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# The human p50$^{csk}$ tyrosine kinase phosphorylates p56$^{lck}$ at Tyr-505 and down regulates its catalytic activity

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**Protein tyrosine kinases participate in the transduction and modulation of signals that regulate proliferation and differentiation of cells. Excessive or deregulated protein tyrosine kinase activity can cause malignant transformation. The catalytic activity of the T cell protein tyrosine kinase p56$^{lck}$ is normally suppressed by phosphorylation of a carboxyl-terminal tyrosine, Tyr-505, by another cellular protein tyrosine kinase. Here we characterize a human cytosolic 50 kDa protein tyrosine kinase, p50$^{csk}$, which specifically phosphorylates Tyr-505 of p56$^{lck}$ and a synthetic peptide containing this site. Phosphorylation of Tyr-505 suppressed the catalytic activity of p56$^{lck}$. We suggest that p50$^{csk}$ negatively regulates p56$^{lck}$, and perhaps other cellular *src* family kinases.**

*Key words:* p50$^{csk}$/p56$^{lck}$/pp60$^{c\text{-}src}$/tumour suppressor/ tyrosine phosphorylation

## Introduction

Phosphorylation of proteins on tyrosine residues is implicated in the regulation of cell proliferation and differentiation (Hunter and Cooper, 1985). The *src* family of non-receptor protein tyrosine kinase (PTK) genes consists of eight known members, c-*src*, c-*yes*1, c-*fgr*, *fyn*, *lyn*, *lck*, *hck* and *blk* (Hunter and Cooper, 1985; Kawakami *et al.*, 1986; Semba *et al.*, 1986; Dymecki *et al.*, 1990; Yamanishi *et al.*, 1987; Ziegler *et al.*, 1987; Marth *et al.*, 1985). The *lck* gene is expressed only in lymphoid cells, particularly T lymphocytes (Marth *et al.*, 1985). The 56 kDa PTK encoded by this gene, p56$^{lck}$, is bound to the cytoplasmic surface of the plasma membrane (Marchildon *et al.*, 1984) and is physically and functionally associated with at least three important transmembrane T cell proteins; CD4 (Rudd *et al.*, 1988), CD8 (Veillette *et al.*, 1988) and the 75 kDa $\beta$-chain of the interleukin 2 receptor (Hatakeyama *et al.*, 1991). Recently, *ras*-GAP (GTPase activating protein) was demonstrated to be a substrate for p56$^{lck}$ (Ellis *et al.*, 1991; Amrein *et al.*, 1992). The physiological function of p56$^{lck}$ is presumably to transuce signals related to T lymphocyte development and proliferation via these molecules (Bolen and Veillette, 1989; Mustelin and Altman, 1989, 1991; Sefton, 1991).

The PTK activity of p56$^{lck}$ is tightly regulated *in vivo*. The extensive phosphorylation of a carboxyl-terminal tyrosine, Tyr-505 (Amrein and Sefton, 1988; Marth *et al.*, 1988; Hurley and Sefton, 1989; Veillett *et al.*, 1989), seems to play a major role in the control of p56$^{lck}$ activity (Amrein and Sefton, 1988; Marth *et al.*, 1988). NIH3T3 cells transfected with a Tyr-505 → Phe-505 mutant *lck* expression vector contained greatly elevated levels of phosphotyrosine, compared with cells transfected with a wild type construct, and underwent neoplastic transformation, indicating that the altered p56$^{lck}$ was constitutively active and oncogenic (Amrein and Sefton, 1988; Marth *et al.*, 1988). Tyr-505 is not phosphorylated by p56$^{lck}$ itself (Casnellie *et al.*, 1982, 1983; Marth *et al.*, 1988), but apparently by another cellular PTK.

The human *csk* gene (first denoted *cyl* for consensus tyrosine lacking) was originally identified using a probe from a cDNA sequence isolated among several novel PTKs obtained by a polymerase chain reaction-based strategy (Partanen *et al.*, 1990). The sequence of the full-length human and rat *csk* cDNAs contain several unusual features (Partanen *et al.*, 1991; Nada *et al.*, 1991). It encodes a ~50 kDa PTK containing essentially only SH2 (src-homology) and SH3 regions and a tyrosine kinase domain. Notably, no membrane attachment, tyrosine autophosphorylation or obvious regulatory carboxyl-terminal sequences are present. The rat *csk* sequence displays 98% identity with its human counterpart at the amino acid level.

