We produced and purified recombinant proteins in the systems which allow detection of transient interaction. Binding of Csk to Src kinases has been detected. We family protein tyrosine kinases. Up to now, no stable regulatory, carboxyl-terminal tyrosine of most of the Src genes: c-src, c-yes, fyn, lyn, lck, blk, hck, c-fgr, yrk (1). The kinase activity of Src PTKs is regulated by phosphorylation of two highly conserved tyrosine (Tyr) residues. One of these residues, located in the catalytic domain, is involved in positive regulation of the kinase activity upon autophosphorylation (2). Contrary to their viral counterparts, the products of the cellular genes have their activity repressed by phosphorylation of their COOH-terminal Tyr (3) which probably generates a binding domain for their own Src homology 2 (SH2) domain, thereby placing the molecule in an inactive conformation (4, 5). A widely expressed PTK isolated initially by Okada and Naka-gawa in 1988, Csk, has been shown in vitro to phosphorylate the negative regulatory Tyr of c-Src (6, 7), Lyn, Fyn (8), Lck (9), and c-Fgr (10).

Csk is related to Src PTKs but does not contain a myristylation site, or the conserved autophosphorylated Tyr and the regulatory COOH-terminal Tyr (6, 11). Thus, the mechanism by which Csk function is regulated is not known. Similar to Src kinases, Csk contains one SH3 and one SH2 domain, which both are found in a number of catalytic and non catalytic signal transduction molecules and mediate protein-protein interactions (12). In Src kinases, the SH2 domain is considered to be involved in the intramolecular suppression of the kinase activity (4, 5) and in the binding to sequence specific tyrosine phosphorylated proteins (13, 14), whereas the SH3 domain is involved in the recruitment of substrates containing proline-rich sequences (15).

Several systems provide evidence for the involvement of Csk in the regulation of cell activation through down-regulation of Src PTKs. Thus in vivo, (i) overexpression of Csk in v-Crk/c-Src transformed fibroblast cells causes reversion to normal phenotype (16), (ii) overexpression of Csk in a T cell line inhibits TCR-induced tyrosine protein phosphorylation and lymphokine production (17), (iii) the kinase activity of Lyn is constitutively activated in Csk-negative B-cell clones (18), and (iv) coexpression of Csk counteracts cell death caused by expression of c-Src in Schizosaccharomyces pombe (19) and Saccharomyces cerevisiae (20). Furthermore, Csk-deficient mouse embryos are developmentally arrested at the somite stage, and the kinase activity of several members of the Src family is greatly enhanced in these embryos (21, 22).

Although Src is a substrate of Csk, no stable binding of Csk to c-Src or v-Src has been detected (11, 23). Therefore, we postulated that the binding of Src PTKs to Csk must be transient with a rapid off-rate, and decided to study the interaction between Csk and one of its substrate Lck, a Src PTK involved

The COOH-terminal Src kinase (Csk) is responsible for the phosphorylation of the conserved, negative regulatory, carboxyl-terminal tyrosine of most of the Src family protein tyrosine kinases. Up to now, no stable binding of Csk to Src kinases has been detected. We therefore decided to analyze this interaction using two systems which allow detection of transient interaction. We produced and purified recombinant proteins in the glutathione S-transferase prokaryotic expression system. First, using real-time biospecific interaction analysis (BIAcore™), we detected in vitro a specific interaction between Csk and one of its substrates Lck, a lymphocyte-specific member of the Src family. This interaction requires the autophosphorylation of Lck on tyrosine 394 (the phosphorylation of which is correlated with an increase of the kinase activity) and involves a functional Csk SH2 domain. Second, using the yeast two-hybrid system, we confirmed in vivo the physical interaction between Csk and Lck. Furthermore, in vitro we showed that autophosphorylation of Lck on tyrosine 394 enhances the phosphorylation of Lck by Csk on the negative regulatory site, tyrosine 505, suggesting that activated Lck serves preferentially as substrate for Csk. These findings might explain the mechanism(s) by which Csk interacts with most of Src kinases to down-regulate their kinase activity.

