Purification of Bovine Thymus Cytosolic C-Terminal Src Kinase (CSK) and Demonstration of Differential Efficiencies of Phosphorylation and Inactivation of $p56^{lyn}$ and $pp60^{c-src}$ by CSK†

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ABSTRACT: The C-terminal src kinase (CSK) is a ubiquitously expressed, cytosolic enzyme capable of phosphorylating and inactivating several plasma membrane-bound src-family protein tyrosine kinases *in vitro* [Nada, S., Okada, M., MacAuley, A., Cooper, J. A., & Nakagawa, H. (1990) *Nature 351*, 69−72; Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P., & Alitalo, K. (1992) *EMBO J. 11*, 2919−2924]. We purified CSK to apparent homogeneity from bovine thymus cytosol to study *in vitro* how the purified enzyme recognizes the various src-family kinases as its substrates. A novel assay method was developed for assaying the ability of CSK to inactivate src-family tyrosine kinases. With this assay method, we demonstrated that CSK inactivated $p56^{lyn}$ with a significantly higher efficiency than $pp60^{c-src}$. Phosphopeptide mapping of CSK-phosphorylated $p56^{lyn}$ and $pp60^{c-src}$ shows that the consensus tyrosine residue (also termed tail tyrosine) in the C-terminal regulatory domain of $p56^{lyn}$ was phosphorylated by CSK with an efficiency much higher than that of $pp60^{c-src}$. Thus, the higher efficiency of inactivation of $p56^{lyn}$ by CSK is a result of the ability of $p56^{lyn}$ to serve as a better substrate of CSK. The synthetic peptides derived from the C-terminal portion of $p56^{lyn}$ and $pp60^{c-src}$ were much poorer substrates than the intact src-family kinases for CSK, indicating that the local structure around the tail tyrosine is not sufficient to direct efficient phosphorylation of $p56^{lyn}$ by CSK. Nevertheless, the slightly higher efficiency displayed by CSK in phosphorylating the peptide derived from the C-terminal portion of $p56^{lyn}$ than that from $pp60^{c-src}$ suggests that the structural differences between the C-terminal portions of $p56^{lyn}$ and $pp60^{c-src}$ contribute to the differential efficiencies displayed by CSK in phosphorylating the two kinases. Determination of the CSK-phosphorylation site in the src-C-terminal peptide by phosphopeptide mapping reveals that the whole C-terminal regulatory domain and an adjacent part of the protein kinase domain contain some of the structural determinants directing CSK to phosphorylate the consensus tail tyrosine of the src-family kinases.

Among the known non-receptor-like protein tyrosine kinases, the src-family protein tyrosine kinases are the best characterized. They are protein products of the src family of protooncogenes and oncogenes including *src, lck, fyn, lyn, hck, fgr, blk, yes,* and *yrk* [reviewed in Cooper (1990), Sukegawa et al. (1987), Katemine et al. (1988), Quintrell et al. (1987), Trevillyan et al. (1986), Dymecki et al. (1990), and Sudol et al. (1993)]. Using homologous recombination to generate null alleles of some members of the src-family protooncogenes, mice deficient in *src* only, *lck* and *fyn*, *fyn* only, or *lyn* only display a wide variety of abnormalities ranging from osteopetrosis to changes in long term potentiation, suggesting that the src-family kinases are involved in a number of different signalling pathways in different cell types [reviewed in Cooper (1990), Courtneidge (1994), Varmus and Lowell (1994), and Hibbs (1995)]. However, the exact functions and the regulatory mechanisms of the src-family kinases are not fully understood. Among all known regulatory mechanisms, autophosphorylation of a tyrosine residue in the protein kinase domain and phosphorylation of the consensus tyrosine residue [also termed tail tyrosine by Courtneidge (1994)] in the C-terminal regulatory domain play the most pivotal roles. Autophosphorylation is responsible for autoactivation in a number of src-family kinases, while phosphorylation of the consensus C-terminal regulatory tyrosine leads to inactivation (Figure 1) [reviewed in Cooper (1990) and Sotirellis et al. (1995)]. A tyrosine kinase termed C-terminal src sinase (CSK), expressed ubiquitously but predominantly in lymphoid tissues and neonatal brain, has been implicated to be the upstream regulatory tyrosine kinase by virtue of its ability to inactivate

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FIGURE 1: Amino acid sequences of the C-terminal portions of all members of the src family of protein tyrosine kinases. The sequences contain the consensus C-terminal regulatory domain and part of the kinase domain. The boundaries of the kinase domain were defined previously by Hanks and Quinn (1991) on the basis of homology of the sequences in all known protein tyrosine kinases. The C-terminal boundary of the kinase domain is set at a hydrophobic residue (Leu in the src family of tyrosine kinases) that lies 10 residues downstream of the invariant Arg (underlined). The consensus tail tyrosine is marked by an asterisk. The numbers shown in each sequence represent the residue numbers of the amino acids in the sequence of kinase.

several src-family kinases by phosphorylating their tail tyrosine residue *in vitro* (Okada & Nakagawa, 1989; Okada et al., 1991; Bergman et al., 1992; Ruzzene et al., 1994). Several lines of evidence implicate phosphorylation of src-family kinases by CSK *in vivo*. Firstly, the kinase activity of $pp60^{c-src}$, $p59^{fyn}$, and $p56^{lyn}$ was greatly enhanced and phosphorylation of the tail tyrosine was greatly reduced in CSK-deficient mice (Nada et al., 1993; Imamoto & Soriano, 1993). Secondly, coexpression of CSK and $pp60^{c-src}$ in budding yeast *Saccharomyces cerevisiae* results in phosphorylation of $pp60^{c-src}$ at the tail tyrosine and suppression of the $pp60^{c-src}$-induced growth inhibition of the yeast cells (Murphy et al., 1993). However, none of these studies provides evidence for direct phosphorylation of src-family kinases by CSK *in vivo*.

The primary structures of human and rat CSK have been determined by protein sequencing and molecular-cloning methods (Nada et al., 1991; Okada et al., 1991; Partanen et al., 1991). CSK shares a very high sequence homology with the src-family kinases (approximately 59% similarity) (Courtneidge, 1994). It contains the SH2, SH3, and kinase domains, lacking, however, the myristoylation domain, the consensus autophosphorylation site, and the tyrosine in the C-terminal tail (Nada et al., 1991). These structural features suggest that CSK should localize in the cytosol and that it cannot be regulated by autophosphorylation and C-terminal tyrosine phosphorylation in the same fashion as the src-family kinases.

Although CSK was first purified from membrane extracts of neonatal rat brain and adult rat spleen (Nada et al., 1991; Okada et al., 1991), subsequent studies revealed that the majority of the enzyme is in the cytosol [Bergman et al., 1992; see Courtneidge (1994) for review]. The lack of the myristoylation domain accounts for the predominant distribution of CSK in cytosol. Since all src-family kinases are membrane-bound, mechanisms must exist *in vivo* for recruiting CSK from the cytosol to the plasma membrane and directing CSK to phosphorylate the tail tyrosine of src-family kinases. The structural basis of these mechanism remains to be elucidated.

Using PCR-based molecular-cloning procedures to isolate tyrosine kinases on the basis of their consensus kinase sequences (Wilks, 1989), several isoforms (Matk/HYL,[1] Ctk/Batk, and Ntk) of a CSK-related protein tyrosine kinase have been discovered (Bennet et al., 1994; Sakano et al., 1994; Klages et al., 1994; Kuo et al., 1994; Chow et al., 1994; Avraham et al., 1995). Comparison of their cDNA sequences suggests that they were products of alternative translation initiation *in vivo*. While the expression of CSK is ubiquitous with the highest expression in lymphoid tissues and neonatal brain, tissue expression of the various isoforms of this CSK-related kinase is highly restricted. Matk/HYL is expressed exclusively in platelets, megakaryocytes, lung, and brain (Bennett et al., 1994; Sakano et al., 1994), while the expression of Ctk/Batk is restricted to the brain. Ntk was found to be expressed in the brain, thymus, and spleen. Since members of the src-family kinases are differentially expressed in various tissues [reviewed in Courtneidge (1994)], it is very likely that CSK and the CSK-related tyrosine kinase phosphorylate and regulate different subsets of src-family tyrosine kinases *in vivo*. In this context, identification of the src-family members that serve as *in vivo* substrates of CSK is an avenue to elucidate the cellular functions of CSK and the mechanism of regulation of src-family kinases. One approach to address this problem is to identify the structural features in substrate proteins that are recognized by CSK as substrate specificity determinants. Once these determinants are identified, the distribution of these structural determinants in various src-family kinases would indicate which of the src-family kinases are likely *in vivo* substrates of CSK.

In the present study, we report the purification of CSK from bovine thymus cytosol. Using a newly developed assay and phosphopeptide mapping, we have demonstrated that CSK displays different efficiencies in phosphorylation and inactivation of $pp60^{c-src}$ and $p56^{lyn}$ *in vitro*. Both our result and the extensive overlap of tissue distributions of CSK and $p56^{lyn}$ support the notion that CSK phosphorylates and regulates $p56^{lyn}$ *in vivo*. Synthetic peptides with sequences almost identical to the C-terminal portion of $p56^{lyn}$ and $pp60^{c-src}$ were phosphorylated by CSK with different efficiencies. The peptide derived from the C-terminal portion of $p56^{lyn}$ was proven to be a more effective substrate. This indicates that some of the structural features that are preferentially recognized by CSK as substrate specificity determinants are located in a short segment encompassing the tail tyrosine in the C-terminal portion of $p56^{lyn}$. Further studies with these peptides reveal that the whole C-terminal regulatory domain and an adjacent portion of the protein kinase domain contain some structural determinants directing CSK to phosphorylate exclusively the tail tyrosine.

[1] Abbreviations: Batk, brain-associated tyrosine kinase; Ctk, CSK-type protein tyrosine kinase; Ntk, nervous tissue and T-lymphocyte kinase; Matk, megakaryocyte-associated tyrosine kinase; HYL, hematopoietic consensus tyrosine-lacking kinase. Batk and Ctk are names given to the same protein kinase. Matk and HYL are names given to the same protein kinase.

