CSK: a Protein-tyrosine Kinase Involved in Regulation of src Family Kinases*

(Received for publication, July 29, 1991)

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The functions of src family protein-tyrosine kinases are thought to be regulated negatively by the phosphorylation of highly conserved tyrosine residues close to their carboxyl termini. Recently we have purified and cloned a protein-tyrosine kinase (designated as CSK) that can specifically phosphorylate the negative regulatory site of ~60"~". To elucidate the relationship between CSK and other types of src family kinases, we investigated the tissue distribution of CSK and examined whether CSK could phosphorylate the negative regulatory sites of src family kinases other than p60"~". Western blot analysis indicated that CSK was enriched at the highest level in lymphoid tissues in which the expression of p60"~" is considerably lower than those of other types of src family kinases. CSK phosphorylated p56"~" and p59"~", which are known to be expressed in lymphoid tissues at a relatively high level. The putative regulatory site, tyrosine 508, was found to be essential for phosphorylation in p56"~", and the kinase activities of these src family kinases were repressed by phosphorylation with CSK. These findings raise the possibility that CSK might act as a universal regulator for src family kinases.

The protooncogene products of the src gene family are membrane-associated phosphoproteins that possess intrinsic protein-tyrosine kinase activity (1). Although several members of the src family kinase are found to be associated with certain signal transduction systems (2-4), the genuine functions of the kinases still remain to be clarified. Outside of the highly conserved catalytic domain, the src family kinases also show strong sequence homology within their carboxyl-terminal 15-17 residues (5). This sequence contains a tyrosine residue that has been shown to be phosphorylated in p60"~" (6) and p56"~" (7). Phosphorylation at these tyrosine residues (Tyr-527 in p60"~", Tyr-505 in p56"~") has been shown to be involved in the negative regulation of kinase activity (7-12). Other members of the src family also have the putative phosphorylation site. Therefore they are probably regulated negatively in a similar manner to that of p60"~" and p56"~".

Since p60"~" has only low autophosphorylation activity for Tyr-527 (8, 13-15), phosphorylation has been thought to be mediated by a distinct protein-tyrosine kinase (16). Recently we have found a novel protein-tyrosine kinase that specifically phosphorylates Tyr-527 of p60"~" in vitro and cloned a cDNA that encodes the enzyme (15, 17). The enzyme (designated as CSK) also phosphorylates Tyr-527 of p60"~" even in intact yeast cells where CSK and p60"~" are co-expressed. Thus, CSK is considered to be a strong candidate for the kinase involved in the negative regulation of p60"~".

The carboxyl-terminal sequences of src family kinases are conserved, indicating that CSK might be involved in the regulation of src family kinases in addition to p60"~". To test this possibility, we investigated the tissue distribution of CSK in relation to those of src family kinases and examined if CSK also phosphorylates putative negative regulatory sites of p56"~" and p59"~".

EXPERIMENTAL PROCEDURES

Purification of CSK from Neonatal Rat Brain and Adult Rat Spleen—The purification procedures were essentially as described previously (15, 17). In brief, the eluate from the first DE52 column was dialyzed against Buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM mercaptoethanol, 10% glycerol, and 0.1% Nonidet P-40) and applied to a poly(Glu,Tyr)-Sepharose CL-4B column (50 ml). The column was washed, and fractions containing enzyme activity were eluted with 0.1 M NaCl in Buffer A. The eluate was then dialyzed and applied to a MonoQ column, which was eluted with a linear gradient of 0-0.35 M NaCl. CSK eluted at about 0.25 M NaCl and then purified further on a Sephacryl S200HR column. Finally, the active fractions were dialyzed, applied to a MonoS column, and eluted with a linear gradient of 0-0.2 M NaCl. The yield was 100 μg of highly purified CSK/300 g of neonatal rat brain or 50 μg/100 g of spleen.

Western Blot Analyses—Rat tissues (perfused with saline) were homogenized in 0.25 M sucrose (0.32 M for brain) containing 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 units/ml aprotinin. The homogenates (60 μg of protein/tissue) were subjected to SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was probed with anti-CSK antiserum raised against bacterially expressed protein (17). Immunoreactive proteins were stained by the peroxidase-antiperoxidase complex procedure described previously (15, 17).

Presentation of src Family Kinases—p60"~" was purified from yeast cells carrying p60"~" expression plasmid with monoclonal antibody 327 (17). p59"~" was purified from rat spleen with an antiserum generated against a bacterially expressed peptide corresponding to amino acid residues 25-141 of the human fyn protein sequence (18). p56"~" was purified from rat spleen with a monoclonal antibody raised against the amino-terminal sequence of human lyn protein (19). The microsomal fractions prepared from rat spleen (1 g) were solubilized in 1 ml of RIPA buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin) at 4 °C, and the clarified supernatant was then incubated with each antibody at 4 °C for 1 h. The immune complex formed (rabbit anti-mouse IgG was added as a secondary antibody

* This work was supported in part by Grants-in-aid for Scientific Research 02780177, 03780179, and 01440024 from the Ministry of Education, Science and Culture of Japan and by a grant from the Yamamoto Foundation for Research on Metabolic Disorders. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: p60"~", a protooncogene product of c-src; p59"~", a protooncogene product of lyn; p56"~", a protooncogene product of c-yes; p56"~", a protooncogene product of lck; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FSBA, 5'-p-fluorosulfonylbenzoyladenosine.