The protein tyrosine kinases (PTKs) of the Src family are nonreceptor kinases which are involved in cell proliferation and differentiation. This family comprises nine proto-oncogenes: c-src, c-yes, fyn, lyn, lck, blk, hck, c-fgr, yrk (1). The kinase activity of Src PTKs is regulated by phosphorylation of two highly conserved tyrosine (Tyr) residues. One of these residues, located in the catalytic domain, is involved in positive regulation of the kinase activity upon autophosphorylation (2). Contrary to their viral counterparts, the products of the cellular genes have their activity repressed by phosphorylation of their COOH-terminal Tyr (3) which probably generates a binding domain for their own Src homology 2 (SH2) domain, thereby placing the molecule in an inactive conformation (4, 5). A widely expressed PTK isolated initially by Okada and Nakagawa in 1988, Csk, has been shown in vitro to phosphorylate the negative regulatory Tyr of c-Src (6, 7), Lyn, Fyn (8), Lck (9), and c-Fgr (10).

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**Detection of a Physical and Functional Interaction between Csk and Lck Which Involves the SH2 Domain of Csk and Is Mediated by Autophosphorylation of Lck on Tyrosine 394**

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in T cell signaling (24), using real-time biospecific interaction analysis (BIAcore™) (25). Toward this end, we produced recombinant Csk and Lck proteins in bacteria using the glutathione S-transferase (GST) expression system (26). We demonstrated that Csk interacts physically with Lck through its SH2 domain and that this interaction requires the phosphorylation of Tyr394 of Lck. We confirmed the interaction between Csk and Lck in vivo using the yeast two-hybrid system which also allows to detect transient interaction (27). Furthermore, through in vitro phosphorylation assays, we demonstrated that Lck phosphorylation on Tyr505 by Csk is enhanced by autophosphorylation of Lck on Tyr394.

EXPERIMENTAL PROCEDURES

Expression and Purification of GST Fusion Proteins—Wild-type (WT) full-length or point-mutated (Y394F and Y505F) full-length or point-mutated (K273E) deleted from the first 33 amino acid residues human LCK cDNAs were subcloned in the pGEX-2T prokaryotic expression system as described previously (28). Wild-type full-length or wild-type deleted (SH3 domain, SH2 domain, and SH3/SH2 domains) rat csk cDNAs (29) and mutated (S108C) deleted (SH2 domain) chicken csk cDNAs (30) were subcloned in the pGEX-2T vector. The expression and purification of GST fusion proteins were performed as described elsewhere (29). Purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and the concentrations of the recombinant proteins were estimated by Coomassie Blue staining.

The Csk-Lck Interaction with the BIAcore™—The principle of operation of the BIAcore™ biosensor (Pharmacia BiOsensor AB, Uppsala, Sweden) has been described previously (25). Purified Csk (without GST) was immobilized within the flow-cell matrix as described previously (31) (as shown in Brunati et al. (32)). Two hybrid System Methods—S. cerevisiae strain L40 ([MATa trp1 leu2 his3 lys2-801 trp1 leu2 his3 URA3::lexA-lacZ]) (33) was grown at 30°C in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose. The full-length rat csk cDNA was fused to the DNA-binding domain of (LexA-DB) in plJ10, pBMT116 plasmid (34) in which the frame of the cloning site has been modified.2 Human LCK cDNA was cloned in fusion with Gal4-activating domain (Gal4-AD; pGAD-GH) (35). Plasmid DNA transformations were carried out using the lithium acetate method (36). Double transformants were plated on yeast drop-out medium lacking tryptophan, histidine, and leucine, and histidine, or on Whatman 40 filters and tested for α-galactosidase activity (37). Plasmids pLexA-Ras2-12 (33) and pGAD-Raf (38) were used as controls.

In Vitro Kinase Assay—In vitro phosphorylation of c-Fgr: 500 nM of purified c-Fgr (39) was incubated in a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 100 μM of cold ATP for 30 min at 30°C. In vitro phosphorylation of GST-Lck.WT by Csk: 100 nM of purified GST-Lck.WT was incubated with 100 nM of purified recombinant Csk (without GST) and with various concentrations of Csk-Y394 peptides (Y394: H-RILIEDENY™STAREGAK-OH and pY394: H-RILIEDENY™PO2JSTAREGAK-OH). The reaction was terminated by the addition of SDS-PAGE buffer. The labeled peptides were analyzed by autoradiography. The labeled peptides were excised from the gel, and radioactivity was determined by Cerenkov radiation.