EXPERIMENTAL PROCEDURES

Materials

DEAE-Sepharose CL6B, Sephacryl S-200 superfine, phenyl-Sepharose, Mono-Q anion exchange (HR5/5), and Mono-S cation exchange columns (HR5/5) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxylapatite was from BioRad (Hercules, CA). [γ-^{32}P]ATP was from Bresatec (Adelaide, South Australia). The horseradish peroxidase-linked sheep anti-rabbit IgG and the alkaline phosphatase-linked sheep anti-rabbit IgG were from Silenus Laboratory (Victoria, Australia). Keyhole limpet hemocyanin, (*m*-maleimidobenzoyl)-*N*-hydroxysuccinimide, *p*-nitroblue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) were from Calbiochem-Novabiochem (San Diego, CA). Immobilon (PVDF) membranes were from Millipore (Bedford, MA). The reverse phase C_{18} Sep-Pak cartidges were from Waters (Millford, MA). Phosphoserine, phosphothreonine, and phosphotyrosine were from Sigma (St. Louis, MO). Microcrystalline cellulose Macherey-Nagel TLC plates were from Alltech (Deerfield, IL). Poly(Glu,Tyr), a random copolymer of glutamate and tyrosine (Glu:Tyr = 4:1), was also purchased from Sigma. The anti-CSK antibody against a synthetic peptide derived from residues 433−450 at the C terminus of human CSK was a gift from Dr. Mathias Bergman from the University of Helsinki. The same antibody was later prepared by us using procedures similar to those described by Bergman et al. (1992). Recombinant mouse p56^{lyn} was purified from *Spodoptera frugierda* 9 (Sf9) insect cells infected with the baculovirus carrying the *lyn* gene using the procedures described previously (Sotirellis et al., 1995). Recombinant human pp60^{c-src} was prepared and purified by immunoaffinity chromatography as described previously (Feder & Bishop, 1990) followed by FPLC Mono-Q ion exchange chromatography (Bjorge et al., 1995). Both the recombinant p56^{lyn} and pp60^{c-src} preparations were shown by immunoblot with anti-phosphotyrosine antibody to contain less than 5% of tyrosine-phosphorylated kinases, indicating that both the consensus autophosphorylation site and the tail tyrosine of more than 95% of p56^{lyn} and pp60^{c-src} were in the nonphosphorylated state (Bjorge et al., 1995; Sotirellis et al., 1995).

Assay for the Poly(Glu,Tyr) Kinase Activity of CSK

For purification of CSK, activity of protein tyrosine kinases in bovine thymus cytosolic extract during purification was determined by measuring the incorporation of PO_3^{2-} from [γ-^{32}P]ATP into poly(Glu,Tyr), a random copolymer of glutamate and tyrosine. Routine enzyme assays were carried out at 30 °C in a 25 μL volume containing 20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM Na_3VO_4, 0.3 μM PKI(5−22) peptide, 100 μM ATP (specific radioactivity, 300−400 cpm/pmol), and 0.5 mg/mL poly(Glu,Tyr). The reaction was initiated by the addition of ATP and then terminated by spotting a 17 μL aliquot of the reaction mixture onto a Whatman 3MM paper square. The paper squares were washed in 5% trichloroacetic acid and 0.5% H_3PO_4 six times for 10 min each wash and once with ethanol and then dried. Radioactivity in the strips was monitored by Cerenkov counting.

Kinetic Analysis of CSK Phosphorylation of Synthetic Peptides

Fyn(503−537) and lyn(480−512) peptides of various concentrations were phosphorylated by CSK (0.21 μM) in the presence of 100 μM [γ-^{32}P]ATP (specific radioactivity, 300−500 cpm/pmol) and the assay buffer [20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 μM Na_3VO_4] at 30 °C for 40 min. The reaction was stopped by the addition of 30 μL of 50% acetic acid. Aliquots of the reaction mixture were spotted onto phosphocellulose paper squares and processed as described in the previous section. The initial rate of peptide phosphorylation was measured because in all cases less than 5% of the substrates was consumed at the end of the reaction. The K_m and V_{max} of the reaction were determined by Lineweaver-Burke plot.

Assay for the Ability of CSK to Phosphorylate and Inactivate pp60^{c-src} and p56^{lyn}

Figure 4 depicts the sequence of steps required for the inactivation assay. The assay was performed in two steps: (i) inactivation of pp60^{c-src} or p56^{lyn} by CSK and (ii) measurement of the remaining tyrosine kinase activity of pp60^{c-src} and p56^{lyn} using the [K^{19}]cdc2(6−20) peptide as substrate. For step (i), inactivation of pp60^{c-src} or p56^{lyn} by CSK was carried out at 30 °C for 30 min in a 15 μL volume containing 20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM Na_3VO_4, 100 μM [γ-^{32}P]ATP (specific radioactivity, 300−500 cpm/pmol), and 0.48 μM purified pp60^{c-src} or p56^{lyn} and in the absence (control reaction) or presence (inactivation reaction) of CSK with concentrations ranging from 56.7 nM to 2.08 μM. In step (ii), the remaining tyrosine kinase activity of pp60^{c-src} and p56^{lyn} was determined by measuring the incorporation of phosphate from [γ-^{32}P]ATP into [K^{19}]cdc2(6−20) peptide. The peptide kinase activity assay of p56^{lyn} and pp60^{c-src} was initiated by the addition of 4 μL of the mixture from the control reaction or the inactivation reaction into an assay mixture with the various components of the assay buffer, [γ-^{32}P]ATP, and [K^{19}]cdc2(6−20) peptide to give a final reaction volume of 50 μL. The final concentrations of the components are 19.1 nM p56^{lyn} or pp60^{c-src}, 20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 50 μM Na_3VO_4, 100 μM [γ-^{32}P]ATP (specific radioactivity, 300−500 cpm/pmol), 300 μM [K^{19}]-cdc2(6−20) peptide, and 0−0.08 μM CSK. The peptide phosphorylation reaction was allowed to proceed for 6 or 10 min (6 min for pp60^{c-src} and 10 min for p56^{lyn}) at 30 °C and was terminated by the addition of 30 μL of 50% (v/v) acetic acid. A 36 μL aliquot of the mixture was spotted onto a phosphocellulose paper square and processed as described in the previous section. The initial rate of peptide phosphorylation was measured because in all cases less than 10% of the ATP was consumed at the end of the reaction.

Since [K^{19}]cdc2(6−20) is a very poor substrate of CSK but a very efficient and specific substrate for src-family tyrosine kinases (refer to Results for the specific enzyme activities of the kinases), phosphorylation of this peptide is a reliable measure of the residual tyrosine kinase activity of p56^{lyn} or pp60^{c-src} in both the control reaction and inactivation reaction. This is confirmed because, when pp60^{c-src} and p56^{lyn} were omitted from the mixture of the inactivation reaction, it was determined that less than 3% of the

phosphorylation of [K^{19}]cdc2(6−20) peptide was contributed by CSK. The proportion of $p56^{lyn}$ or $pp60^{c-src}$ inactivated by CSK (% inactivation) was calculated using the following formulas:

fraction (F) of active $p56^{lyn}$ or $pp60^{c-src}$ remaining in the inactivation reaction after phosphorylation by CSK = (peptide kinase activity of the inactivation reaction mixture)/(peptide kinase activity of the control reaction mixture)

$$\% \text{ inactivation} = 100\% \times (1 - F)$$

The % inactivation is a measure of the ability of CSK to inactivate the src-family kinases.

Determination of the Relative Degree of Autophosphorylation and CSK Phosphorylation in $p56^{lyn}$ or $pp60^{c-src}$ by Tryptic Phosphopeptide Mapping

Purified $pp60^{c-src}$ or $p56^{lyn}$ (0.48 μM) was incubated at 30 °C for 30 min with CSK with concentrations ranging from 0 to 6.24 μM in the presence of 100 μM [γ-^{32}P]ATP (specific radioactivity, 3000−4000 cpm/pmol), 20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 μM Na_3VO_4 in a final volume of 30 μL. The reaction was terminated by the addition of 20 μL of 5 × SDS−PAGE sample buffer (Laemmli, 1970). The phosphorylated $pp60^{c-src}$ or $p56^{lyn}$ was separated from CSK and free [γ-^{32}P]ATP by SDS−PAGE. The proteins on the gel were then electrotransferred to nitrocellulose (Millipore) and the phosphorylated kinases located by autoradiography.

Prior to tryptic digestion, the strips corresponding to the phosphorylated $pp60^{c-src}$ and $p56^{lyn}$ were excised and processed as previously described (Aebersold, 1989). Briefly, the nitrocellulose strips were incubated for 30 min at 37 °C with 1 mL of 0.5% PVP-40 dissolved in 100 mM acetic acid. Excess PVP-40 was removed by washing with 10 × 1 mL of Milli-Q H_2O. For tryptic digestion, the nitrocellulose strips were incubated with 18 μg of TPCK-treated trypsin (Sigma) in 180 μL of 100 mM NH_4HCO_3 (pH 7.9)/acetonitrile (95:5, v/v) at 30 °C for 15 h. After digestion, the supernatant was removed and saved; the nitrocellulose strips were rinsed with 2 × 100 μL of Milli-Q H_2O/acetonitrile (90:10, v/v). The supernatant and the rinse were pooled and lyophilized to dryness. The dry sample was resuspended with 200 μL of Milli-Q H_2O and relyophilized. Finally, the dry sample was resuspended in 15−20 μL of Milli-Q H_2O before it was applied onto a TLC plate. Separation of the tryptic fragments containing the autophosphorylation site from that containing the CSK-phosphorylation site was accomplished by thin layer chromatography in the TLC buffer (*n*-butanol, pyridine, acetic acid, and H_2O in a ratio of 15:10:3:12). The radioactive spots on the TLC plate were located by autoradiography. This method has been used previously to separate the tryptic phosphopeptide containing the autophosphorylation site from that containing the CSK-phosphorylation site in $p56^{lyn}$ (Okada et al., 1991; Hata et al., 1994). The fast-migrating spot corresponds to the autophosphorylation site peptide, and the slow-migrating spot is the tryptic phosphopeptide containing the CSK-phosphorylated tail tyrosine (Figures 5−7).

Effects of Varying the Time of Reaction and the Concentration of Lyn on Its Phosphorylation and Inactivation by CSK

For the time course study, 0.48 μM $p56^{lyn}$ was incubated with 0.35 μM CSK for 10, 20, 30, and 40 min at 30 °C in the presence of 100 μM [γ-^{32}P]ATP (specific radioactivity, 3000−4000 cpm/pmol), 20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 μM Na_3VO_4 in a final volume of 30 μL. An aliquot (4 μL) was taken out for [K^{19}]cdc2-(6−20) peptide kinase activity assay, and the remaining portion was subjected to SDS−PAGE and then tryptic phosphopeptide mapping to determine the relative levels of autophosphorylation and CSK phosphorylation as described in the previous section. As a control, $p56^{lyn}$ was incubated with [γ-^{32}P]ATP under exactly the same conditions except that CSK was omitted in the reaction mixture. The peptide kinase activity and the degree of autophosphorylation of $p56^{lyn}$ were determined as described in the previous section.

