.

To examine the function of Csk *in vivo*, mice carrying mutations at the *csk* locus were generated through targeted disruption in embryonic stem (ES) cells. The embryos homozygously carrying a disrupted *csk* gene actually showed enhanced kinase activity of not only p60$^{c\text{-}src}$, but also p59^{fyn} and p53/56^{lyn}. In addition, they showed a developmental defect at the neurula stage. These results demonstrate that Csk is an indispensable negative-regulator of Src family kinases *in vivo*, and plays an important role in normal development of mouse embryo.

Results

Production of Mice with Targeted Disruption at *csk* Allele

To introduce mutations into *csk* gene by homologous recombination in ES cells, two types of targeting vector were constructed (Figure 1). The pCZ-DT vector has *lacZ* and *neo*^{r} (neomycin phosphotransferase) genes inserted into an NcoI site in the exon encoding the kinase domain of Csk; the homologous recombination should yield a fusion protein of 150 kDa in which β-galactosidase was fused to the N-terminal region, SH3 and SH2, of Csk (from 1 to 301 amino acids). This insertion has disrupted the kinase domain, so that the kinase activity of Csk should be completely destroyed. This vector was designed to examine the effect of loss of kinase activity of Csk. In the second vector, pNZ-DT, the DNA fragment from a KpnI site in the 5'-noncoding exon to an NcoI site in the kinase domain was replaced by *lacZ* and *neo*^{r} genes. Since the translational initiation codon was located in the deleted DNA fragment, homologous recombination with this targeting vector should generate a null mutation of *csk* gene. The *lacZ* reporter gene was introduced into each vector in order that expression of *csk* gene could be monitored with β-galactosidase staining. In the targeting vectors, the length of the homologous region was either 6 kb (pCZ-DT) or 10 kb (pNZ-DT) on 5' side of the *lacZ-neo*^{r} insert and 0.6 kb on 3' side. To increase the frequency of homologous recombination, AT-rich mRNA-destabilizing sequence (Shaw and Kamen, 1986), "pause" signal (Resnekov and Aloni, 1989) and DT-A (diphtheria toxin A fragment) gene (Yagi *et al.*, 1990) were utilized in negative selection (Yagi *et al.*, 1933b).

The targeting vectors were introduced by electroporation into male TT2 ES cells (XY) that was established from a C57Bl/6 by CBA embryo (Yagi *et al.*, 1993d). The frequency of homologous recombinants was 11

out of 120 G418 resistant clones for pCZ-DT vector and 2 out of 35 using the pNZ-DT vector determined by polymerase chain reaction (PCR) analysis (data not shown). Southern blot analysis confirmed the presence of the targeted allele in these homologous recombinants.

Five of 11 homologous recombinant clones obtained with pCZ-DT and two with pNZ-DT were screened for multipotency by injecting the cells into about fifty ICR 8-cell embryos. Screening was done by determining whether the mutant ES clones yielded offspring with ES-derived agouti coat color. The 100% chimeras were yielded from three ES clones obtained with pCZ-DT and one with pNZ-DT; the ES clones were CZ-M, CZ-S, CZ-V and NZ-2. All of the 100% chimeras from CZ-M, CZ-S and CZ-V were male, and those from NZ-2 were female; the loss of Y chromosome was confirmed in this ES clone. The 100% chimeras were test-mated with albino Balb/C females or males and confirmed to yield ES-derived agouti offspring exclusively. These mice were crossed with C57Bl/6J females or males to obtain heterozygous mice through germline transmission (Figure 1C). Immunoblot analysis of spleen of heterozygous offspring showed that the level of Csk protein was indeed decreased to nearly half that found in control mouse. In addition, the expression of $p150^{cz}$, the fusion protein derived from csk^{cz}, or β-galactosidase from csk^{nz} was detected (Figure 1D). Unexpectedly, the amounts of these proteins were very low even in spleen, where high Csk expression is normally seen; and no tissue was stained for β-galactosidase activity in heterozygous mice. Though the reason for this is not clear at present, it is possible that some sequences regulating Csk expression were disrupted during the targeting.

Death of Homozygous Mutant Embryos at Neurula Stage

The following experiments were performed in mutant mice derived from the CZ-S clone, and similar results were obtained in other csk^{cz} or csk^{nz} mutants. The CZ-S heterozygous mice were intercrossed, and pups were born at the 1:2 ratio of wild-type to heterozygous, but no homozygous mutant pups were obtained from any litter (Table 1). When fetuses were surveyed for the presence of homozygous mutants, none were found among embryos 11.5-13.5 days post coitum. The presence of absorbed embryos was apparent in these stages, however, and some of those genotype by PCR analysis proved to be homozygous mutants (data not shown). When examined at embryonic (E)10.5 days, about 22% of the embryos were very small, and "turning" of these embryos had not occurred (Figure 2); in normal embryos the lordotic curvature of the trunk turns into a strong dorsal, kyphotic bend at E8.5-9.0 days (Rugh, 1990; Theiler, 1989). PCR analysis identified these retarded embryos as homozygous mutants. At E9.5 days, homozygous mutant embryos were also small and were not turned. At E8.5 days, homozygous mutant embryos were grossly normal, though somewhat smaller; at this stage normal embryos have a dorsal flexure and are not yet turned. The embryonic lethal phenotype was not peculiar to the mutants derived from CZ-S, but was also seen in the mutants derived from CZ-M and CZ-V. Thus it was not caused by a mutation indifferent to *csk* gene which had accidentally occurred during the isolation of the ES clones. Nor was there any difference in the phenotype of mutant embryos derived from NZ-2, indicating that the expression of Csk SH2 and SH3 in fusion with β-galactosidase had no effect. The question of whether the phenotype might be caused by the expression of β-galactosidase either alone or in a fusion rather than by the *csk* mutation per se was not ruled out by the present studies. However, this is less likely given the low level of β-galactosidase expression, as noted above. The absence of Csk protein in these

homozygous mutant embryos was confirmed by immunoblot analysis as described below (Figure 5B).

A preliminary examination of the phenotypic abnormalities in Csk mutant mice was performed using embryos derived from the intercross of heterozygous mice. By E10.5 days the homozygous mutant embryos were systemically necrotic and were no longer alive. Growth retardation was already apparent in the neural tube of the E8.0th day embryos. The closure of the neural tube had not yet occured at the level of the 4th and 5th somites, although the morphology and the size of the neural tube was similar to those of control littermates (Figure 3A). At E9.5 days the embryos were still alive, but the number of somites remained at the 10 to 12 stage. The neural tube was being closed, but the closure remained at the cervico-cranial boundary; the anterior and posterior neuropores remained open, though normally they close at E9 days, by the 19 somite stage. At this stage of normal embryogenesis, the entire neural tissue undergoes differentiation, and there are many mitosis in the ependymal layer of the neural tube. In contrast, in homozygous mutant embryos, the trunk region of the neural tube showed an irregular shape, reduced thickness of the neuroepithelium, great number of necrotic cells and reduction in the number of mitotic figures (Figure 3C); cell necrosis in the neural tube had already started at E8.5 days (Figure 3B). In the cranial region, necrosis was not evident in the neuroepithelium but a reduced thickness and a reduction in the number of mitotic figures was observed (Figure 3D). The formation of the cranial ganglia derived from neural crest cells was greatly impaired, and most of the ganglion cells exhibited necrosis.