Tryptic Phosphopeptide Mapping—2–25P-Labeled labeled corresponding to in vitro phosphorylated GST-Lck.WT by Csk was excised from polyacrylamide gel. The slice of the dried gel was rehydrated with 200 μl of 50 mM Tris-HCl, pH 8.8, and exhaustively digested with trypsin (50 μg).

RESULTS

Production and Purification of Csk, Lck, and c-Fgr—In our study, we used Lck and Csk purified as recombinant proteins using the GST prokaryotic expression system which allows production of highly pure and active fusion proteins (as shown in Jullien et al. (28) and Bougeret et al. (29)). We purified full-length Lck wild-type, or with a point mutation in the autophosphorylation site (Y394F), or with a point mutation in the negative regulatory site (Y505F), or with a point mutation in the ATP binding site (K273E) (28). In addition, we also purified full-length Csk, and Csk SH3 and SH2 domains either alone or combined, wild-type or with a point mutation in the SH2 domain (S108C) which abolishes interaction with phosphotyrosine proteins (29, 30). Another Src PTK used in this study, c-Fgr, has been purified to homogeneity from rat spleen (as shown in Brunati et al. (39)).

We have previously shown that with the exception of the inactive mutant (GST-Lck.K273E), all other GST-Lck proteins were tyrosine phosphorylated in vivo in bacteria due to autophosphorylation. We have previously determined that Tyr394 was phosphorylated in the GST-Lck.WT and GST-Lck.Y505F mutant, and Tyr505 was phosphorylated in the GST-Lck.WT and GST-Lck.Y394F mutant (28). Therefore, in our binding and phosphorylation experiments, these GST-Lck proteins were used without prior in vitro phosphorylation. On the contrary, the purified c-Fgr was not tyrosine-phosphorylated in vivo. It has been shown that c-Fgr auto-phosphorylates in vitro on the conserved autophosphorylation site, Tyr402 (10). Thus, for our binding studies, we used c-Fgr either nonphosphorylated or after in vitro autophosphorylation.

Binding of Recombinant GST-Lck and Purified c-Fgr to Immobilized Csk—Csk-Lck interaction was studied in real time using a biosensor instrument, BIAcore™ (Pharmacia), that relies upon surface plasmon resonance (SPR) to measure changes in refractive index upon binding of a soluble analyte (GST-Lck) to an immobilized ligand (Csk). The SPR signal is expressed as a sensorgram, in RU plotted as a function of time (25). The SPR signal displays in overlay format the real-time measurement of association and dissociation phases of different GST-Lck proteins with immobilized full-length Csk. In order to avoid protein dimerization through GST/GST interaction, we immobilized a purified Csk protein treated with thrombin which removes the GST part of the recombinant protein. During the injection of GST-Lck (association phase), binding of GST-Lck to Csk can be followed as a gradual rise of the resonance signal. When buffer replaces GST-Lck solution, dissociation of the GST-Lck/Csk complex can be seen (dissociation phase). The difference between the baseline value and the value obtained at the end of the injection indicates binding of GST-Lck proteins to Csk surface. We observed that GST-Lck.WT and GST-Lck.Y505F proteins bound to immobilized Csk (350 and 940 RU, respectively). In contrast, we did not detect significant binding of the inactive GST-Lck.K273E mutant (10 RU). Since this mutant is not phosphorylated on tyrosine, this suggests that Csk-Csk interaction requires previous tyrosine phosphorylation of Lck. In addition, no significant binding with the GST-Lck.Y394F mutant which does not contain the Tyr394 was observed (20 RU), indicating that the previous tyrosine phosphorylation of Lck which is required for binding of Lck to Csk might occur on Tyr394.

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ously shown that GST-Lck.Y505F purified from bacteria is more phosphorylated on Tyr$^{394}$ than GST-Lck.WT (28). This might explain the higher binding of the mutant form of Lck (940 RU) compared to the wild-type form (350 RU), and is further evidence for the involvement of Tyr$^{394}$ of Lck in the interaction with Csk. Altogether, these results suggest that Lck interacts with Csk via its phosphorylated Tyr$^{394}$ residue.