For the study of the effect of varying the $p56^{lyn}$ concentration on its phosphorylation and inactivation by CSK, 0.24, 0.48, and 0.8 μM $p56^{lyn}$ was incubated for 30 min at 30 °C with 100 μM [γ-^{32}P]ATP (specific radioactivity, 3000−4000 cpm/pmol), 20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 μM Na_3VO_4 in a final volume of 30 μL in the presence and absence of 0.35 μM CSK. The [K^{19}]cdc2-(6−20) peptide kinase activity and tryptic phosphopeptide mapping were performed as described in the previous section.

Protein Concentration Determination

The protein concentrations of the purified CSK, the recombinant $pp60^{c-src}$, and the recombinant $p56^{lyn}$ were determined using bovine serum albumin as the standard after SDS−PAGE and staining with Coomassie Blue R-250. Briefly, the purified protein kinases were run on SDS−PAGE along with various concentrations of bovine serum albumin. Following electrophoresis, the gel was stained with Coomassie Blue R-250 and then destained. The gel was scanned with a laser densitometer and the protein concentration determined by comparison of the areas of the $pp60^{c-src}$ protein bands with the areas of bovine serum albumin standards.

Determination of the Phosphorylation Site in Fyn-(503−537) Phosphorylated by CSK

Fyn(503−537) (300 μM) was phosphorylated by CSK (2.5 μM) in a volume of 50 μL in the presence of 100 μM [γ-^{32}P]-ATP (specific radioactivity, 1000−2000 cpm/pmol) and the assay buffer [20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 μM Na_3VO_4] at 30 °C for 30 min. The reaction was stopped by the addition of 1 mL of 0.1% trifluoroacetic acid, and the mixture was applied to a prehydrated reverse phase C_{18} Sep-Pak cartridge (Millipore). The Sep-Pak cartridge was washed with 100 mL of 0.1% trifluoroacetic acid to remove the free radioactive ATP, and the radioactive phosphopeptide was then eluted from the Sep-Pak cartridge with acetonitrile. After removal of acetonitrile, the phosphopeptide was purified by reverse phase HPLC on a Vydac 218TP54 C_{18} column. After the sample was loaded onto the column, the peptides were eluted from the column with a gradient of 100% solvent A (0.1% TFA in H_2O) and 0% solvent B (0.1% TFA in acetonitrile) to 30% solvent A

and 70% solvent B in 70 min at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected. The phosphopeptide was then lyophilized to dryness. To the lyophilized phosphopeptide was added 20 μg of TLCK-treated chymotrypsin in 20 μL of 0.1 M ammonium bicarbonate (pH 7.9). After 8 h of incubation at 37 °C, another aliquot (20 μL of 1 mg/mL) of chymotrypsin was added and the digestion was allowed to proceed for another 12 h. The digest was analyzed by reverse phase HPLC on a Vydac C_{18} column using the TFA/acetonitrile solvent system and the same gradient. The purified chymotryptic phosphopeptide fragment was covalently attached to aminophenyl-coated glass disks, and the ^{32}P-labeled phosphorylation site was identified by automated sequencing with methods described by Aebersold et al. (1991) and Wettenhall et al. (1991).

HPLC Analyses of the Degree of Phosphorylation of Fyn(503−537) and Fyn(524−537)

Phosphorylation of the peptides by CSK was carried out at 30 °C for 30 min in a final volume of 50 μL containing the assay buffer [20 mM Tris-HCl (pH 7), 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 50 μL of Na_3VO_4], 300 μM peptide substrates, 3.1 μM CSK, and 100 μM [γ-^{32}P]ATP (specific radioactivity, 500−1000 cpm/pmol). The reaction was stopped by the addition of 200 μL of 50% acetic acid and the mixture subsequently processed with a C_{18} Sep-Pak cartridge (Waters) to remove the free ATP. The peptides were chromatographed on a Vydac 218TP54 C_{18} column. The column was first washed with 15 mL of solvent A (0.1% TFA), and the peptides were eluted from the column with a gradient of 100% solvent A and 0% solvent B (0.1% TFA in acetoniltrile) to 0% solvent A and 100% solvent B in 50 min at a flow rate of 1 mL/min. One minute fractions were collected, and the radioactivity in each fraction was monitored by Cerenkov counting.

Immunoblot Analysis

Immunoblot analysis was performed according to the method of Towbin et al. (1979) using either alkaline phosphatase-linked secondary antibody and *p*-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate as color substrate or horseradish peroxidase-linked secondary antibody and the enhanced chemiluminescence detection kit from Amersham.

Preparation of Synthetic Peptides

All synthetic peptides used were synthesized with the Applied Biosystems Model A431 automated peptide synthesizer using Fmoc-based chemistry. After cleavage of the peptides from the solid support with trifluoroacetic acid in the presence of the appropriate scavengers, the crude peptide preparations were purified by gel filtration chromatography on a Sephadex G-10 column and an Alltech Econosil C_{18} reverse phase HPLC column (22.5 mm × 250 mm). Purity exceeding 95% and authenticity of the synthetic peptides were shown by analytical reverse phase HPLC, amino acid composition analysis, and mass spectrometry. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry of the peptide was performed by the Finnigan MAT lasermat mass analyser using α-cyano-4-hydroxycinnamic acid as the matrix (Beavis et al., 1992).

Purification of CSK

Extraction of Tyrosine Kinases from Bovine Thymus. Fresh bovine thymuses were obtained from a local slaughterhouse and transported to the laboratory on ice. All procedures were carried out at 4 °C unless otherwise indicated. Three hundred grams of thymus was routinely used in the procedure. After removal of the excess fat, the thymuses were ground in a meat grinder and then further homogenized in 900 mL of homogenization buffer containing 25 mM Hepes (pH 7), 1 mM EDTA, 0.154 mg/mL dithiothreitol, 0.2 mg/mL benzamidine, 0.1 mg/mL phenylmethanesulfonyl fluoride, and 0.1 mg/mL soybean trypsin inhibitor in a Waring blender. The homogenate was centrifuged at 2000*g* for 45 min. The supernatant was filtered through glass wool and then centrifuged at 100000*g* for 40 min.

DEAE-Sepharose CL-6B Chromatography. The 100000*g* supernatant was applied to a 300 mL DEAE-Sepharose CL-6B column (5 × 16 cm) which was pre-equilibrated with the homogenization buffer. The column was washed with 4 × bed volume of the buffer and then eluted with a linear gradient (gradient size, 2 L) of 0 to 0.15 M NaCl in homogenization buffer. The column was further eluted with 500 mL of 0.3 M NaCl in homogenization buffer. Fractions of 17 mL were collected, and those containing poly(Glu,-Tyr) tyrosine kinase activity (Figure 2a) and α-CSK immunoreactivity (Figure 2f) were pooled.

Hydroxylapatite Column Chromatography. Fractions pooled from the DEAE CL-6B column were applied to a 50 mL hydroxylapatite column (2.5 × 23 cm) pre-equilibrated with the homogenization buffer. The column was washed with 100 mL of buffer and eluted with a linear gradient (gradient size, 500 mL) of 0.0 to 0.3 M potassium phosphate in homogenization buffer (pH 7). Fractions of 10 mL were collected, and those containing poly(Glu,Tyr) tyrosine kinase activity (Figure 2b) and α-CSK immunoreactivity (Figure 2g) were pooled.

Phenyl-Sepharose Column Chromatography. Pooled fractions from the hydroxylapatite column were adjusted to 1 M potassium phosphate (pH 7) and 0.1% Nonidet P40 and applied to a 50 mL phenyl-Sepharose column (2.5 × 23 cm) equilibrated with 1 M potassium phosphate in buffer A containing 25 mM Hepes (pH 7), 1 mM EDTA, 0.154 mg/mL dithiothreitol, 0.2 mg/mL benzamidine, 0.1 mg/mL phenylmethanesulfonyl fluoride, and 0.1% Nonidet P40. The column was washed with 50 mL of 1 M potassium phosphate (pH 7) in buffer A and eluted with a linear gradient (gradient size, 500 mL) of 1.0 to 0.0 M potassium phosphate in buffer A followed by a further 100 mL of buffer A. Fractions of 10 mL were collected, and those containing poly(Glu,Tyr) tyrosine kinase activity (Figure 2c) and α-CSK immunoreactivity (Figure 2h) were pooled. The pooled fractions from the phenyl-Sepharose column were concentrated using an Amicon PM-10 membrane with 40 psi nitrogen gas to a final volume of 10 mL.

Sephacryl S-200 Superfine Column Chromatography. A column of Sephacryl S-200 Superfine (2.1 × 90 cm) was equilibrated in buffer A containing 200 mM NaCl. The sample was applied to the column, and the column was eluted with the same buffer. Fractions of 6 mL were collected, and those containing poly(Glu,Tyr) tyrosine kinase activity