Similarly, sense organ primordia remained at the 10 somite stage in E9.5th day homozygous mutant embryos; lens placode was not formed, olfactory placode was not apparent, and otic vesicles had not closed over.

Blood islands and capillaries were numerous in the yolk sac of normal E9th day embryos, but were not in that of homozygous mutant E9.5th day embryos. However, some tissues apparently developed beyond the 12 somite stage in the E9.5th day homozygous mutant embryos. In particular, the development of the placenta was indistinguishable from that of normal E9.5th day embryos. The hearts of homozygous mutant embryos were almost four-chambered and beat occasionally. The original wide opening of the gut into the yolk sac was narrowed, and the hind gut was formed (Figure 3C). The liver primordium was formed, and muscle and epithelium appeared to be normal.

Expression of Csk and p60$^{c\text{-}src}$ at Neurula Stage

In normal embryos examined by immunoblot analysis, the expression of Csk was already apparent at E8.5 days and increased over E10.5 days (compare +/+ lanes of Figure 5B). The expression of p60$^{c\text{-}src}$ was low at E8.5 days and increased more dramatically over E10.5 days (Figure 5C), and the kinase activity of total p60$^{c\text{-}src}$ increased concomitantly (Figure 5D). Thus, the expression of p60$^{c\text{-}src}$ coincided with the stages when embryonic development was retarded in the homozygous mutant embryos.

At first I intended to examine cell types expressing Csk based on the β-galactosidase activity in heterozygous mutants. As noted above, however, no tissue, including neonatal brain which ordinarily express high level of Csk, could be stained with the chromogenic substrate X-Gal. The spatial distribution of *csk* mRNA was subsequently examined by whole mount *in situ* hybridization to the E8.5th day embryos with digoxigenin-labeled cRNA probes (Figure 4). A low level of expression was seen throughout the entire body, but there was strong expression in the neuroepithelium, especially of the prospective head region and in

cranial ganglia. These features were also apparent in *in situ* hybridization on the tissue sections. The *csk* expression in the E9.5 embryos was ubiquitous and high in the actively dividing neuroepithelial layer of the closed neural tube and in neural crest cells.

Activation of Src Family Kinases in Homozygous Mutant Embryos

Based on experiments testing the specificity of Csk *in vitro*, we have previously postulated that Csk specifically phosphorylates negative-regulatory tyrosine residue of Src family members and thereby inhibits their kinase activities (Okada *et al.*, 1991), but it has remained for the present study to be confirmed whether Csk indeed plays this role *in vivo*. To address this question, the kinase activity of p60$^{c\text{-}src}$ in the E8.5th to E10.5th day embryos of +/+, +/*csk*^{cz} and *csk*^{cz} /*csk*^{cz} genotype was measured. In the homozygous mutant embryos Csk was actually absent (Figure 5B). In the normal embryo, the kinase activity of p60$^{c\text{-}src}$ to the exogenous substrate enolase increased rapidly with the progression through embryonic stages (compare +/+ lanes in Figure 5D) and this increase was accompanied by an increase in the amount of p60$^{c\text{-}src}$ (+/+ lanes in Figure 5C). Thus the specific activity of p60$^{c\text{-}src}$ did not apparently change in these stages. In the homozygous mutant embryos, the activity was much higher than that of wild-type or heterozygous embryos in all these stages (Figure 5D), although the amount of p60$^{c\text{-}src}$ in homozygous mutant embryos was markedly less than that of wild-type or heterozygous embryos at each stage (Figure 5C). This suggests that the increase in p60$^{c\text{-}src}$ kinase activity in the homozygous mutant embryos was due to the elevation of its specific activity.

The increase in apparent specific activity was further assessed on the E9.5 day for p60$^{c\text{-}src}$, p59*fyn* and p53/56*lyn*. The expression of

these Src family members in the whole embryo extracts was also estimated by immunoblot analysis. As was the case for p60$^{c\text{-}src}$, the protein amounts of p59*fyn* and p53/56*lyn* in homozygous mutant embryos were less than those of wild-type or heterozygous embryos; they were similar to those of the E8.5th day wild-type or heterozygous embryos (data not shown). The kinases were immunoprecipitated from the extracts containing equivalent amounts of each protein, and then subjected to enolase phosphorylation assay. Actually the increase in the relative incorporation of ^{32}P to enolase by p60$^{c\text{-}src}$, p59*fyn* and p53/56*lyn* was about 14.7, 3.9 and 8.5 fold, respectively, for wild-type embryo (Figure 6).

An increase in intrinsic kinase activity of Src family kinases in homozygous mutant embryos is indicative of an increase in those phosphorylated proteins which may be substrates of these kinases at the neurula stage. The E8.5th to E10.5th day embryos were subjected to immunoblot analyses with anti-phosphotyrosine antibody to detect these proteins. In the homozygous mutant embryos, several novel bands appeared when compared with wild-type or heterozygous embryos (Figure 5A). The increase in the phosphorylation of several proteins at around 85 and 120 kDa was especially notable through the E8.5 to E10.5 day.

Discussion

Csk-deficient mice were produced to examine whether Csk indeed acts as a unique negative regulator for Src family kinases *in vivo*. In accordance with this hypothesis the kinase activity of p60$^{c\text{-}src}$, p59^{fyn} and p53/56^{lyn} was indeed elevated in the Csk-deficient embryos. The enolase-phosphorylation activity of p60$^{c\text{-}src}$ immunoprecipitated from Csk-deficient embryos was about 14.7 times that from normal embryos. It is reported that removal of about 80% of the phosphorous group from Tyr527 of p60$^{c\text{-}src}$ by acid phosphatase treatment increases 10 to 20 fold the kinase activity to enolase (Cooper and King, 1986). Mutational replacement of Tyr527 by phenylalanine also results in 13-fold increase in specific activity (Kmiecik *et al.*, 1987). Thus the result obtained in this study is consistent with the possibility that the elevation of the kinase activity of p60$^{c\text{-}src}$ in Csk-deficient embryos is due to substantial reduction of phosphate on Tyr527. Since the Tyr527 can be somewhat autophosphorylated by p60$^{c\text{-}src}$ itself (Jove *et al.*, 1987; Cooper and MacAuley, 1988) and the possibility that as-yet unidentified another kinase is also involved in the phosphorylation cannot be excluded in this study, it is likely that the Tyr527 is still phosphorylated to some extent in Csk-deficient embryos. As mentioned above, however, the increase in the kinase activity of p60$^{c\text{-}src}$ in Csk-deficient embryo was almost comparable to the maximal increase achieved in the *in vitro* experiments. Therefore it is suggested that Csk plays an essential role in the negative regulation of p60$^{c\text{-}src}$ *in vivo*.