In a search for modified sequences surrounding the autophosphorylation site of Src family PTKs, we noticed that c-Fgr is the only Src PTK to have a different motif at the autophosphorylation site (Tyr-Asn-Pro-Cys instead of Tyr-Thr-Ala-Arg) (41). Therefore we tested the interaction of purified c-Fgr with immobilized Csk (Fig. 1B). We did not detect any binding of c-Fgr to Csk either nonphosphorylated (30 RU) or after in vitro autophosphorylation (32 RU), whereas, on the same Csk surface, a strong binding of GST-Lck.Y505F was measured (750 RU). This suggests that the conserved sequence at the autophosphorylation site is required for Lck interaction with Csk.

To determine which part of Csk was involved in the interaction with Lck, we injected GST-Lck.Y505F either alone or pre-
incubated with different subdomains of Csk over a Csk immobilized surface. The results are summarized in Table I. Complete inhibition of the binding was observed when GST-Lck.Y505F was preincubated either with full-length Csk (purified without GST) or with GST-Csk SH3/SH2 fusion protein. However, the recombinant GST-Csk SH3/SH2.S108C protein with the point-mutation in the SH2 domain which abolishes its binding to phosphotyrosine proteins (30), did not prevent Lck interaction with Csk. Furthermore the binding was not affected by preincubation of GST-Lck.Y505F with GST-Csk SH3 fusion protein. These results suggest that Csk interacts with Lck via its SH2 domain. Surprisingly, the GST-Csk SH2 fusion protein was unable to inhibit this interaction. By in vitro binding assays, we have observed that Csk SH2 domain alone was unable to bind phosphotyrosine proteins, whereas Csk SH3/SH2 domains clearly displayed such an interaction3 (30). These results suggest that the SH3 domain of Csk participates in the interaction, presumably by allowing the SH2 domain to have the required steric conformation.

Kinetic Analysis of Recombinant GST-Lck.WT and GST-Lck.Y505F Interaction with Immobilized Csk—To compare the kinetic parameters for GST-Lck.WT and GST-Lck.Y505F binding to Csk, we determined association and dissociation constants using different GST-Lck concentrations (Fig. 2, A and B). The association phase (600 s of injection) was used to determine association constants $k_a$ (Fig. 2, C and D) and the dissociation phase (200 s after the end of the injection) was used to determine dissociation constants $k_d$ (Fig. 2, E and F). Equilibrium affinity constants $K_D$ were obtained by Scatchard analysis (Fig. 2, G and H). The results are summarized in Table II. The apparent affinity constants (calculated either from $k_a/k_d$ or from equilibrium data) for binding of GST-Lck.WT and GST-Lck.Y505F to Csk are similar to those reported for binding of phosphotyrosine peptides to SH2 domains using the same experimental approach (42, 43). Interestingly, whereas the $k_a$ for GST-Lck.WT and GST-Lck.Y505F are similar, there is a significant difference in the $k_d$ for GST-Lck.WT and GST-Lck.Y505F (15-fold higher). Thus, the GST-Lck.Y505F mutant binds to Csk with a 9-fold higher affinity than GST-Lck.WT due to a much higher association rate. The lower association rate observed for GST-Lck.WT might be explained by the heterogeneity of GST-Lck.WT protein which is phosphorylated either on Tyr394 or on Tyr505, or on both (28). As phosphorylated Tyr505 can interact with Lck SH2 domain, this may decrease the accessibility of Csk SH2 domain to the phosphorylated Tyr394.

Interaction between Csk and Lck in Vivo—To confirm the interaction between Csk and Lck in vivo, we used the yeast two-hybrid system (27). Full-length wild-type csk and lck genes were fused to LexA-DB in pV.L10 and to Gal4-AD in pGAD-GH, respectively. If the two proteins interact, the reporter strain is expected to grow in the absence of histidine and to produce β-galactosidase. Using Western blot analysis with a monoclonal anti-phosphotyrosine antibody, we confirmed that Lck expressed in yeast is phosphorylated on tyrosine residue(s) (data not shown). Fig. 3 shows that only pLexA-Csk/pGAD-GH conferred on L40 yeast cells the ability to grow in the absence of histidine, but not any negative controls. In addition, only this strain was able to transactivate the lacZ reporter gene (data not shown). L40 pLexA-Ras12/pGAD-Raf was used as a positive control (33, 38). Therefore, we confirmed in vivo that there is a direct interaction between Csk and Lck.