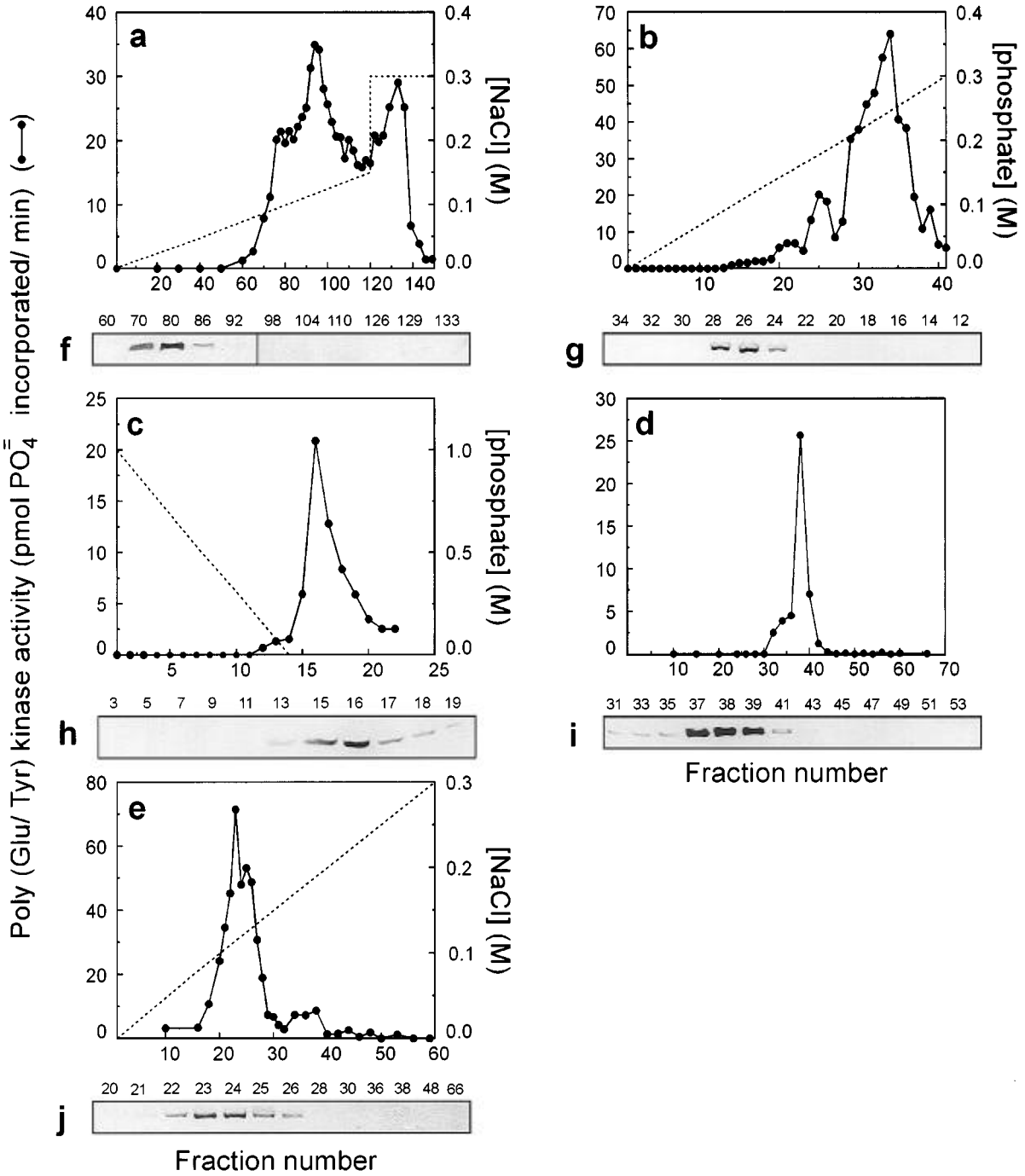

FIGURE 2: Coelution of a poly(Glu,Tyr) kinase activity with the α-CSK immunoreactivity from DEAE, hydroxylapatite, phenyl-Sepharose, S-200 gel filtration, and Mono-S cation exchange columns. Aliquots (5−10 μL) of the column fractions from DEAE (a and f), hydroxylapatite (b and g), phenyl-Sepharose (c and h), S-200 gel filtration (d and i) and Mono-S cation exchange column (e and j) chromatographic steps were assayed for poly(Glu,Tyr) kinase activity (a−e) and anti-CSK immunoreactivity by immunoblots (f−j).

(Figure 2d) and α-CSK immunoreactivity (Figure 2i) were pooled.

FPLC Mono-S Cation Exchange Column Chromatography. The pooled fractions of the Sephacryl S-200 Superfine column were dialyzed against buffer B which contained 25 mM Hepes (pH 7), 1 mM EDTA, 0.154 mg/mL dithiothreitol, 0.1 mg/mL phenylmethanesulfonyl fluoride, and 0.1% Nonidet P40, and the dialyzed sample was applied to a Pharmacia Mono-S HR5/5 cation exchange column pre-equilibrated with buffer B. The column was washed with 4 mL of buffer B and then eluted with a linear gradient (gradient size, 30 mL) of 0 to 0.3 M NaCl in buffer B. The column was further eluted with 0.5 M NaCl in buffer B. Fractions of 0.5 mL were collected, and those containing poly(Glu,Tyr) tyrosine kinase activity (Figure 2e) and α-CSK immunoreactivity (Figure 2j) were pooled.

FPLC Mono-Q Anion Exchange Column Chromatography. The pooled fractions of the Mono-S column were dialyzed against buffer C which contained 25 mM Hepes (pH 7), 0.154 mg/mL dithiothreitol, 0.1 mg/mL phenylmethanesulfonyl fluoride, 0.2 mg/mL benzamidine, and 0.1% Nonidet P40, and the dialyzed sample was applied to a Pharmacia Mono-Q HR5/5 cation exchange column pre-equilibrated with buffer C. The column was washed with 4 mL of buffer C and then eluted with a linear gradient (gradient size, 30 mL) of 0 to 0.3 M NaCl in buffer C. The column was further eluted

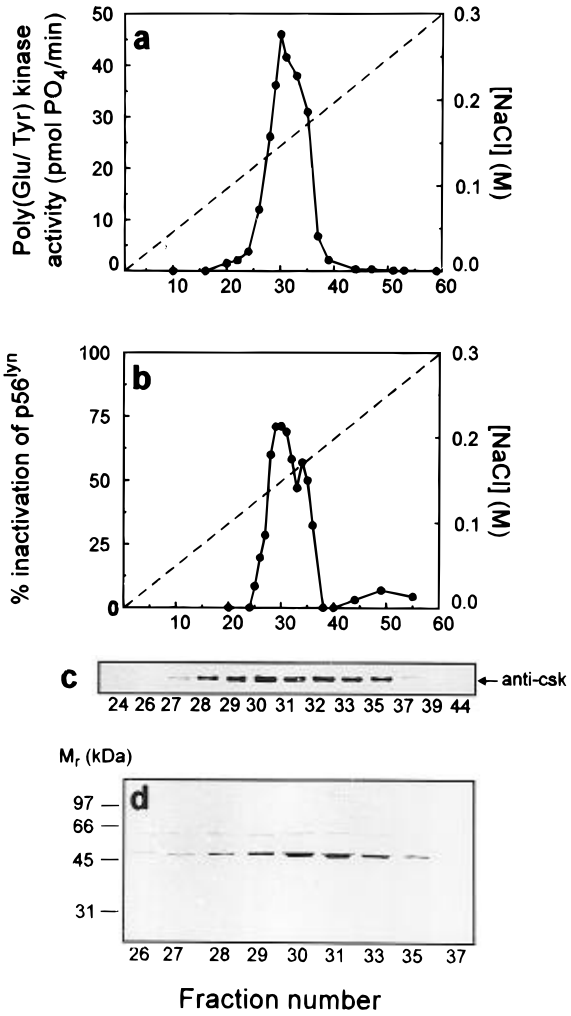

FIGURE 3: Poly(Glu,Tyr) kinase activity, α-CSK immunoreactivity, $p56^{lyn}$ inactivation, and protein profiles of the final step of purification of CSK from bovine thymus cytosol. The fractions from the Mono-S column (Figure 2e,j) displaying poly(Glu,Tyr) kinase activity and α-CSK immunoreactivity were pooled and applied to a Mono-Q anion exchange column. Proteins bound to the column were eluted with a salt gradient: (a) profile of the poly(Glu,Tyr) kinase activity, (b) profile of the ability of proteins in the column fractions to inactivate $p56^{lyn}$, (c) α-CSK immunoreactivity profile, and (d) Coomassie Blue-stained protein profile. Thirty microliters out of 0.5 mL from each of the indicated column fractions was analyzed for purity by SDS−PAGE and Coomassie Blue staining.

with 0.5 M NaCl in buffer C. Fractions of 0.5 mL were collected and assayed for poly(Glu,Tyr) tyrosine kinase activity (Figure 3a), α-CSK immunoreactivity (Figure 3c), and the ability to inactivate $p56^{lyn}$ (Figure 3b). The protein profile (Figure 3d) of the column fractions was determined by SDS−PAGE of aliquots of the column fractions followed by staining with Coomassie Blue and destaining.

RESULTS

Purification of CSK from Bovine Thymus Cytosol. Since lymphoid tissues such as spleen and thymus contain the most abundant quantity of CSK (Okada et al., 1991; Sabe et al., 1992), we attempted purification of CSK from bovine thymus. The cytosolic fraction of thymus was chosen because the majority of the α-CSK immunoreactivity was found in the cytosolic fraction (data not shown), and we wanted to investigate if the cytosolic CSK displays the same enzymatic properties as those previously reported for the membrane-bound CSK (Okada & Nakagawa, 1989; Okada et al., 1991).

Three assays were used in the purification procedures to monitor the elution of CSK from columns: (i) protein tyrosine kinase activity using the nonspecific substrate poly(Glu,Tyr), (ii) immunoblot assay using an α-CSK antibody, and (iii) a newly developed assay of the ability of CSK to inactivate the src-family tyrosine kinase $p56^{lyn}$. From the chromatographic profiles of the DEAE and hydroxylapatite columns, it is evident that CSK is not the predominant tyrosine kinase in bovine thymus cytosol because α-CSK immunoreactivity (Figure 2f,g) was detected only in column fractions corresponding to the minor poly(Glu,Tyr) tyrosine kinase activity peaks (Figure 2a,b). Consecutive chromatography of the thymus extract through the DEAE and hydroxylapatite columns effectively separated CSK from other protein tyrosine kinases. For the subsequent purification steps, the tyrosine kinase activity (Figures 2c−e and 3a) coeluted from the columns with the α-CSK immunoreactivity (Figures 2h−j and 3c). Characterization of the column fractions of the final purification steps shows that the α-CSK immunoreactivity (Figure 3c), the poly(Glu,Tyr) tyrosine kinase activity (Figure 3a), and the activity measured by the inactivation of $p56^{lyn}$ (Figure 3b) coeluted from the Mono-Q column, demonstrating that we purified a protein tyrosine kinase capable of inactivating $p56^{lyn}$ and cross-reacting with the α-CSK antibody. SDS−PAGE of the column fraction and then staining with Coomassie Blue revealed a major protein band of 50 kDa which strongly cross-reacted with the α-CSK antibody, indicating that CSK was purified to apparent homogeneity (Figure 3d).

Convenient Assay for the Ability of CSK To Inactivate Src-Family Tyrosine Kinases. The conventional method for assaying the CSK inactivation of src-family kinases requires separation of the src-family kinase from CSK by immunoprecipitation (Okada & Nakagawa, 1989; Bergman et al., 1992). The immunoprecipitated src-family kinase is then assayed with the heat- and acid-treated enolase as the substrate. After the phosphorylation reaction, enolase and the src-family kinase in the immunoprecipitate are separated by SDS−PAGE and the degree of phosphorylation of enolase is monitored by autoradiography. This method is quite cumbersome as it involves several manipulations. In this report, we describe a convenient method to assay the ability of CSK in inactivating src-family tyrosine kinases *in vitro*. This assay method has been successfully used to characterize the effects of CSK phosphorylation on the kinase activity of two transforming mutants of $pp60^{c-src}$ (Bjorge et al., 1995).