An elevation of the kinase activities of p59^{fyn} and p53/56^{lyn} was also observed by 3.9 and 8.5 fold, respectively. Besides these Src family members, Csk can phosphorylates p56^{lck} and p62$^{c\text{-}yes}$ at their C-terminal negative regulatory tyrosines *in vitro* (Okada *et al.*, 1991;

Bergman *et al.*, 1992). Csk also showed high selectivity for conserved amino acid sequences of C-terminal region of Src family kinases (MacAuley *et al.*, 1993). Furthermore, the pattern of Csk expression corresponds to that of Src family kinases in general (Okada *et al.*, 1991), and no homologous gene has been found in the vertebrate genome (Sabe *et al.*, 1992). Taken these findings together with the lack of complementation of Csk-deficiency by other tyrosine kinases, it is strongly suggested that Csk may be a unique and universal negative-regulator for all Src family kinases *in vivo*.

An increase in the phosphorylation of proteins at around 85 and 120 kDa was observed in Csk-deficient embryos. Similarly sized phosphoproteins have previously been reported in several cell lines expressing p60$^{v\text{-}src}$ and may represent possible substrates of p60$^{c\text{-}src}$ (Kanner *et al.*, 1989, 1990; Linder and Burr, 1988; Hamaguchi *et al.*, 1988; Reynolds *et al.*, 1989). Some of these proteins have recently been identified; p80/85 is a cytoskeleton-associated protein which has tandemly repeated sequences, a helical region, a proline rich region and an SH3 region (Wu *et al.*, 1991). p125^{FAK} is a protein-tyrosine kinase which localizes to focal adhesions and is suggested to be a cytoplasmic mediator of cell attachment through integrin (Schaller *et al.*, 1992). p120 has β-catenin-related sequences, and its phosphorylation is suggested to be directly correlated with morphological transformation of fibroblast cells (Linder and Burr, 1988; Reynolds *et al.*, 1989, 1992). To test the possibility that these proteins can be substrate of activated p60$^{c\text{-}src}$ *in vivo*, these three proteins were immunoprecipitated with the individual antibodies form Csk-deficient embryo, and their tyrosine phosphorylation was determined. Only p80/85 was revealed to be phosphorylated, while the other two proteins were not (data not shown). However, the physiological meanings of tyrosine phosphorylation of p80/85, its

correlation with the phenotype of Csk-deficiency, and identification of other phosphorylated proteins must await future studies.

The Csk deficiency reported herein caused characteristic defect in the development of the neural tissues and the defect occurred at a stage when the expression of p60$^{c\text{-}src}$ increased. The development of the placenta was apparently normal and the tissues of the ectoderm and mesoderm were relatively unaffected. Although no detailed expression of Src family kinases has previously been reported at the neurula stage in mammals, the mice in which *fyn* gene has been replaced by the *lacZ* reporter gene (Yagi *et al.*, 1993a, 1993c) were generated. In these animals, the expression of *fyn* at the neurula stage could be easily monitored by β-galactosidase activity. p59*fyn* was expressed in a marginal zone of the closed neural tube, composed of developing neural fibers, but it was not expressed in dividing neuroepithelial cells in the ependymal zone. It was also expressed in neural fibers of dorsal root ganglia, but not in migrating neural crest cells. The expression was not detected in other tissues at these stages. A similar pattern of expression of *c-src* has been reported during neurulation in chicken (Maness *et al.*, 1986; Fults *et al.*, 1985). In contrast, *csk* RNA was detected in dividing neuroepithelial cells. The simplest explanation for the developmental defect observed in Csk-deficient embryos is that this is a direct consequence of the enhanced activity of Src family kinases in dividing ependymal cells. However, the proliferation of neuroectoderm is dependent upon a series of complex tissue interactions, and the identification of the primary sites that trigger developmental arrest at the 10 to 12 somite stages and inhibit the embryos' ability to turn remain to be determined. In addition, the present work cannot rule out the possibility that there are other as-yet unknown substrates for Csk that may play essential roles at this stage.

Finally, it is intriguing to note that Csk may function as an anti-oncogene, through its negative regulation of Src family kinases. Fibroblast-like cells cultured from E9th day Csk-deficient embryos showed no enhanced proliferation, and the activation of Src family kinases which observed in this study was not sufficient to immortalize these cells. Such cells showed no morphological alterations *in vitro*, failed to form colonies in soft agar and were not tumorigenic when injected into nude mice. Furthermore, mice heterozygous for the *csk* mutation have not develop spontaneous turmors over an observation period of eighteen months. Nevertheless, it will be worthwhile to examine whether tumor develops in chimeras between Csk-deficient and normal embryos.

Experimental Procedures

Targeting Vector

The mouse *csk* gene was cloned from a C57Bl/65 mouse genomic library (Okano *et al.*, 1991) by standard colony hybridization procedure using rat Csk cDNA (Nada *et al.*, 1991) as a probe. The *LacZ-neo*^{r} cassette consisted of *TrpS-LacZ* (Maekawa *et al.*, 1991), *hprt* polyA, SV40 early gene polyA and PGKneo, in which *neo*^{r} gene was driven under the *pgk-1* promoter (Boer *et al.*, 1990). The DT-A cassette for negative selection consisted of AT-rich sequence (Shaw and Kamen, 1986), "pause" signal (Resnekov and Aloni, 1989), Bluescript DNA and DT-A gene with MC1 promoter (Yagi *et al.*, 1990); the *neo*^{r} and DT-A genes lacked polyadenylation signal, expecting the acquisition of polyadenylation signal for their expression. The MVM "pause" signal and Bluescript DNA were inserted in order to prevent the interference of downstream DT-A expression by the upstream promoter of the *neo*^{r} gene in case of random integration (Resnekov and Aloni, 1989). The mRNA destabilizing signal was also inserted to instabilize "read through" transcription from *neo*^{r} gene in case of random integration (Shaw and Kamen, 1986). The details of the construction of these cassettes were described previously (Yagi *et al.*, 1993b).

ES Cell Culture and Chimera Production

TT2 ES cells were established from a F1 blastocyst between C57Bl/6 and CBA mice (Yagi *et al.*, 1993d) and cultured in high glucose Dulbecco's modified Eagle medium (BRL) supplemented with 20% fetal bovine serum (Flow Laboratories), 1,000 units/ml leukemia inhibitory factor (AMRAD), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 1x nonessential amino acids (Flow Laboratories) on mitomycin C (Sigma)-

treated primary fibroblasts raised from the E14th day mouse embryos carrying a *neo*^{r} gene. 10^7 cells were suspended in 500 µl HBS (25 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM glucose) containing 12 nM linearized targeting vector, and electroporated with a Gene Pulser (BioRad) at an electric pulse of 250 V and 960 µF. On the 2nd day after electroporation selection was started in medium containing 150 µg/ml G418 (Sigma). Colonies were picked after one week of selection and trypsinized; two thirds of the cell suspension were plated on new feeder cells and the rest was subjected to PCR analysis to detect homologous recombinants. Chimeras were produced by injecting about ten ES cells into a single 8-cell stage ICR embryo and transplanting the embryos into the uterus of pseudopregnant females (Tokunaga and Tsunoda, 1992).