Analysis of Recombinant GST-Lck.WT Phosphorylation by Csk—The finding of Csk interaction with Lck through BIAcore™ studies and using the yeast two-hybrid system was further confirmed by an independent experiment in which we determined phosphorylation of GST-Lck.WT by Csk (purified without GST). In an initial analysis, concentrations of GST-Lck.WT and Mn2+ were varied to have low level of Lck autophosphorylation. Under these conditions, the level of GST-Lck.WT autophosphorylation (Fig. 4A, lane 1) was at least 10-fold lower than the phosphorylation of GST-Lck.WT by Csk (Fig. 4A, lane 2). To identify the phosphorylation sites on Lck, tryptic digestion was performed on GST-Lck.WT phosphorylated by Csk. A single phosphopeptide was obtained (Fig. 4B, lane 1) which comigrates with a synthetic tryptic peptide containing the phosphorylated Tyr505 of Lck (Fig. 4B, lane 2). Furthermore, we did not detect any phosphorylation of GST-Lck.Y505F incubated with Csk (data not shown). Together these results show that Tyr505 of Lck is the phosphorylation site by Csk. Next, the different GST-Lck fusion proteins were phosphorylated in vitro by Csk (Fig. 4C). The phosphorylation of GST-Lck.WT was 13-fold higher than the phosphorylation of GST-Lck.K273E and 3-fold higher than the phosphorylation of GST-Lck.Y394F, suggesting that previous phosphorylation of Lck on Tyr394 facilitates the phosphorylation of Tyr505 by Csk. The phosphorylation of GST-Lck.Y394F by Csk might be due to binding of phosphorylated Tyr505 of Lck to Csk SH2 domain. As we did not detect any interaction between Csk and GST-Lck.Y394F in our BIAcore™ studies, this binding might be of very low affinity and irrelevant in vivo. The fact that the inactive GST-Lck.K273E mutant, which is not tyrosine phosphorylated, is much less phosphorylated by Csk is consistent with our hypothesis that Lck phosphorylation by Csk is enhanced by previous tyrosine phosphorylation of Lck. Finally, the GST-Lck.WT protein was phosphorylated in vitro by Csk either alone or in the presence of peptide corresponding to the sequence surrounding the Tyr394 of Lck (Fig. 4D). We observed that the addition of Lck-Tyr394 phosphopeptide inhibits the phosphorylation of GST-Lck.WT by Csk, in a dose dependent manner. This inhibition was not observed with the nonphosphorylated peptide. Furthermore, no inhibition was observed with a synthetic tryptic peptide containing the Tyr505 of Lck phosphorylated used at the same concentrations (data not shown), showing the specificity of the inhibition observed with the Lck-Tyr394 phosphopeptide. Therefore, the blocking of the binding site for Lck on Csk occurring with the Lck-Tyr394 phosphopeptide, but not with the nonphosphorylated one, impinges on the phosphorylation of Lck by Csk, suggesting that Lck phosphorylated on Tyr394 binds more efficiently to Csk.

**DISCUSSION**

Using two systems which allow to detect transient interaction, the in vitro real-time interaction analysis (BIAcore™) and the in vivo yeast two-hybrid system, we have shown that
Csk interacts physically with Lck. Through BIAcore™ experiments, we noticed the absence of detectable binding of Lck.K273E and Lck.Y394F to Csk, and the inhibition of Lck-Csk interaction by pre-incubation of Lck with Csk SH3/SH2 domains, which is not observed with a mutant of Csk SH3/SH2 protein (S108C) unable to bind phosphotyrosine proteins. This strongly suggests that the autophosphorylated Tyr394 of Lck interacts with the SH2 domain of Csk. Furthermore, using a Y505F mutant of Lck we observed an increase in the rate of association but no change in the rate of dissociation compared to wild-type Lck. On the one hand, the fact that the Lck.Y505F mutant which corresponds to an hyperactive form due to the absence of the negative regulatory site, binds to Csk with a higher rate of association, suggests that the binding of Csk to Lck is enhanced by an increase of the kinase activity of Lck through a yet unknown mechanism. On the other hand, the fact that we did not observe any change in the dissociation rate of the Lck.Y505F mutant which is not phosphorylated by Csk, suggests that the release of Lck after interaction with Csk is not due to phosphorylation by Csk, but might be due to the transience of the interaction. Thus, the off-rate might be too