We have previously shown that the cdc2(6−20) peptide and a substitution analog [K^{19}]cdc2(6−20) derived from the cell cycle controlling protein kinase $p34^{cdc2}$ are specific and efficient substrates for src-family tyrosine kinases (Cheng et al., 1992; Litwin et al., 1991). When the [K^{19}]cdc2(6−20) peptide kinase activities of CSK, $p56^{lyn}$, and $pp60^{c-src}$ were assayed under the same conditions (incubation of each kinase with 100 μM [γ-^{32}P]ATP, 300 μM [K^{19}]cdc2(6−20), and the assay buffer at 30 °C for 6 min in a final volume of 50 μL), the specific enzyme activity of CSK [2.8×10^{-4} μmol of PO_3^{2-} incorporated min^{-1} (mg of kinase)$^{-1}$] was much lower than those of $p56^{lyn}$ (0.05 μmol of PO_3^{2-} incorporated min^{-1} mg of kinase)$^{-1}$] and $pp60^{c-src}$ (0.4 μmol of PO_3^{2-} incorporated min^{-1} mg of kinase)$^{-1}$]. Therefore, this peptide was used as the substrate for src-family kinases

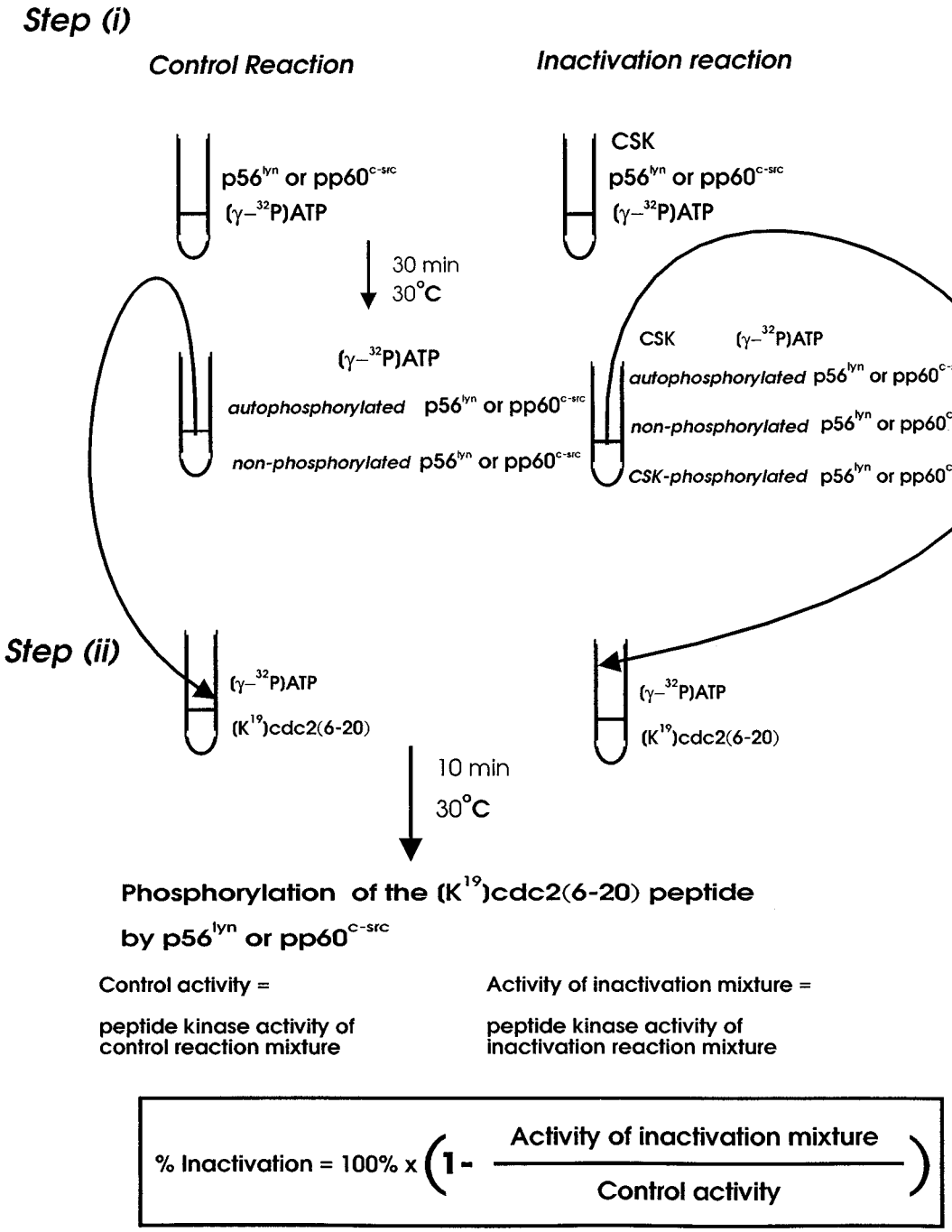

FIGURE 4: Schematic diagram showing the procedures of the assay of the ability of CSK to inactivate $p56^{lyn}$ and $pp60^{c-src}$. The assay was performed in two steps. In step (i), CSK phosphorylates $p56^{lyn}$ or $pp60^{c-src}$ on the consensus C-terminal regulatory tyrosine and thereby inactivates the kinases (inactivation reaction). As a control, CSK is omitted from the reaction (control reaction). After 30 min of incubation at 30 °C, the inactivation reaction mixture should contain the autophosphorylated, nonphosphorylated, and CSK-phosphorylated forms of $p56^{lyn}$ and $pp60^{c-src}$ in varying proportions. The control reaction mixture should contain the autophosphorylated and nonphosphorylated forms of $pp60^{c-src}$ or $p56^{lyn}$ only. In step (ii), an aliqout was taken from each of the reaction mixtures and it is added to a kinase activity assay mixture consisting of [γ-^{32}P]ATP, assay buffer, and the [K^{19}]cdc2(6−20) peptide which can only be phosphorylated by the active forms of $pp60^{c-src}$ and $p56^{lyn}$. The rate of [K^{19}]cdc2(6−20) peptide phosphorylation is a measure of the amount of the active forms of $pp60^{c-src}$ or $p56^{lyn}$ in the control and inactivation reaction mixtures. By comparison of the rate of phosphorylation of the peptide by the control reaction mixture (control activity) and that by the inactivation reaction mixture (activity of the inactivation mixture), the % inactivation which is a measure of the ability of CSK to inactivate $p56^{lyn}$ and $pp60^{c-src}$ can be calculated.

in our newly developed assay of the ability of CSK to inactivate src-family kinases.

This assay consists of two steps. In step (i), $p56^{lyn}$ or $pp60^{c-src}$ was incubated with ATP in the presence (inactivation reaction) and absence (control reaction) of CSK. In step (ii), the residual tyrosine kinase activity of $p56^{lyn}$ or $pp60^{c-src}$ was assayed by monitoring the rate of phosphorylation of the [K^{19}]cdc2(6−20) peptide (Figure 4). In the control reaction, $p56^{lyn}$ and $pp60^{c-src}$ can only undergo autophosphorylation. Our previous study and a study by Reuter et al. (Sotirellis et al., 1995; Reuter et al., 1990) have demonstrated that the autophosphorylation reaction follows an intermolecular mechanism, and therefore, the reaction is dependent upon the concentration of the nonphosphorylated src-family kinases. More importantly, autophosphorylation induces autoactivation of both $pp60^{c-src}$ and $p56^{lyn}$. Two reactions

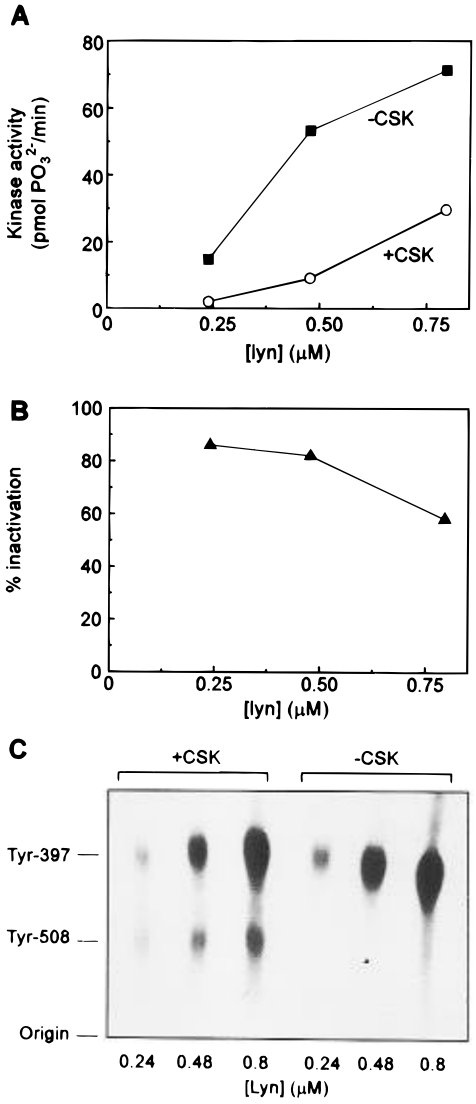

FIGURE 5: Effects of varying the $p56^{lyn}$ concentration in the inactivation reaction on its autophosphorylation, phosphorylation, and inactivation by CSK. (A) Kinase activity of $p56^{lyn}$ at the concentrations indicated in the presence and absence of CSK. (B) The data in panel A were plotted as % inactivation of $p56^{lyn}$ by CSK. (C) Tryptic phosphopeptide mapping of the autophosphorylation site and CSK-phosphorylation site of $p56^{lyn}$.

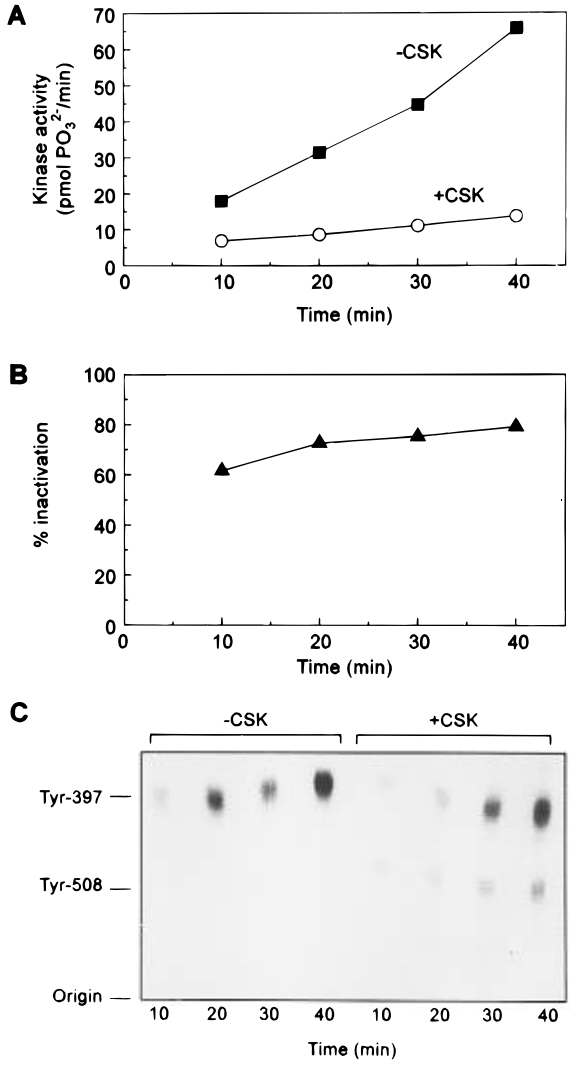

FIGURE 6: Effects of varying the time of incubation of $p56^{lyn}$ with CSK on its autophosphorylation, phosphorylation, and inactivation by CSK. (A) Kinase activity of $p56^{lyn}$ at the times indicated in the presence and absence of CSK. (B) The data in panel A were plotted as % inactivation of $p56^{lyn}$ by CSK. (C) Tryptic phosphopeptide mapping of the autophosphorylation site and CSK-phosphorylation site of $p56^{lyn}$.