PCR Analysis

Three primers were used to determine the genotype. The primer, prC1, was derived from the 3' region of the *csk* gene not included in the targeting vector (5'-AGCAGGGCACCAGATCTGTAATCTCCGAGG-3'). The primer, prC0, was derived from the sequences upstream from the NcoI site in the *csk* gene (5'-CGCAGGGGAGTTTGGTGGACTATCTTCGAT-3'). The primer, prN, was derived from sequences in *neo*^{r} gene (5'-TCGTGCTTTACGGTATCGCCGCTCCCGATT-3'). The PCR analysis was performed using these three primers together, and 900 bp and 800 bp products were detected in homologous recombinants or heterozygotes; the former was amplified from the wild-type *csk* allele between prC0 and prC1, and the latter from the targeted allele between prN and prC1. Samples were processed as follows: Cell pellets, tissue fragments or small pieces of tails were boiled in 50 to 100 µl water for 10 min, digested with

50 μg/ml proteinase K at 55 °C for 120 min and boiled again. Ten to 20 μl of these samples were included in a reaction mixture of 50 μl (0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.1 μM prC0 and prN, 0.2 μM prC1, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100 and 2.5 units Taq DNA polymerase (Promega)). The PCR was performed at 30 cycles of reaction at 94 °C for 45 sec, at 60 °C for 25 sec and at 72 °C for 3 min. The products were analyzed by 2% agarose or 5% polyacrylamide gel electrophoresis.

Histological Analysis

Embryos were fixed overnight with Bouin's solution, dehydrated, and embedded in paraffin. They were sectioned to 5 μm thickness and stained with hematoxylin-eosin.

Whole Mount *In Situ* Hybridization

The E8.5th day embryos were prefixed overnight in phosphate buffered saline (PBS) containing 4% paraformaldehyde at 4 °C. They were treated twice with methanol for 5 min and twice with PBS containing 0.1% Tween 20 (PBST) for 5 min, with 50 μg/ml proteinase K for 5 min at room temperature, and postfixed in PBS containing 4% paraformaldehyde for 20 min. The embryos were prehybridized at room temperature for 2 h in 5x SSC containing 0.1% Tween 20, 50 μg/ml heparin, 100 μg/ml sonicated and boiled salmon sperm DNA, 10 μg/ml yeast tRNA and 50% deionized formamide and hybridized with a digoxigenine (DIG)-labeled RNA probe (100 ng/ml) at 55 °C for 12 h . They were then washed with 5x SSC briefly, with 2x SSC containing 50% formamide at 65 °C for 1 h and three times with 2x SSC at 37 °C for 10 min, and treated with 20 μg/ml RNase A at 37 °C for 1 h. Embryos were further washed three times with 2x SSC at 37 °C for 10 min, with 2x SSC

containing 50% formamide at 65 °C for 1 h, with 2x SSC at 55 °C for 15 min and with 0.2x SSC at 55 °C for 15 min, and rinsed with PBST. Embryos were then processed for immunological detection of DIG-probe. After 1 h incubation with blocking solution (0.2% Tween 20, 0.2% Triton X-100 and 2% fetal calf serum in PBS), embryos were incubated overnight at 4 °C with alkaline phosphatase (AP)-conjugated Fab fragment of anti-DIG polyclonal antibody (1:8,000 dilution in blocking solution; Boehringer). They were washed three times with blocking solution for 30 min and three times with AP-reaction buffer (100 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20 and 1 mM Levamizole) for 10 min, and then stained in AP-reaction buffer containing NBT and X-phosphate (DIG-probe detection kit; Boehringer) for several hours. The staining reaction was stopped by extensive washing of embryos with AP-reaction buffer. DIG-labeled sense and antisense RNA probes were synthesized according to the manufacturer's instruction (DIG RNA labeling kit; Boehringer). The template was a linearized plasmid (Bluescript) containing rat *csk* cDNA fragment.

Immunoblot Analysis

Mouse tissues or whole embryos were homogenized in 0.25 M sucrose containing 2 mM EDTA, 10 mM Tris-HCl pH 7.5 and 100 units/ml aprotinine. The homogenates (20 μg protein/lane in the analysis of Figure 1 and 10 μg protein/lane in the analysis of Figure 5) were subjected to 8% gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane. The membrane was probed with anti-phosphotyrosine antibody (PY20; ICN), anti-Csk antiserum (Nada *et al.*, 1991), MAb327 (Lipsich *et al.*, 1983; purchased from Oncogene Science)

or affinity purified anti-β-galactosidase polyclonal antibody (5Prime->3Prime Inc.), and further probed with a peroxidase-conjugated second antibody. Immunoreactive proteins were visualized with an Enhanced Chemiluminescence (ECL) detection system (Amersham) on X-ray film.

Immunoprecipitation and Kinase Assay

For immunoprecipitation, proteins of whole embryos were solubilized in TNE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM Na_3VO_4, 50 μM Na_2MoO_4, 1% Nonidet P-40 and 100 units/ml aprotinine). One μg of appropriate antibody was added to each protein sample (in Figure 5D, the amount of protein used in an assay was 10 μg, while in Figure 6, it was 5 to 10 μg) in 100 μl TNE buffer; for immunoprecipitation of p60*c-src* MAb327, for p59*fyn* rabbit polyclonal antibody fyn2 recognizing N-terminal region of human *fyn* gene product (MBL) and for p53/56*lyn* monoclonal antibody Lyn-8 recognizing N-terminal region of human *lyn* gene product (Okada *et al.*, 1991) were used. After adsorbing to protein-G Sepharose (Pharmacia), immunoprecipitates were washed five times with TNE buffer and twice with kinase assay buffer (50 mM Tris-HCl pH 7.4, 3 mM $MnCl_2$ and 0.1 mM Na_3VO_4). In a kinase assay, immunoprecipitate gained from 5 to 10 μg of total proteins, 2.5 μg acid-treated enolase (Cooper *et al.*, 1984) and 4 nmol/0.74 MBq of [γ-^{32}P]ATP were included in 25 μl kinase assay buffer. Phosphorylation was allowed to proceed at 25 ^{o}C for 20 min, and phosphoproteins were resolved by 10% gel SDS-PAGE followed by autoradiography or image analysis by BAS2000 system (Fuji Film). In this assay condition, phosphorylation against enolase was linear for up to 30 min (data not shown).