Vol. 266, No. 36, Issue of December 25, pp. 3424-3432, 1991
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Printed in U.S.A.
for mouse monoclonal antibody) was precipitated with 20 μl of Pan- sorbin (Calbiochem) and washed in RIPA buffer extensively. Prior to the phosphorylation reaction, the immunoprecipitate was washed with 10 mM sodium phosphate, pH 7.2, containing 10% glycerol and 0.01% Nonidet P-40 and suspended in the same buffer (400 μl), then divided into two portions. One half was incubated with 2 mM FSBA at room temperature for 60 min to destroy the kinase activities of src family kinases (15). Then, the immunoprecipitate was washed with 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, and 0.1% Nonidet P-40. The other half was incubated without FSBA, washed, and then subjected to autophosphorylation as described previously (17).

p56\textsuperscript{Lyn} was generated by introducing a mutation in the codon for Tyr-508 so that it specified phenylalanine. The Hgal-BamHI fragment (nucleotides 1781–1949) of human lyn cDNA (19) was replaced with a synthetic oligonucleotide so that the codon for Tyr-508 was changed to that for phenylalanine. The resulting fragment (lyn-F508) was then introduced in a yeast expression vector (pHM209), and expression of the p56\textsuperscript{Lyn} protein was induced by the method described previously (17).

Phosphorylation Assay—Phosphorylation of src family kinase by CSK was determined as described previously (15, 17). The reaction mixture (10 μl) contained 50 mM Tris-HCl, pH 7.4, 3 mM MgCl\textsubscript{2}, 0.1 mM Na\textsubscript{2}VO\textsubscript{4}, 1 μM [γ-\textsuperscript{32}P]ATP (18.5 MBq/nmol), src, lyn, or lyn immunoprecipitate, and CSK. After incubation for 10 min at 30 °C, the reaction product was analyzed by SDS-PAGE and autoradiography. For the enolase phosphorylation assay, 2.5 μg of acid-treated enolase was included in the reaction mixture as described previously (15).

Peptide Mapping—Tryptic digestion of labeled p56\textsuperscript{Lyn} was done essentially by the method described previously (15). Phosphorylated p56\textsuperscript{Lyn} was eluted from the gel, acid-precipitated, oxidized with performic acid, and digested with trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone. The digest was separated in one dimension on cellulose thin-layer plates by ascending chromatography with a solvent system of 1-butanol/pyridine/acetic acid/water (75:50:15:60).

RESULTS AND DISCUSSION

The expression of CSK in various rat tissues was estimated by Western blot analysis (Fig. 1A). It can be seen that the immunoreactive protein was enriched at the highest level in spleen. Neonatal brain, thymus, lung, and liver also contained relatively high levels, while other tissues contained only a limited amount of the proteins. To further confirm that the immunoreactive proteins in spleen cells were actually CSK, we purified a protein possessing CSK activity from spleen using the same method we employed previously to purify CSK from neonatal brain (15, 17). The results were consistent with the spleen kinase and neonatal brain CSK being quite similar if not identical. Both reacted with anti-CSK antibody (Fig. 1C) and had identical chromatographic behaviors, molecular weight estimated on SDS-PAGE (Fig. 1B), and specificity for src family kinases (described below). The specificity of the spleen kinase was confirmed using mutant p60\textsuperscript{Src} and a previously described method (17). As can be seen in Fig. 2A, the spleen kinase phosphorylated p60\textsuperscript{Src}, but not p60\textsuperscript{527} or p527, indicating that the spleen kinase, as shown previously with the brain CSK (17), specifically phosphorylates Tyr-527 of p60\textsuperscript{Src}.

In lymphoid tissues including spleen, the expression of p60\textsuperscript{Src}, a known substrate for CSK, is substantially lower.

FIG. 1. Expression of CSK protein in various rat tissues. A, Western blot analysis with an anti-CSK antiserum. Homogenates of indicated rat tissues were probed with an anti-CSK antibody as described under “Experimental Procedures.” Lane 1, neonatal rat brain; lane 2, adult brain; lane 3, thymus; lane 4, spleen; lane 5, lung; lane 6, liver; lane 7, heart; lane 8, kidney; lane 9, testis. B, silver-stained SDS-PAGE gel of highly purified CSK from neonatal brain (lane 1) and adult spleen (lane 2). C, Western blot analysis of the highly purified CSK from neonatal brain (lane 1) and spleen (lane 2).