occur in the inactivation reaction mixture: (i) autoactivation of $p56^{lyn}$ and $pp60^{c-src}$ by autophosphorylation (Sotirellis et al., 1995; Reuter, 1990) and (ii) phosphorylation and inactivation of the src-family kinases by CSK. The inactivation of both src-family kinases by CSK can be a result of the following mechanisms: mechanism 1, inactivation of the src-family kinases resulted from CSK phosphorylation of the tail tyrosine only; or mechanism 2, inactivation of the src-family kinases by CSK phosphorylation of their consensus C-terminal regulatory tyrosine residue, together with a consequential decrease in the effective concentration of the nonphosphorylated src-family kinases that can be activated by autophosphorylation. Experiments were designed to elucidate which of the above mechanisms is responsible for CSK inactivation of $p56^{lyn}$ *in vitro*.

In the absence of CSK, increasing the $p56^{lyn}$ concentration resulted in an increase in its rate of phosphorylation of [K^{19}]-cdc2(6−20). However, in the presence of CSK, the kinase activity of $p56^{lyn}$ was significantly suppressed (Figure 5A). The % inactivation of $p56^{lyn}$ decreased as its concentration increased (Figure 5B). With the increase in $p56^{lyn}$ concentration, the levels of both autophosphorylation and phosphorylation of its C-terminal regulatory tyrosine by CSK were elevated (Figure 5C), indicating that CSK is capable of phosphorylating $p56^{lyn}$ in a concentration-dependent manner. Furthermore, comparison of the levels of $p56^{lyn}$ autophosphorylation in the presence and absence of CSK reveals that autophosphorylation was significantly suppressed by CSK (Figure 5C).

In the absence of CSK, increasing the time of incubation of $p56^{lyn}$ with ATP led to an increase in the level of autophosphorylation and a concomitant increase in tyrosine kinase activity (Figure 6A,C). In the presence of CSK, both autophosphorylation and CSK phosphorylation increased with the time of incubation with ATP (Figure 6C). However, the increase in the extent of both autophosphorylation and tyrosine kinase activity was much attenuated in the presence of CSK (Figure 6A,C). The time-dependent increase in CSK phosphorylation and decrease in autophosphorylation account for the time-dependent increase in % inactivation of $p56^{lyn}$ (Figure 6B).

Taken together, our results support the notion that CSK phosphorylation of the C-terminal regulatory tyrosine residues of the src-family kinases results in inactivation and hence a decrease in the effective concentration of the nonphosphorylated src-family kinases that can be activated by autophosphorylation. Thus, both the tail tyrosine phosphorylation and the decreased efficiency of autophosphorylation contribute to the inactivation of p56^{lyn} by CSK, as decribed in mechanism 2.

Comparison of the Efficiency of Phosphorylation and Inactivation of pp60^{c-src} and p56^{lyn} by CSK. There are several reports on phosphorylation and inactivation of src-family tyrosine kinases, including pp60^{c-src}, p56^{lck}, p60^{yes}, p56^{lyn}, p59^{fyn}, and p56^{c-fgr}, by CSK *in vitro* (Okada & Nakagawa, 1989; Nada et al., 1991; Okada et al., 1991; Ruzzene et al., 1994). However, comparison of the efficiency of CSK in inactivating the various src-family tyrosine kinases has not been documented. The expression of pp60^{c-src} is ubiquituos with its high level of expression in the brain and platelets, while p56^{lyn} is expressed in placenta, platelets, and B-lymphocytes, with B-lymphocytes expressing the highest concentration of p56^{lyn}. The expression pattern of CSK and that of p56^{lyn} overlap significantly because CSK is also expressed predominantly in lymphoid tissues [see Courtneidge (1994) for review]. The expression patterns of CSK, p56^{lyn}, and pp60^{c-src} infer that CSK may not be the major upstream regulatory kinase of pp60^{c-src} *in vivo*. Furthermore, the overlapping expression pattern of CSK and p56^{lyn} strongly suggests that p56^{lyn} may be a preferred substrate of CSK *in vivo*. For this reason, we chose to compare the efficiency of phosphorylation and inactivation of p56^{lyn} and pp60^{c-src} by CSK. The newly developed assay for CSK inactivation of src-family kinases was used in this comparative study.

As shown in Figure 7, when equimolar amounts (0.48 μM) of p56^{lyn} and pp60^{c-src} were autophosphorylated in the absence of CSK, pp60^{c-src} displayed a kinase activity much higher than that of p56^{lyn}. Panels A and C of Figure 8 show the stoichiometry of phosphorylation of pp60^{c-src} and p56^{lyn} in the absence and presence of varying concentrations of CSK. In the absence of CSK, pp60^{c-src} and p56^{lyn} undergo autophosphorylation at Tyr-419 and Tyr-397, respectively (panels B and D of Figure 8). The extent of pp60^{c-src} autophosphorylation was twice that of p56^{lyn} autophosphorylation (panels A and C of Figure 8). Both the higher kinase activity and the higher degree of autophosphorylation of pp60^{c-src} indicate that its catalytic efficiency is higher than that of p56^{lyn}. In the presence of increasing concentrations of CSK, the extent of autophosphorylation of p56^{lyn} significantly decreased while phosphorylation of the tail tyrosine (Tyr-508) increased (Figure 8B). The result gives further support to our claim that CSK inactivation of p56^{lyn} is a consequence of two events: (i) CSK phosphorylation of Tyr-508 and (ii) decreased autophosphorylation of Tyr-397. For pp60^{c-src}, the increase in CSK concentration also resulted in an increase in phosphorylation of the tail tyrosine (Tyr-530). However, CSK was significantly more efficient in phosphorylating the tail tyrosine of p56^{lyn} than that of pp60^{c-src} (panels B and D of Figure 8).

The effect of CSK on pp60^{c-src} autophosphorylation (Tyr-419) was somewhat complicated. The extent of pp60^{c-src} autophosphorylation is higher when CSK was absent. However, when the concentration of CSK was increased from

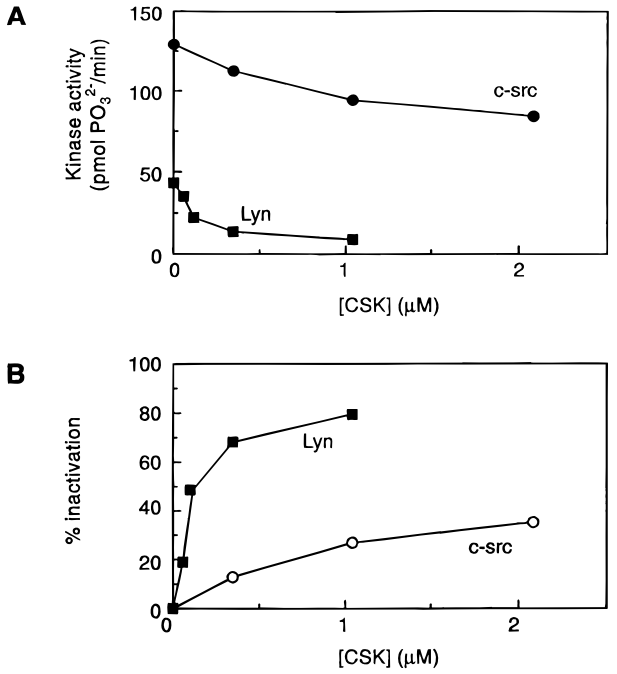

FIGURE 7: Comparison of the efficiency of inactivation of p56^{lyn} and pp60^{c-src} by CSK. (A) Kinase activity of p56^{lyn} and pp60^{c-src} in the absence and presence of the indicated concentrations of CSK. (B) The data in panel A were plotted as % inactivation of p56^{lyn} and pp60^{c-src} by the indicated concentrations of CSK.

0.35 to 2.08 μM, the extent of pp60^{c-src} autophosphorylation also increased. The reason for the enhanced autophosphorylation of pp60^{c-src} at higher CSK concentrations is not known. The result indicates that the decrease in kinase activity of pp60^{c-src} in the presence of CSK is solely a result of phosphorylation of its tail tyrosine (Tyr-430) by CSK (i.e. mechanism 1 as detailed in Convenient Assay for the Ability of CSK to Inactivate Src-Family Tyrosine Kinases).

Obviously, it is worthwhile to determine the K_m and V_{max} of CSK phosphorylation of p56^{lyn} and pp60^{c-src} to ascertain if the higher degree of phosphorylation of the tail tyrosine of p56^{lyn} by CSK was a result of a higher affinity of CSK for p56^{lyn} and/or a higher turnover number of CSK when p56^{lyn} was the substrate. As the phosphopeptide-mapping procedure used to differentiate autophosphorylation and C-terminal tail tyrosine phosphorylation in the two src-family kinases involves several consecutive manipulations of the phosphorylated kinases, accurate kinetic analyses of phosphorylation of the tail tyrosine in p56^{lyn} and pp60^{c-src} by CSK are currently technically unattainable. We will generate mutants of p56^{lyn} and pp60^{c-src} with the consensus autophosphorylation site replaced by a phenylalanine and conduct kinetic analyses of CSK phosphorylation of these mutants.