Figures and Legends

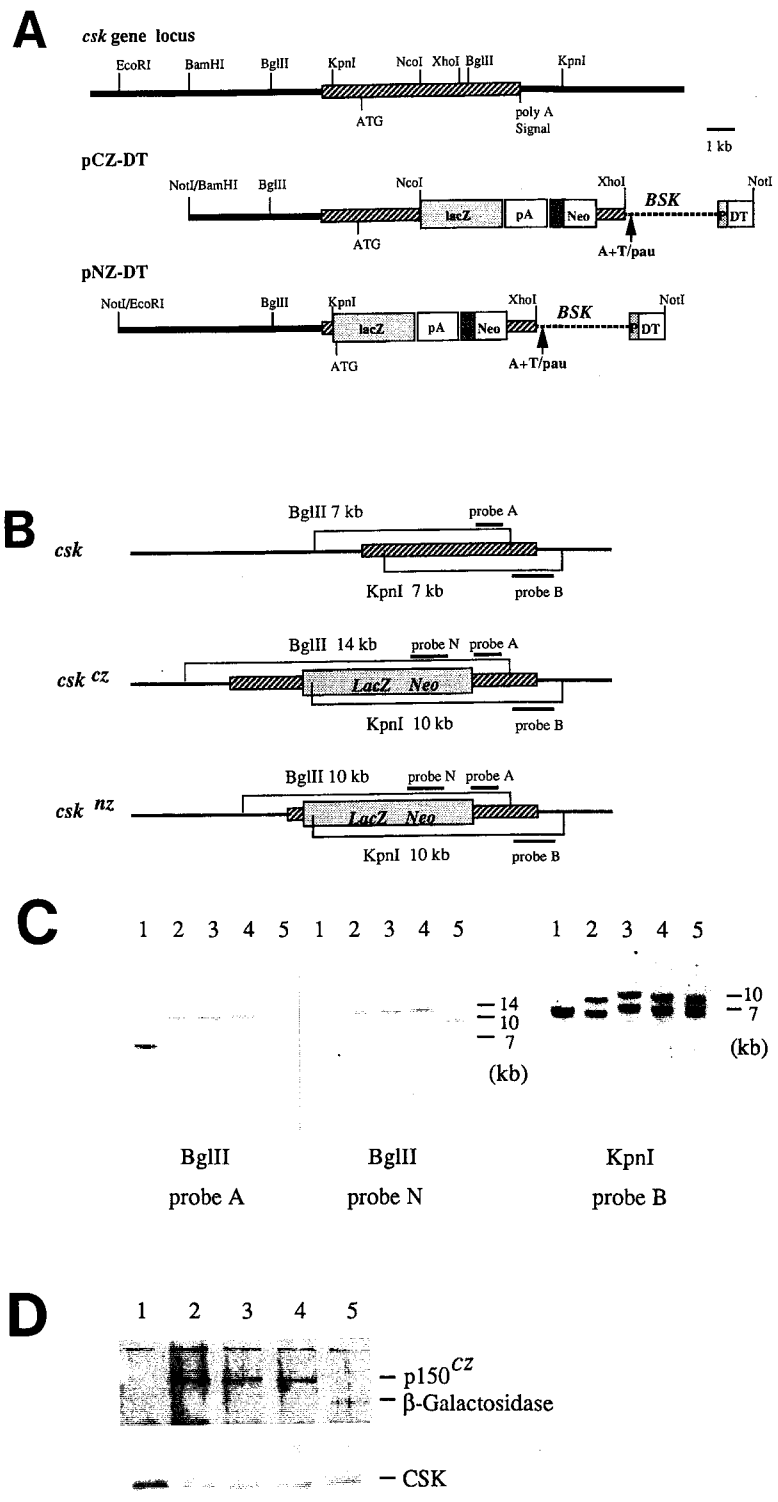

Figure 1. Schematic Representation of the Mouse *csk* Gene and Its Disruption by Homologous Recombination

(A) Structure of the *csk* locus and targeting vectors. The coding region is indicated by the hatched box. This region was identified by hybridization with full-length *csk* cDNA cloned from rat brain (Nada et al., 1991), but the exon–intron structure has not been determined; the NcoI site is present in the exon encoding a part of the kinase domain. A targeting vector, pCZ-DT, was constructed by inserting the *lacZ–neo*r cassette into this NcoI site to disrupt the *csk* gene. A 6 kb fragment encompassing the BamHI to NcoI sites was used as the 5′ homologous region, and a 0.6 kb fragment from the NcoI to XhoI sites was used as the 3′ homologous region. At the 3′ terminal XhoI site of the homologous region, a DT-A cassette was placed, which was composed of the mRNA-destabilizing sequence (A+T) of the granulocyte macrophage colony-stimulating factor gene (Shaw and Kamen, 1986), a pause signal (pau) of MVM (minute virus of mouse) (Resnekov and Aloni, 1989), Bluescript DNA, and MC1DT-A (the DT-A gene with the MC1 promoter) (Yagi et al., 1990); this was done to negatively select out random integrants. In the targeting vector pNZ-DT, the *lacZ–neo*r cassette was replaced with the KpnI–NcoI fragment of the *csk* gene, and a 10 kb fragment from the EcoRI to KpnI sites was used as the 5′ homologous region; similar manipulations were made as described for pCZ-DT.

(B) Structure of *csk*, *csk*^{cz}, and *csk*^{nz} alleles. For Southern blot analysis, probes A and B, specific to the *csk* gene, and probe N, specific to the *neo*r gene, as well as restriction enzymes used and the size of fragments expected, are shown.

(C) Genomic Southern blot analysis on four lines of heterozygous mice. Genomic DNAs were extracted from liver and subjected to Southern analysis. Lane 1, wild type; lane 2, +/*csk*^{cz} mouse derived from CZ-M; lane 3, +/*csk*^{cz} mouse from CZ-S; lane 4, +/*csk*^{cz} mouse from CZ-V; lane 5, +/*csk*^{cn} mouse from NZ-2.

(D) Immunoblot analysis of four lines of heterozygous mice. Homogenates were prepared from spleen, and 20 μg of total protein was applied to SDS–polyacrylamide gels, followed by electrotransfer to nitrocellulose membrane and probing with anti-β-galactosidase (upper) or anti-Csk (lower) antibody. After another probing with horseradish peroxidase–conjugated second antibody, positive signals were detected by the enhanced chemiluminescence detection system (Amersham).

Table 1 Genotype analysis of conceptuses obtained by intercrosses of +/*csk*^{cz} mice

Stage	Total	+/+	+/*csk*^{cz}	*csk*^{cz}/*csk*^{cz}	absorbed*
P3W	93	31 (33%)	62 (67%)	0 (0%)	-
P2	8	3 (38)	5 (63)	0 (0)	-
E13.5	17	5 (29)	9 (53)	0 (0)	3 (18%)
E12.5	18	2 (11)	8 (44)	0 (0)	8 (44)
E11.5	23	6 (26)	9 (39)	0 (0)	8 (35)
E10.5	60	14 (23)	26 (43)	13 (22)	7 (12)
E9.5	65	11 (17)	34 (52)	14 (22)	6 (9)
E8.5	47	8 (17)	28 (60)	10 (21)	1 (2)

*"absorbed" refers to the number of embryos being absorbed. The +/*csk*^{cz} mice of the CZ-S line were intercrossed and conceptuses were genotyped at various developmental stages by PCR. P3W, 3 week postnatal; P2, 2 day postnatal; E8.5 to 13.5, embryonic day 8.5 to 13.5.