**Fig. 2.** Kinetic analysis of recombinant GST-Lck.WT and GST-Lck.Y505F interaction with immobilized Csk. Purified GST-Lck.WT and GST-Lck.Y505F proteins were used at various concentrations (conc.) ranging from 625 nM to 1000 nM and allowed to interact with immobilized Csk for 10 min (sensorgrams A and B, respectively). The association phase was analyzed in a dR/dt versus R plot for each GST-Lck concentration. The slope values obtained are plotted against GST-Lck.WT and GST-Lck.Y505F concentrations (C and D, respectively). The dissociation phase was followed in continuous buffer flow during 200 s after the end of the injection and was analyzed in a ln (Rf/Rn) versus time plot for GST-Lck.WT and GST-Lck.Y505F (E and F, respectively). Equilibrium values were determined by fitting the steady state binding values (Req) to the binding equation by Scatchard analyses, giving rise to a Req/GST-Lck concentration versus Req plot for GST-Lck.WT and GST-Lck.Y505F (G and H, respectively).

| Table II | Kinetic rate and affinity constants for GST-Lck.WT and GST-Lck.Y505F binding to Csk |
|-----------------|-----------------|-----------------|-----------------|
| Association rate constants $k_a$ were obtained from the slopes in Fig. 2, C and D. Dissociation rate constants $k_d$ were obtained from the slopes in Fig. 2, E and F. Apparent affinity constants $K_{eq}$ were calculated from $k_a/k_d$. Equilibrium affinity constants $K_{eq}$ were calculated from equilibrium data obtained in Fig. 2, G and H. |
| $k_a$ | $k_d$ | $K_{eq}$, calc | $K_{eq}$, eq |
| $\text{M}^{-1}\text{s}^{-1}$ | $\text{s}^{-1}$ | $\text{nM}$ | $\text{nM}$ |
| GST-Lck.WT | $1.28 \times 10^3$ | $1.11 \times 10^{-3}$ | 867 | 1000 |
| GST-Lck.Y505F | $19.6 \times 10^3$ | $1.95 \times 10^{-3}$ | 99 | 141 |

Csk interacts physically with Lck. Through BIAcore™ experiments, we noticed the absence of detectable binding of Lck.K273E and Lck.Y394F to Csk, and the inhibition of Lck-Csk interaction by pre-incubation of Lck with Csk SH3/SH2 domains, which is not observed with a mutant of Csk SH3/SH2 protein (S108C) unable to bind phosphotyrosine proteins. This strongly suggests that the autophosphorylated Tyr394 of Lck interacts with the SH2 domain of Csk. Furthermore, using a Y505F mutant of Lck we observed an increase in the rate of association but no change in the rate of dissociation compared to wild-type Lck. On the one hand, the fact that the Lck.Y505F mutant which corresponds to an hyperactive form due to the absence of the negative regulatory site, binds to Csk with a higher rate of association, suggests that the binding of Csk to Lck is enhanced by an increase of the kinase activity of Lck through a yet unknown mechanism. On the other hand, the fact that we did not observe any change in the dissociation rate of the Lck.Y505F mutant which is not phosphorylated by Csk, suggests that the release of Lck after interaction with Csk is not due to phosphorylation by Csk, but might be due to the transience of the interaction. Thus, the off-rate might be too
rapid to detect a stable complex between Src PTKs and Csk by
conventional binding assays (11, 23). Finally, through in vitro
phosphorylation experiments, we have also demonstrated that
Lck phosphorylated on Tyr394 is more efficiently phosphoryl-
ated on Tyr394 by Csk. Similarly, another group has observed that Csk phosphorylates cellular proteins, including Lck, in a phosphotyrosine dependent manner, suggesting that previous substrate tyro-
sine phosphorylation may be critical for the substrate selection of Csk.4 Interestingly, we have shown by immunofluorescence microscopy that upon T cell activation the cytoplasmic Csk (revealed with fluorescent tagged monoclonal anti-Csk antibody) translocates to plasma membrane where most of Lck is
localized.3 This is compatible with the hypothesis that after T
 cell stimulation, membrane bound Lck is activated, phosphory-
lated on Tyr394 which creates a temporary binding site for
Csk. We propose that the binding of Csk SH2 domain to the
autophosphorylated Tyr of the Src kinases facilitates phospho-
rylation of the COOH-terminal Tyr by Csk thereby repressing
the kinase activity. This mechanism may be a general feature
for down modulation of the kinase activity of most Src PTKs.