## Results

Antisera were raised against two synthetic peptides corresponding to the amino- and carboxyl-termini of the predicted human *csk* product ($\alpha$CskN and $\alpha$CsKC respectively; Figure 1A). To identify such a protein and verify the specificity of our antibodies, the *csk* cDNA was transcribed *in vitro* and the resulting RNA translated in a reticulocyte lysate. This resulted in one major polypeptide of ~50 kDa and several smaller products (Figure 1B, lane 1). The $\alpha$CskC antiserum immunoprecipitated all these bands (lane 2), while $\alpha$CskN immunoprecipitated only the 50 kDa band (lane 3), indicating that the open reading frame of the *csk* cDNA encodes the predicted 50 kDa protein (p50$^{csk}$) and that the smaller products have an intact carboxyl-terminus but lack amino-terminal sequences, apparently produced by internal translational initiation from downstream initiation codons. To substantiate the specificity of the antibodies further, Cos cells were transfected with pSV*csk*, encoding the full-length human *csk* cDNA. Both antisera immunoprecipitated a protein of $M_r$ ~50 from the [$^{35}$S]methionine-labelled transfected cells (Figure 1C, lane 2 and data not shown), but only a very weak band (presumably endogenous p50$^{csk}$) from Cos cells transfected with a

control cDNA (lane 4). Similarly, immunoblotting of extracts with both antisera revealed a strong band at 50 kDa in the *csk*-transfected cells (Figure 1C, lanes 5 and 7) and a much weaker band in untransfected Cos cells (lanes 6 and 8). The recognition of this polypeptide was efficiently blocked by addition of the specific peptides to the sera (Figure 1B and C, lanes 4 and 3 respectively).

A ~50 kDa polypeptide was also seen in all human and murine cell types tested by immunoblotting with the αCskC or αCskN antisera (Figure 1D). The intensity of this band varied considerably between cell types and was strongest in human platelets. In some cells, such as the erythroleukemia HEL, the band appeared as a closely spaced doublet. In peripheral blood T cells ~80 and 20% of $p50^{csk}$ was present in cytosolic and membrane fractions respectively. The kinase was readily extracted by detergents and was barely detectable in detergent-insoluble cytoskeletal preparations (Louie *et al.*, 1988; data not shown).

To test if $p50^{csk}$ possessed PTK activity (as predicted from the cDNA), the protein was immunoprecipitated from lymphocytes and platelets and tested for kinase activity using various protein and peptide substrates in the presence of [γ-$^{32}$P]ATP and $Mn^{2+}$. No autophosphorylation of $p50^{csk}$ or significant phosphorylation of possibly coimmunoprecipitated proteins was detected in these experiments (Figure 2A, lane 1). Enolase, a good substate for many PTKs (Cooper *et al.*, 1984), and bovine serum albumin were also poor substrates (Figure 2A, lanes 2 and 3). In contrast, recombinant wild-type $pp60^{c\text{-}src}$ and recombinant kinase-inactive (Lys-295 → Arg-295 mutant) $pp60^{c\text{-}src}$ proteins