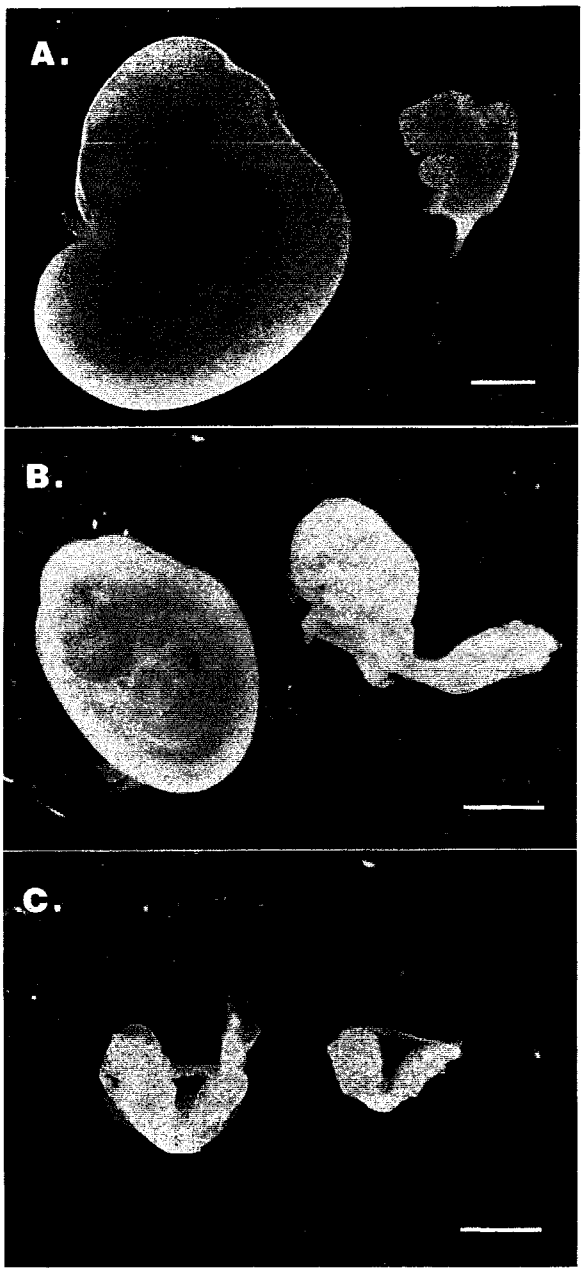

Figure 2. External Forms of *csk*^{cz} /*csk*^{cz} Embryos
Embryos were recovered from pregnant +/*csk*^{cz} females mated with +/*csk*^{cz} males and genotyped by PCR with yolk sac. Left, wild-type embryo; right, *csk*^{cz} /*csk*^{cz} embryo. (A), E10.5th day; (B), E9.5th day; (C), E8.5th day. White bar in each panel represents 1 mm.

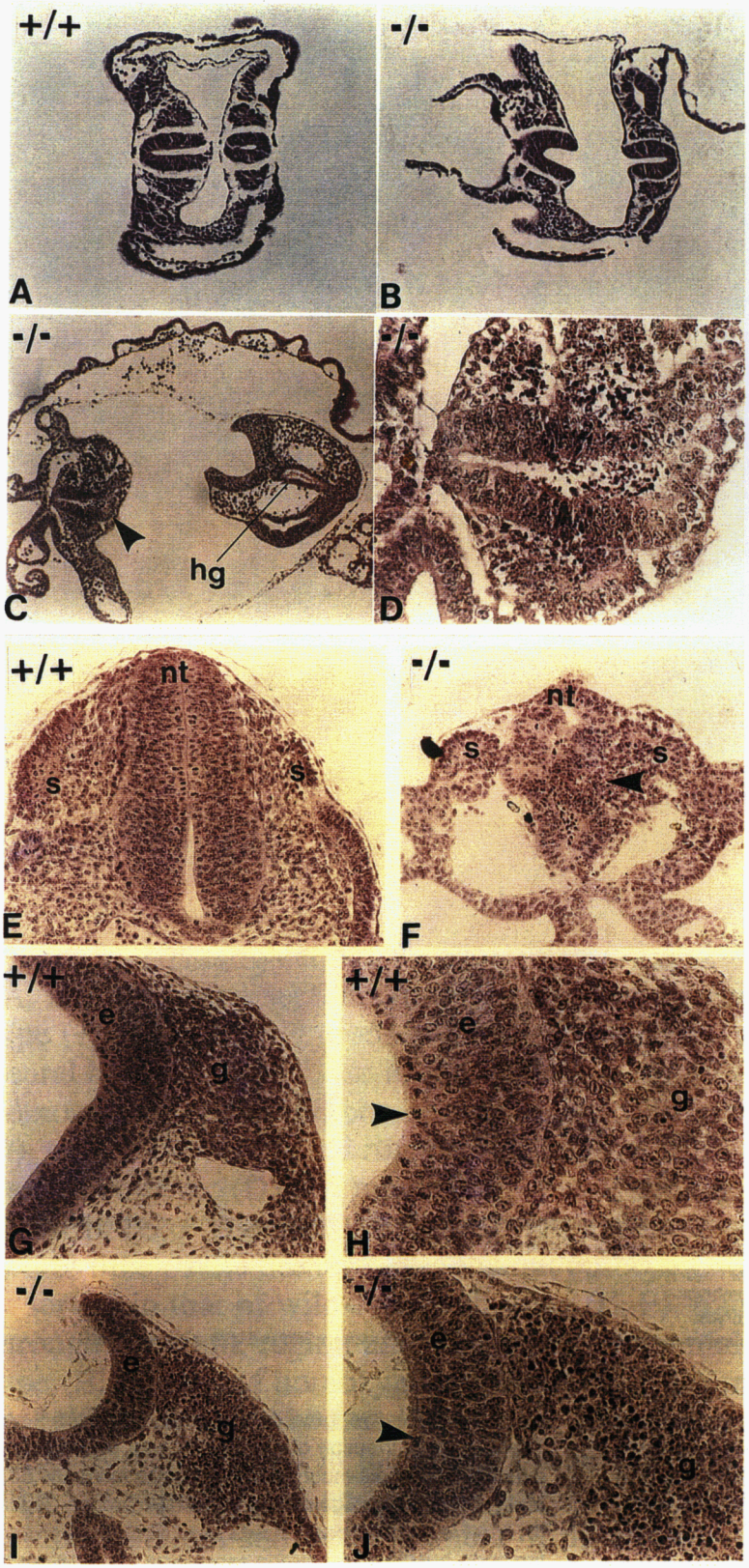

Figure 3. Histological Appearances of *csk*^{cz}/*csk*^{cz} Embryos

(A and B) Transverse sections through the trunk region of E8.0 wild-type (+/+) and homozygous mutant (−/−) embryos, respectively. The neural tube began to close in the wild-type embryo, but not in the homozygous mutant embryos; otherwise, no difference was apparent.