FIG. 2. Phosphorylation of src family kinases by CSK. A, phosphorylation of p60\textsuperscript{Src} by spleen CSK. p60\textsuperscript{527}, p60\textsuperscript{527} or p527 were expressed in yeast cells and immunoprecipitated with monoclonal antibody 327 by the method described previously (17). The immunoprecipitates were incubated with (lanes 3 and 5) or without (lanes 1, 2, and 4) spleen CSK in the reaction system described under “Experimental Procedures.” B, phosphorylation of p56\textsuperscript{Lyn} by spleen CSK. p56\textsuperscript{Lyn} was immunoprecipitated from rat spleen, and one half of the immunoprecipitate was treated with FSBA. After washing, the immunoprecipitate was incubated with (lane 3) or without (lane 2) spleen CSK. Another half of the immunoprecipitate was subjected to autophosphorylation (lane 1). The immunoreactive 53K protein seen in this experiment was found to be an alternative spliced product of the lyn gene. C, phosphorylation of p59\textsuperscript{Lyn} by spleen CSK. p59\textsuperscript{Lyn} was immunoprecipitated from rat spleen, and the immunoprecipitate was assayed for autophosphorylation without (lane 1) or with (lane 2) FSBA treatment, or assayed for phosphorylation by spleen CSK after treatment with FSBA (lane 3). In our immunoprecipitation system, an immunoreactive species migrating as a 56K protein was detected. The nature of the protein is still unclear. Additional experimental details are described under “Experimental Procedures.”

FIG. 3. Carboxyl-terminal sequences of src family kinases. Carboxyl-terminal sequences of gene products of c-src (23) fyn (24), c-yes (25), c-fgr (26), lck (21), btk (27), hck (28), and lyn (19) were aligned for maximum homology. Only the amino acids deviated from the c-src product are indicated. Numbers on top of the aligned sequences indicate amino acid number of the c-src product. The negative regulatory site (Tyr-527 in the c-src product) is indicated by boldface.
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Thus the difference in size of the transcripts probably results from an alternative splicing that does not affect the activity or apparent molecular size of CSK, or the difference in the utilization of poly(A) additional signals.

To address the phosphorylation site in p56\textsuperscript{ vn}, autophosphorylated p56\textsuperscript{ vn} and that phosphorylated by CSK (Fig. 2B) were eluted from the gel, digested with trypsin, and separated in one dimension on thin-layer chromatography (Fig. 4A). Autophosphorylated p56\textsuperscript{ vn} gave one major and two minor phosphopeptides. The major phosphopeptide was considered to contain the autophosphorylation site (Tyr-394). In contrast, p56\textsuperscript{ vn} phosphorylated by CSK gave a single phosphopeptide which corresponded to one of the minor phosphopeptides obtained from autophosphorylation. This indicates that CSK phosphorylation and autophosphorylation occur at two distinctly different sites. To determine if the putative regulatory site, Tyr-508, is important in phosphorylation by CSK, we generated a mutant p56\textsuperscript{ vn} in which Tyr-508 was replaced by phenylalanine. The mutant (p56\textsuperscript{ vn}) was expressed in yeast cells, purified, and treated with FSBA as described under "Experimental Procedures." As shown in Fig. 4B, p56\textsuperscript{ vn} was not a substrate for CSK.

Phosphorylation of p56\textsuperscript{ vn} by CSK resulted in a decrease in their abilities to phosphorylate enolase (Fig. 4, C and D). The effects were apparent when the activities were measured in the presence of a limited (1 \mu M) and an excess (10 \mu M) amount of ATP, suggesting that the decrease in activities is due to a decrease in the V\textsubscript{max} values of these src family kinases. However, as previously observed in the case of p60\textsuperscript{ src} (17), the inhibitory effects (40–78\%) were not so large as expected. This might be explained on the basis that autophosphorylation of the src family kinases, which are suggested to rather enhance their own activities, proceeded in parallel with phosphorylation by CSK in our reaction system. Anyway, these results demonstrate that, in addition to p60\textsuperscript{ src}, p56\textsuperscript{ vn} and p59\textsuperscript{ vn} can be regulated negatively by CSK phosphorylation. Since other src family kinases have considerably more homology than p56\textsuperscript{ vn} with p60\textsuperscript{ src} in the carboxyterminal region (Fig. 3), it is likely that other src family kinases would also function as a substrate for CSK. Indeed, we have obtained a line of evidence that CSK can also phosphorylate p62\textsuperscript{ src} and p56\textsuperscript{ src}. Although the amino acid changes in the carboxy-terminal regions among src family kinases are very conservative, heat-denatured p60\textsuperscript{ src} and a synthetic peptide of this region (519–533) were only poor substrates for CSK, indicating that a structure of higher order than that provided merely by the electrostatic properties of the amino acid sequences is important for recognition by CSK.

In this study, we showed that CSK could act as a negative regulator for some, possibly all, src family kinases. This raises the possibility that src family kinases are also regulated positively by specific phosphotyrosine-protein phosphatase(s). Thus to understand the physiological function of src family kinases, it seems essential to find the signal or factor that controls phosphorylation \textit{versus} dephosphorylation in the regulatory unit of src family kinases.
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Acknowledgment—We are grateful to Dr. Leonard A. Fahien (University of Wisconsin) for valuable comments on the manuscript.

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