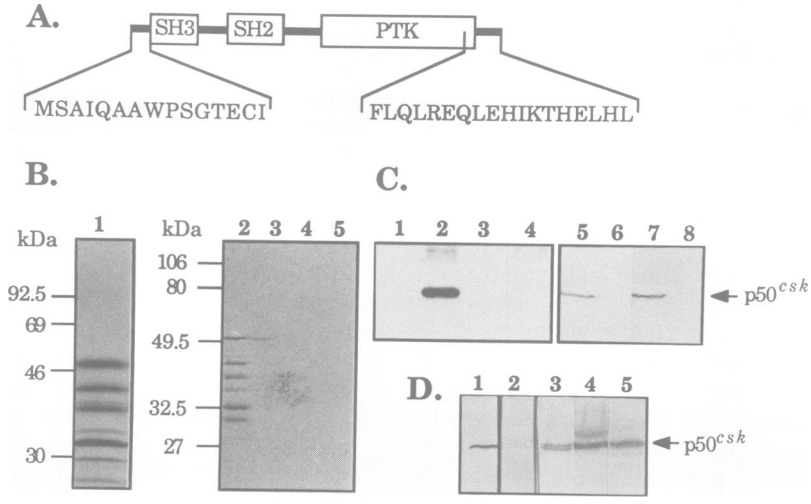


**Fig. 1.** Identification of the *csk*-encoded protein. (**A**) Location and sequences of the unique *csk*-derived peptides used for production of antisera. (**B**) *In vitro* translated polypeptides of a reticulocyte lysate programmed with the *csk* cDNA (lane 1) (exposure time: 2 days). Also shown are immunoprecipitates from the lysate obtained with αCskC (lane 2), αCskN (lane 3), peptide-blocked αCskN (lane 4) or preimmune serum (lane 5) (exposure time for lanes 2–5: 3 days. (**C**) Immunoprecipitation of $p50^{csk}$ from [$^{35}$S]methionine-labelled, pSV*csk*-transfected Cos cells using preimmune serum (lane 1), αCskC (lane 2), peptide-blocked αCskC (lane 3). Immunoprecipitation with αCskC from Cos cells transfected with control plasmid pSVneo (lane 4 (exposure time for lanes 1–4: 3 days). Detection of $p50^{csk}$ by immunoblotting with αCskN and αCskC in *csk*-transfected Cos cells (lanes 5 and 7 respectively) and in untransfected COS cells (lanes 6 and 8). (**D**) Immunoblotting of various cell types with αCskC. Resting human lymphocytes (lane 1), the same sample analyzed with peptide-blocked αCskC (lane 2), HEL cells (lane 3), human thrombocytes (lane 4) and LSTRA cells (lane 5).

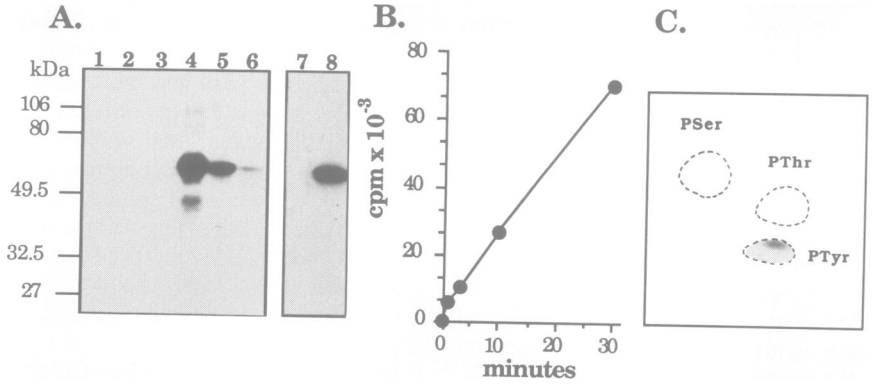


**Fig. 2.** Substrate specificity of the $p50^{csk}$ tyrosine kinase. (**A**) Phosphorylation of various proteins by $p50^{csk}$ immunoprecipitated from human platelets. No addition (lane 1), enolase (4 μg; lane 2), bovine serum albumin (3.5 μg; lane 3), recombinant wild-type $pp60^{c\text{-}src}$, (0.25 μg; lane 4), recombinant kinase-negative $pp60^{c\text{-}src}$ (0.3 μg; lane 5), kinase-negative $pp60^{c\text{-}src}$ (0.3 μg) and purified CD45 (1.6 μg.; lane 6). Autophosphorylation of kinase-negative $pp60^{c\text{-}src}$ (0.3 μg; lane 7) and wild-type $pp60^{c\text{-}src}$ (0.25 μg; lane 8) in the absence of $p50^{csk}$. Exposure time for lanes 1–8, 2 h. (**B**) Phosphorylation of the L505P peptide (CWKERPEDRPTFDFLRSVLEDFFTATEGQYQPQP) by $p50^{csk}$ immunoprecipitated from human lymphocytes. (**C**) Phosphoamino acid analysis of the phosphorylated peptide shown in panel B. Exposure time: 5 days.