(C and D) Transverse sections through the trunk region of a E8.5 homozygous mutant embryo; (D) is a magnified view of the region indicated by an arrowhead in (C). The necrotic figures first appeared at this stage in the neural tube of the homozygous mutant embryos, while they were never observed in that of wild-type embryos.

(E–J) Transverse sections through the trunk region (E and F) and through the cranial region (G–J) of wild-type (E, G, and H) and homozygous mutant (F, I, and J) embryos. (H) and (I) are magnified views. Abnormalities in the E9.5 homozygous mutant embryos were mainly in the neural tissues: necrotic figures in the neural tube (arrowhead in [F]) and cranial ganglia and reduction in size of the neuroepithelium and in the number of mitotic figures (arrowheads in [H] and [J]).

e, neuroepithelium; g, cranial ganglion; hg, hindgut; nt, neural tube; s, somite.

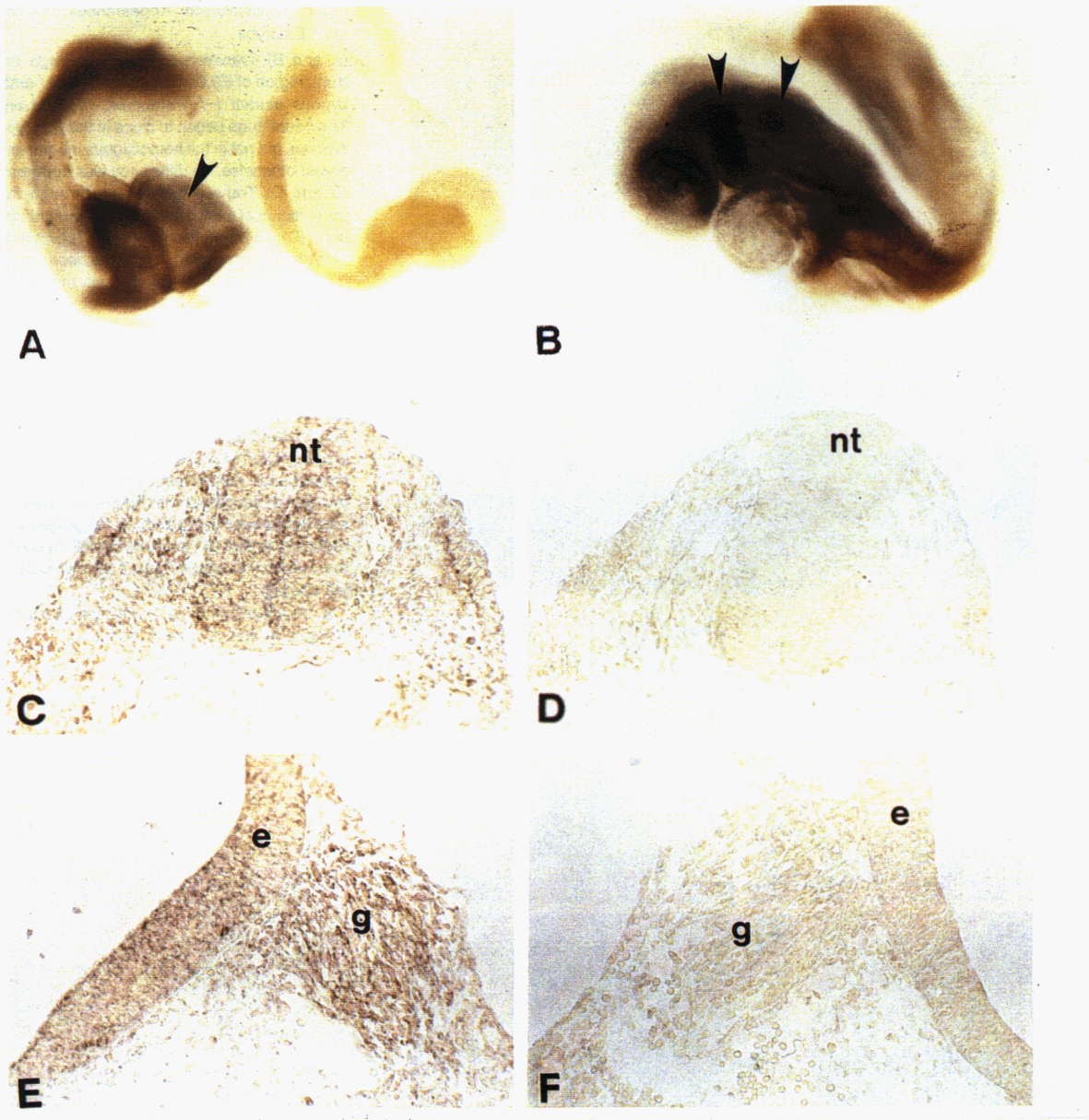

Figure 4. Spatial Expression of *csk* Transcripts by Whole-Mount *In Situ* Hybridization
(A) E8.0th day embryo. The embryo in the left was hybridized with an antisense probe, while that in the right was hybridized with a sense probe. Arrowhead indicates the head neuroepithelium which showed intense hybridization signal. (B) E8.5th day embryo. The arrowheads indicate migrating cranial neural crest cells which are positive for *csk* transcripts.