Comparison of the Efficiency of Phosphorylation of Synthetic Peptides Derived from the Carboxyl-Terminal Portion of Src-Family Kinases. We were interested in elucidating the structural basis for the difference in efficiency of phosphorylation of pp60^{c-src} and p56^{lyn} by CSK. We turned to the sequences around the tail tyrosine because they may contain structural features responsible for the differential phosphorylation of p56^{lyn} and pp60^{c-src} by CSK. Although the C-terminal portions of pp60^{c-src} and p56^{lyn} share a very high degree of sequence similarity, major differences can be found in the sequence downstream from the C-terminal regulatory tyrosine of p56^{lyn} (Y^{508}-QQQP) and that of

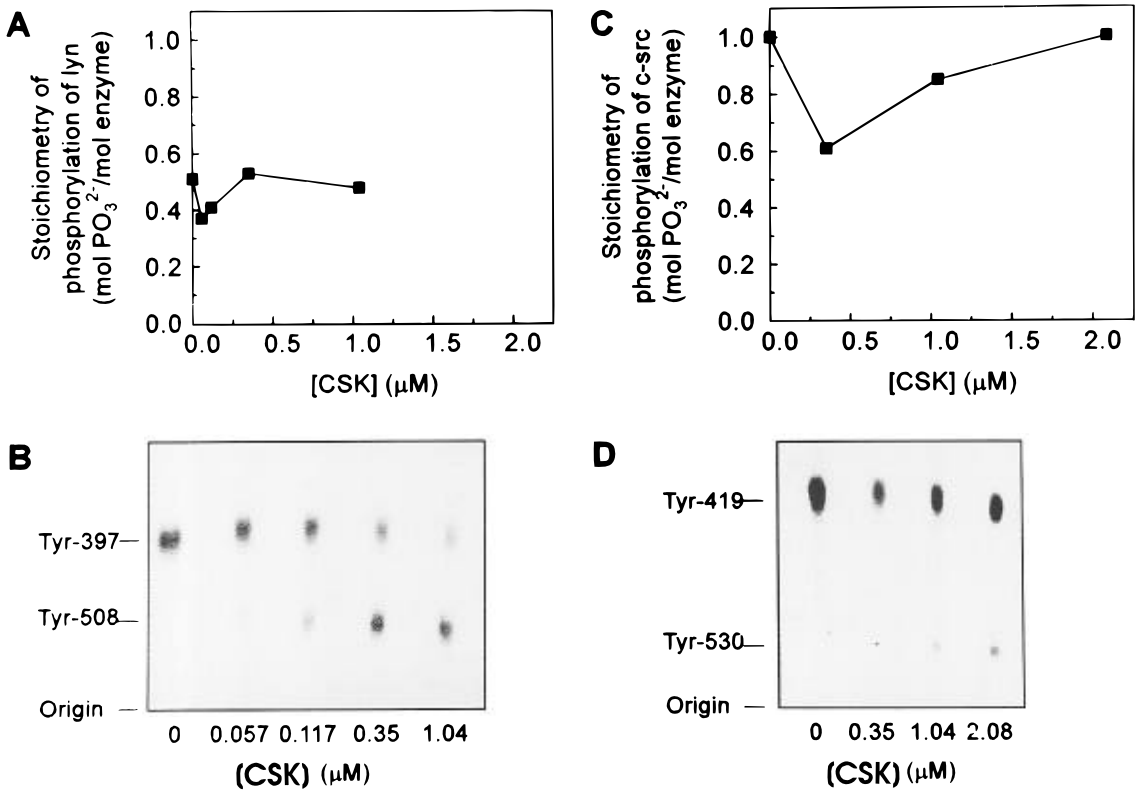

FIGURE 8: Comparison of the efficiency of phosphorylation of $p56^{lyn}$ and $pp60^{c-src}$ by CSK. (A and C) Stoichiometry of $p56^{lyn}$ and $pp60^{c-src}$ (0.48 μM) phosphorylated in the absence and presence of the indicated concentrations of CSK. (B) Tryptic phosphopeptide mapping of the autophosphorylation site (Tyr-397) and the tail tyrosine (Tyr-508) of $p56^{lyn}$ phosphorylated in the absence and presence of the indicated concentrations of CSK. (D) Tryptic phosphopeptide mapping of the autophosphorylation site (Tyr-419) and the tail tyrosine (Tyr-530) of $p60^{c-src}$ phosphorylated in the absence and presence of the indicated concentrations of CSK.

$pp60^{c-src}$ (Y^{530}-QPGENL). To examine if the differences account for the differential phosphorylation and inactivation of $p56^{lyn}$ and $pp60^{c-src}$ by CSK, we synthesized a lyn C-terminal peptide, lyn(480−512), and a fyn C-terminal peptide, fyn(503−537). The sequence of the C-terminal portion of $p59^{fyn}$ differs from that of $pp60^{c-src}$ in that residue 526 of $p59^{fyn}$ is an alanine while a serine residue is located at the corresponding position in $pp60^{c-src}$ (Figure 1). Since fyn(503−537) is almost identical to the C-terminal portion of $pp60^{c-src}$, it can therefore be treated as a peptide derived from the C-terminal portion of $pp60^{c-src}$.

The degree of phosphorylation of the same concentrations (0.48 μM) of lyn(480−512), fyn(503−537), $p56^{lyn}$, and $pp60^{c-src}$ by CSK was compared. No phosphorylation of the two peptides was detected (data not shown), while detectable phosphorylation of Tyr-430 in $pp60^{c-src}$ and very significant phosphorylation of Tyr-508 of $p56^{lyn}$ by CSK were noticed (Figure 8), indicating that the two peptides were much poorer substrates of CSK than the two src-family kinases.

Kinetic analysis shows that, while both peptides were poor substrates for CSK, lyn(480−512) was phosphorylated by CSK with a slightly higher efficiency. Lyn(480−512) was phosphorylated by CSK with a K_m of 3.3 mM and a V_{max} of 19.2 pmol of PO_3^{2-} incorporated min^{-1} (μg of enzyme)$^{-1}$, while fyn(503−537) was phosphorylated with a similar V_{max} but a higher K_m (6.7 mM). The lower K_m of lyn(480−512) phosphorylation by CSK suggests that the YQQQP motif of $p56^{lyn}$ was preferentially recognized by CSK and it may contribute to the higher efficiency displayed by CSK in its phosphorylation and inactivation of $p56^{lyn}$ *in vitro*. However, the poor kinetic parameters of phosphorylation of lyn(480−512) indicate that the C-terminal 480−512 segment is not sufficient to direct the preferential phosphorylation of $p56^{lyn}$ by CSK.

Additional Evidence That Synthetic Peptides Derived from the C-Terminal Portion of Src-Family Kinases Contain Structural Features Recognized by CSK as Substrate Specificity Determinants. The kinetic parameters of CSK phosphorylation of lyn(480−512) and fyn(503−537) suggest that the local structure around the tail tyrosine contributes to the efficient phosphorylation of src-family kinases by CSK. Relevant to this suggestion, an activated transforming mutant $pp60^{c-src}$ with a single-point mutation (Glu-527 → Lys) within the C-terminal regulatory domain was found to be a very poor substrate of CSK (Bjorge, 1995), indicating that Glu-527, located three residues from the tail tyrosine, is recognized by CSK as a substrate specificity determinant.

To investigate further if the local structures around the consensus C-terminal regulatory tyrosine in src-family kinases are recognized by CSK as substrate specificity determinants, we compared the rates of phosphorylation of lyn(480−512) and fyn(503−537) by CSK with those of the synthetic peptide derived from the autophosphorylation site of the src-family kinases and [K^{19}]cdc2(6−20). As shown in Table 1, among the four substrate peptides used in the study, only the lyn(480−512) and fyn(503−537) peptides were preferentially phosphorylated by CSK. Among the four synthetic peptides tested, lyn(480−512) and fyn(503−537) contain more than one tyrosine residue. To ascertain if the higher degrees of phosphorylation of the two peptides by CSK were the result of nonspecific phosphorylation of all

Table 1: Comparison of the Relative Rate of Peptide Phosphorylation by CSK

peptide substrate	sequence	enzyme activity[a] (pmol of PO_3^{2-} incorporated/min)
fyn(503−537)	WKKDPEERTFEYLQSFLEDYFTATEPQYQPGEDL	0.33 (18.2)
LYN(480−512)	WKEKAEERPTFDYLQSVLDDFYTATEGQYQQQP	0.34 (19.1)
src-autophosphorylation site peptide	ADFGLARLIEDNEYTARQG	0.018 (1)
[K^{19}]cdc2(6−20)	KVEKIGEGTYGVVKK	0.074 (4.1)

[a] The number in each set of parentheses indicates the peptide kinase activity relative to that of phosphorylation of the src-autophosphorylation site peptide.

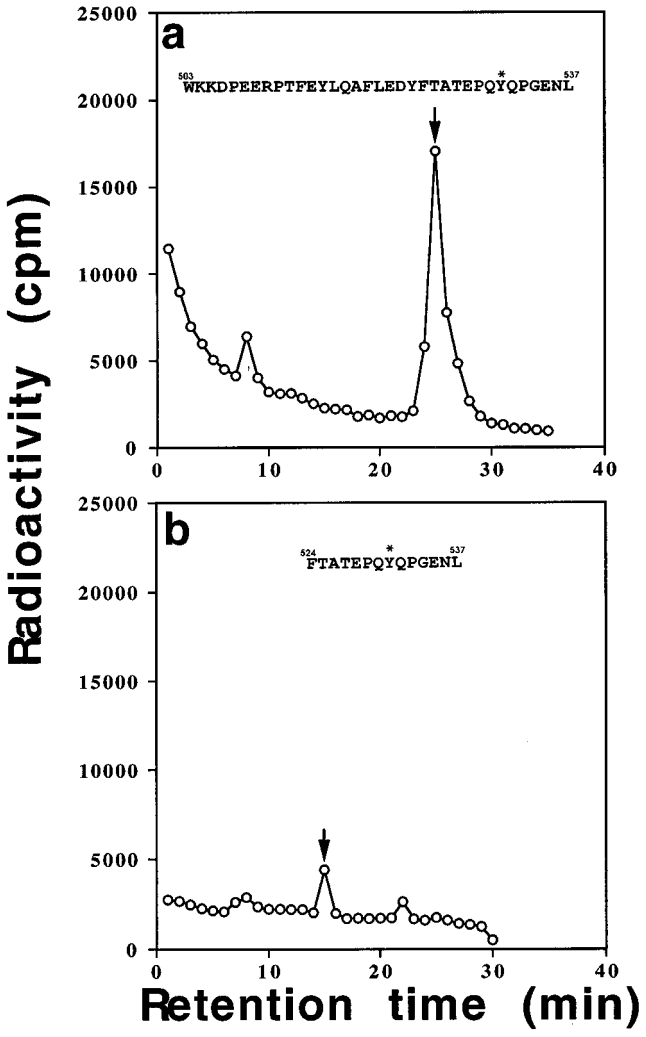

FIGURE 9: HPLC analyses of the degree of phosphorylation of Fyn-(503−537) and fyn(524−537) by CSK. As fyn(524−537) lacks basic amino acid residues which are essential for binding to Whatman P81 phosphocellulose paper used in the standard tyrosine kinase assay (Casnellie, 1991), the efficiencies of peptide phosphorylation were measured by the HPLC method. The peptides (300 μM) were phosphorylated by CSK (3.1 μM) and subsequently processed with C_{18} Sep-Pak cartridges to remove the free ATP before they are chromatographed on an analytical reverse phase C_{18} HPLC column. The degree of phosphorylation of each peptide was monitored by Cerenkov counting of the HPLC fractions. The retention times of the peptides eluted from the column are indicated by arrows. The insets show the amino acid sequences of fyn(503−537) and fyn(524−537). The tail tyrosine (Tyr-531) in the sequences is marked by an asterisk.

three tyrosine residues in the peptides, we compared the rates of phosphorylation of fyn(503−537) and the truncation analog fyn(524−537) by CSK. The degree of phosphorylation of fyn(524−537) by CSK was 8% of that of fyn(503−537) (Figure 9), suggesting that the structural features enhancing phosphorylation of the Tyr-531 by CSK are present in the 503−523 segment of fyn(503−537). Alternatively, the higher rate of CSK phosphorylation of fyn(503−537) might be a result of an unexpected preferential phosphorylation of Tyr-515 and/or Tyr-523 by CSK.