We observed that both the SH3 and the SH2 domains of Csk
are required for the binding to phosphorylated Tyr394 of Lck,
suggesting that the SH3 domain participates in the interaction.
Such a role has been reported by several groups (19, 20, 44)
who found that the SH3 domain of c-Src was required for the
intramolecular binding of the phosphorylated COOH-terminal
Tyr to the SH2 domain. Furthermore, Panchamoorthy et al.
(45) have recently shown that the presence of the adjacent SH3
domain increases the affinity of Fyn SH2 domain for phospho-
4 K. E. Amrein and P. Burn, personal communication.
rylated, c-Fgr is no more susceptible to down-regulation by Csk phosphorylation. Indeed, previous autophosphorylation of c-Fgr does not affect its phosphorylation by Csk and even though autophosphorylated c-Fgr is still phosphorylated by Csk, this phosphorylation does not lead to down-regulation of c-Fgr. The authors propose that autophosphorylation of c-Fgr could induce an intermolecular interaction between the autophosphorylated Tyr and the SH2 domain, resulting in an active homodimeric form (10).

An interaction between Csk SH2 domain and the autophosphorylated Tyr of Src PTKs has been postulated by Songyang et al. (14) who have shown that the sequence motif Tyr(P), Thr/Ala, Lys/Arg, Met/Ile/Val/Arg which is found within the autophosphorylation site of the Src kinases (with the exception of c-Fgr), has high affinity for the SH2 domain of Csk. Furthermore, a functional and physical interaction of Fyn and Csk has been reported and a mutant of Fyn that is highly autophosphorylated on Tyr420 in vivo was shown to form a more stable complex with Csk than with wild-type Fyn (47).

The regulation of Csk-Src PTK interaction proposed in this report does not explain the fact that an inactive form of Src expressed in mouse embryo fibroblasts lacking endogenous Src, is still phosphorylated on Tyr527 (48) and a Lck.Y394F mutant transfected in NIH3T3 cells is still phosphorylated on Tyr505 (5). However, in fibroblasts these phosphorylations might be due either to autophosphorylation, as previously reported for Src in yeast cells (49) and for Lck in bacteria (28), or to phosphorylation by other members of the Csk family. It has been shown that in cell lines established from embryos lacking Csk, the endogenous c-Src is still phosphorylated on Tyr527 (21), suggesting that other kinases may phosphorylate this site in vivo. Several groups have recently described cDNAs encoding a second Csk-related protein tyrosine kinase termed either matk (50), hyl (51), ctk (52), ntk (53), or lsk (54). These kinases might have different affinities for Src PTKs and might be responsible for the regulation of those PTKs which do not bind to Csk. In vitro Ctk/Ntk is capable of phosphorylating Lck at its negative regulatory site (52, 53) and might also phosphorylate the Lck.Y394F mutant. In the same way, Matk can phosphorylate the COOH-terminal Tyr of c-Src (55) and might also phosphorylate the Src.Y416F. Thus, it is likely, that these Csk-related kinases are capable of phosphorylating the COOH-terminal Tyr of Src PTKs in the absence of the autophosphorylated Tyr.

Little is known about the regulation of Csk itself. It has been shown that Csk SH3 an SH2 domains are both required for the suppression of c-Src kinase activity (30) and for its hyperacetylation on Tyr and the SH2 domain, resulting in an active homodimeric form (10).

In sum, we showed that (i) the interaction of Csk with Lck requires the phosphorylated Tyr394 of Lck, the phosphorylation of which is correlated with its kinase activity; (ii) the hyperactive form of Lck (Y505F) has much higher affinity for Csk than the wild-type Lck, and (iii) Lck phosphorylated on Tyr394 is more efficiently phosphorylated by Csk. Altogether these results suggest that activated and autophosphorylated Lck interacts preferentially with Csk. We propose that the modification of Lck accessibility upon activation may be a mechanism for the control of Csk-Lck interaction. Therefore, one might assume that the control of Csk function occurs through its substrate accessibility rather than through an intrinsic mechanism.

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