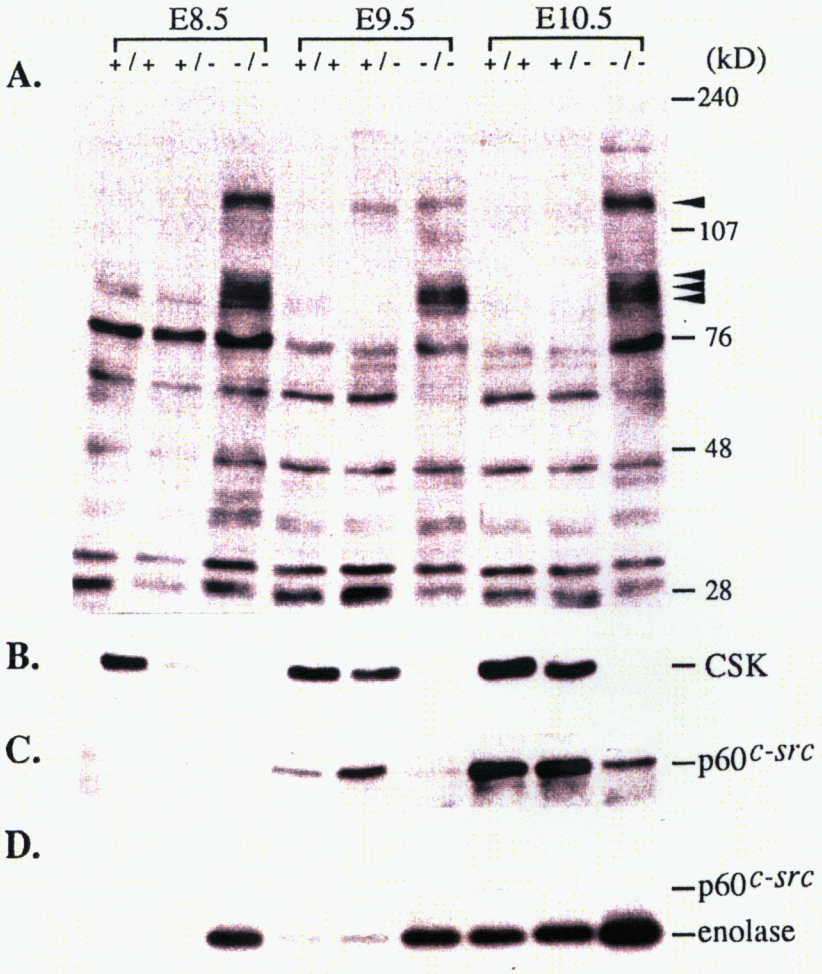

Figure 5. Biochemical Characterization of *csk*^{cz} /*csk*^{cz} Embryos
(A) Tyrosine-phosphorylated proteins, (B) Csk and (C) p60$^{c\text{-}src}$ protein levels and (D) kinase activity of p60$^{c\text{-}src}$ in wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-) embryos of E8.5th, E9.5th and E10.5th day. Ten μg of total proteins obtained from each embryo were subjected to immunoblot analysis with anti-phosphotyrosine (PY20) (A), anti-Csk (B) or anti-p60$^{c\text{-}src}$ (MAb327) (C) antibodies. Arrowheads indicate proteins which were highly phosphorylated in *csk*^{cz} /*csk*^{cz} embryos. (D) Ten μg of aliquot proteins was solubilized in the buffer containing 1% Nonidet P-40, and p60$^{c\text{-}src}$ was immunoprecipitated by MAb327; *in vitro* kinase assay was done by incubating the precipitates in the kinase assay buffer containing acid-treated enolase and [γ-^{32}P]ATP. The embryos from the same litter was subjected to each analysis, and exact stages of embryos were variable to some extents even in normal embryos of the same littermate. The results include this effect; for example, the level of Csk in the 8.5 days heterozygous embryo used in this particular assay was not half of the wild-type embryo, but this heterozygous embryo was morphologically at the E8.0th day stage.

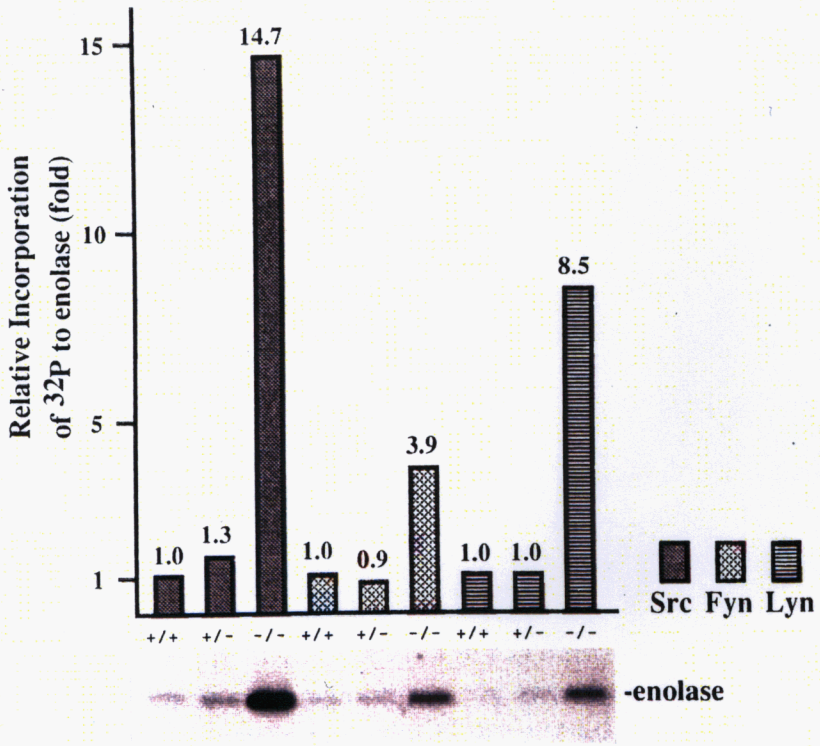

Figure 6. Activation of Src Family Kinases in *csk*^{cz} /*csk*^{cz} Embryos
The +/+, +/*csk*^{cz} and *csk*^{cz} /*csk*^{cz} genotypes are indicated as +/+, +/- and -/-, respectively, under each column. The contents of p60$^{c\text{-}src}$, p59*fyn* and p53/56^{lyn} in the extracts from the E9.5th day whole embryos genotyped by PCR were determined by immunoblot analysis with MAb327, fyn2 and Lyn-8 antibodies, respectively, and then p60$^{c\text{-}src}$, p59*fyn* and p53/56^{lyn} were immunoprecipitated for kinase assay from the extracts containing the same amount of the respective kinases with excess amount of the same antibodies. Immunoprecipitates were incubated in the kinase assay buffer containing acid-treated enolase and [γ-^{32}P]ATP and applied to SDS-PAGE. The incorporation of ^{32}P to enolase was quantitated by BAS2000 image analyzing system. In each experiment the quantity in +/*csk*^{cz} and *csk*^{cz} /*csk*^{cz} samples against that in +/+ sample were calculated as relative values. In the upper histogram, the standard values shown upon each column were determined from five independent assays. Lower figure gives an example of such assay.

Conclusion

In this study, I showed that Csk was a new class of cytoplasmic protein-tyrosine kinase lacking autophosphorylation and regulatory tyrosine residues by cDNA cloning. Purified Csk from neonatal rat brain showed extremely high specificity for Tyr527 of p60$^{c\text{-}src}$ *in vitro* and in yeast cells, and it suppressed the kinase activity of p60$^{c\text{-}src}$ in yeast cells. The deficiency of Csk by gene targeting in embryonic stem cells caused complex phenotypical changes in mouse embryos, such as irregular formation of neural tube, cell death in neuroepithelium and neural crest, and inability to turn. Concomitant with those changes, the activities of Src family kinases, p60$^{c\text{-}src}$, p59^{fyn} and p53/56^{lyn}, were elevated to maximum level in Csk-deficient embryos. All these results indicate that Csk is an indispensable negative regulator for Src family kinases *in vivo*.

However, the problems of why the Csk-deficient embryos die and what is the primary defect by Csk-deficiency are remain unresolved. The physiological roles of Src family in nervous system, the functions of SH3 and SH2 regions in Csk and the regulation mechanism of Csk also must await future studies.

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