To differentiate between these two possibilities, we conducted phosphopeptide mapping to identify the site of CSK phosphorylation in fyn(503−537). Fyn(503−537) was phosphorylated by CSK using [γ-^{32}P]ATP, and the radioactive phosphopeptide was purified by reverse phase HPLC using a C_{18} reverse phase column. The purified phosphopeptide was exhaustively digested by chymotrypsin. Figure 10a shows the potential cleavage sites of chymotrypsin of the peptide. Glu^{514}-Tyr^{515}-Leu^{516}, Glu^{521}-Asp-Tyr^{523}-Phe^{524}, and Thr^{525}-Ala-Thr-Glu-Pro-Gln-Tyr^{531}-Gln-Gly-Glu-Asp-Leu^{537} are the putative phosphotyrosine-containing peptide fragments that can be generated from chymotryptic digestion of the CSK-phosphorylated fyn(503−537). As shown in Figure 10b, only one phosphopeptide fragment was generated, indicating that only one of the three tyrosine residues in fyn(503−537) was phosphorylated by CSK. The chymotryptic phosphopeptide fragment was subjected to solid phase sequencing for determination of the phosphorylation site (Abersold et al., 1991; Wettenhall et al., 1991). The data shown in Figure 10c revealed that most of the radioactivity in the phosphopeptide fragment appeared in the seventh cycle of Edman degradation. Only the fragment corresponding to residues 525−537 has a tyrosine at the seventh position from the N-terminus. Thus, Tyr-531, corresponding to the consensus tail tyrosine of p59^{fyn}, was the only amino acid residue in fyn(503−537) phosphorylated by CSK. From these results, we conclude that the fyn(503−537) peptide contains some structural features directing CSK to target the consensus tail tyrosine.

In summary, the results shown in Table 1, Figure 9, and Figure 10 indicate that the structural features enhancing phosphorylation of the tail tyrosine by CSK are present in both the C-terminal regulatory domain and the adjacent part of the kinase domain of the src-family kinases.

DISCUSSION

In the present study, we have developed a novel assay for the ability of CSK to inactivate src-family tyrosine kinases. Using inactivation of p56^{lyn} by CSK as a model, we demonstrated that the ability of CSK to inactivate a src-family kinase correlates with the degree of CSK phosphorylation of the C-terminal regulatory tyrosine of src-family kinases. We then used this newly developed assay to show that CSK displays a preference for p56^{lyn} over pp60^{c-src} in terms of its ability to inactivate them. Using a phosphopeptide-mapping method, we provide qualitative evidence that the preferential inactivation of p56^{lyn} by CSK is a result

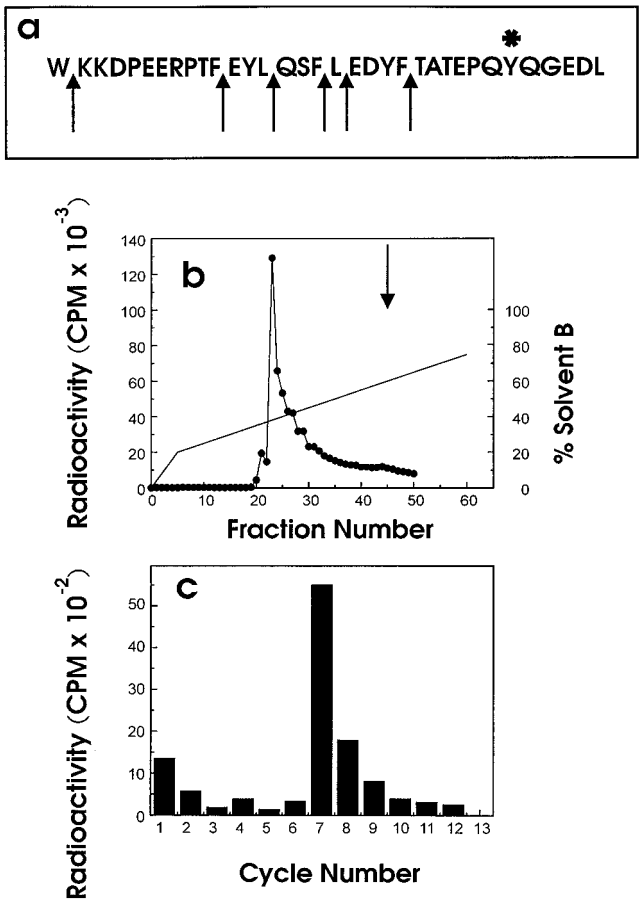

FIGURE 10: Determination of the site of phosphorylation in fyn(503−537) phosphorylated by CSK. Fyn(503−537) was phosphorylated by CSK and subsequently processed with a C_{18} Sep-Pak cartridge to remove the free ATP. The phospho-fyn(503−537) was purified by reverse phase HPLC with a Vydac 218TP54 C_{18} column as described in Experimental Procedures. The purified phosphopeptide was exhaustively digested by chymotrypsin. The phosphopeptide fragment from the chymotryptic digest was purified by reverse phase HPLC. (A) Potential chymotryptic cleavage sites in fyn(503−537) as marked by the arrows. Tyrosine was not included because phosphotyrosine is not a cleavage site for chymotrypsin. (B) The radioactivity profile of HPLC of the chymotryptic digest. The arrow indicates the fraction at which the phospho-fyn(503−537) would be eluted. (C) Solid phase sequencing of the phosphopeptide fragment. Radioactivity associated with the PTH-amino acid generated in each cycle of Edman degradation was measured by Cerenkov counting.

of its ability to phosphorylate the tail tyrosine of p56^{lyn} with greater efficiency. It would be worthwhile to determine the kinetic parameters of CSK phosphorylation of the tail tyrosine of p56^{lyn} and pp60^{c-src}. However, because of the number of steps and manipulations necessary to separate the phosphopeptide containing the autophosphorylation site and that containing the tail phosphotyrosine, an accurate kinetic analysis of the phosphorylation reaction was technically unattainable.

Our study on CSK phosphorylation of synthetic peptides modelled after the C-terminal portion of p56^{lyn} and pp60^{c-src} (and p59^{fyn}) indicates that the C-terminal regulatory domain and an adjacent part of the kinase domain contain some, but not all, of the structural features dictating the different efficiencies of CSK in phosphorylating p56^{lyn} and pp60^{c-src}. Further study with these synthetic peptides reveals that the C-terminal regulatory domain and an adjacent part of the kinase domain contain structural features directing CSK to phosphorylate the consensus tail tyrosine exclusively.

The most interesting finding of our study is the difference in efficiency of phosphorylation and inactivation of pp60^{c-src} and p56^{lyn} by CSK. Our result suggests that p56^{lyn} is a preferred substrate of CSK *in vivo*. The extensive overlap in tissue distribution of CSK and p56^{lyn} gives further support to this suggestion [see Courtneidge (1994) for review]. Moreover, Hata et al. (1994) reported that p56^{lyn} became constitutively active and highly phosphorylated at the autophosphorylation site in CSK-negative chicken B-cell clones, providing further evidence for the functional interaction between CSK and p56^{lyn} *in vivo*.

The tissue distribution of pp60^{c-src} differs significantly from that of CSK in adult mammals. The brain and platelets express the most abundant amount of pp60^{c-src}, while CSK expression in the brain is very low, strongly suggesting that pp60^{c-src} is not phosphorylated and regulated by CSK but instead by other CSK-related tyrosine kinases *in vivo*. All other CSK-related tyrosine kinases, including Matk, Ctk, and Ntk, are expressed predominantly or exclusively in the brain; it would be worthwhile to purify these kinases and examine if they display any substrate preference for pp60^{c-src}. A comparison of the substrate specificity of CSK, Ctk, Matk, and Ntk will provide us with clues to the identity of the upstream regulatory kinase(s) for each of the src-family members. Moreover, it is important to identify the structural features in each of the src-family kinases dictating their differential phosphorylation by CSK or other CSK-related kinases. The inactivation assay introduced in this report will be useful for the comparative study.

The very poor kinetic parameters of CSK phosphorylation of lyn(480−512) indicate that other portions of the src-family kinases, including the unique, SH3, SH2, and kinase domain, may also contribute to the efficient phosphorylation of p56^{lyn} by CSK *in vitro*. Moreover, the mere 2-fold difference in K_m values of CSK phosphorylation of lyn(480−512) and fyn(503−537) cannot fully account for the significantly higher efficiency of CSK in phosphorylating p56^{lyn} and pp60^{c-src}, further suggesting that structural features in other regions of p56^{lyn} must also contribute to its preferential phosphorylation by CSK. Relevant to this suggestion, Bougeret et al. (1996) demonstrated that CSK phosphorylation of the p56^{lck} tail tyrosine was enhanced by p56^{lck} autophosphorylation. The enhancement was possibly a result of interaction of the two kinases mediated by the CSK SH2 domain and the pYTAR motif of the p56^{lck} autophosphorylation site. It is obvious from Figure 8 that the lower efficiency of CSK phosphorylation of pp60^{c-src} was not a consequence of a lower efficiency of p60^{c-src} to undergo autophosphorylation because pp60^{c-src} was autophosphorylated to a higher extent than p56^{lyn} in the CSK-phosphorylation reaction. The presence of the pYTAR motif in the autophosphorylation site of pp60^{c-src} and p56^{lyn} suggests that the autophosphorylated forms of both kinases have the intrinsic ability to bind the SH2 domain of CSK; whether the interaction between CSK and the src-family kinases will occur may depend upon the accessibility of the pYTAR motif in the src-family kinases. We therefore postulate that the difference in accessibility of this pYTAR motif in pp60^{c-src} and p56^{lyn} may account for their differential phosphorylation by CSK. Future attempts to elucidate the structural basis of substrate specificity of CSK should include an examination of the

ability of autophosphorylated $pp60^{c-src}$ and $p56^{lyn}$ to bind the CSK SH2 domain.

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