(kind gift from Dr D.Morgan, University of San Francisco, CA) were efficiently phosphorylated (Figure 2A, lanes 4 and 5). The latter is not capable of autophosphorylation (lane 7), but the former is (lane 8). These results indicate that pp60$^{c\text{-}src}$ is a substrate for the p50$^{csk}$ kinase *in vitro*. Moreover, addition of purified CD45 phosphotyrosine phosphatase (Mustelin *et al.*, 1992) reduced the phosphorylation of the kinase-inactive pp60$^{c\text{-}src}$ (lane 6), indicating phosphorylation of a tyrosine residue. When bound to agarose beads, a synthetic carboxyl-terminal peptide (L505P) corresponding to amino acids 476–509 of p56$^{lck}$ (except that Tyr-489 was changed into Phe) was also efficiently phosphorylated by p50$^{csk}$ (Figure 2B). Phosphoamino acid analysis of the phosphorylated peptide showed that it too was exclusively phosphorylated on tyrosine (Figure 2C). The only tyrosine in L505P corresponds to Tyr-505.

When p50$^{csk}$ immunoprecipitated from human platelets was mixed with recombinant p56$^{lck}$, [$\gamma$-$^{32}$P]ATP and $Mn^{2+}$, the recombinant p56$^{lck}$ was phosphorylated. Since this kinase is catalytically active and autophosphorylates at Tyr-394 [and some additional minor sites; Ramer *et al.* (1991)], we analysed its sites of phosphorylation by tryptic peptide mapping. Most of the label in p56$^{lck}$ incubated without p50$^{csk}$ was recovered in a peptide containing the autophosphorylation site, Tyr-394 (Figure 3, A and B). In contrast, p50$^{csk}$-treated p56$^{lck}$ contained ~50% of its label in a peptide with a higher $R_f$ value in the ascending chromatography and ~50% in the Tyr-394-containing peptide (Figure 3, C and D). For comparison, *in vivo* $^{32}$P-labelled and immunoprecipitated p56$^{lck}$ from LSTRA cells contained two major phosphopeptides (Figure 3E), as described by others (Amrein and Sefton, 1988; Marth *et al.*, 1988; Hurley and Sefton, 1989; Veillette *et al.*, 1989). One of these represents Tyr-394, the other Tyr-505. The migration of the latter was identical to the peptide obtained from p56$^{lck}$ phosphorylated *in vitro* by p50$^{csk}$. Phosphorylated and TPCK-trypsin digested L505P also migrated similarly (Figure 3F). Based on this and the phosphorylation of L505P, we conclude that p50$^{csk}$ specifically phosphorylates Tyr-505 of p56$^{lck}$. Identical results were obtained in several independent experiments and with p50$^{csk}$ immunoprecipitated from resting human lymphocytes.

The phosphorylation of Tyr-505 is thought to have a suppressive effect on the catalytic activity of p56$^{lck}$ (Amrein and Sefton, 1988; Marth *et al.*, 1988) and accordingly the dephosphorylation of Tyr-505 has been shown to activate the kinase (Mustelin *et al.*, 1989; Mustelin and Altman, 1990). To determine if p50$^{csk}$-mediated phosphorylation of Tyr-505 would reduce the catalytic activity of recombinant (unphosphorylated) p56$^{lck}$ *in vitro*, we coincubated the two kinases together in the presence of non-radioactive ATP for various times, removed the p50$^{csk}$ immunoprecipitates by centrifugation and measured the PTK activity of p56$^{lck}$ in the presence of [$\gamma$-$^{32}$P]ATP with enolase as a substrate. These experiments showed a time-dependent decrease in the catalytic activity of p56$^{lck}$ (Figure 4A). The amount of p56$^{lck}$ was similar in all samples as determined by immunoblotting (Figure 4B).

## Discussion

Activation of T lymphocytes by antibodies against the T cell antigen receptor–CD3 complex or mitogenic lectins is associated with a rapid and obligatory tyrosine phosphorylation of a set of cellular proteins (Mustelin *et al.*, 1990;

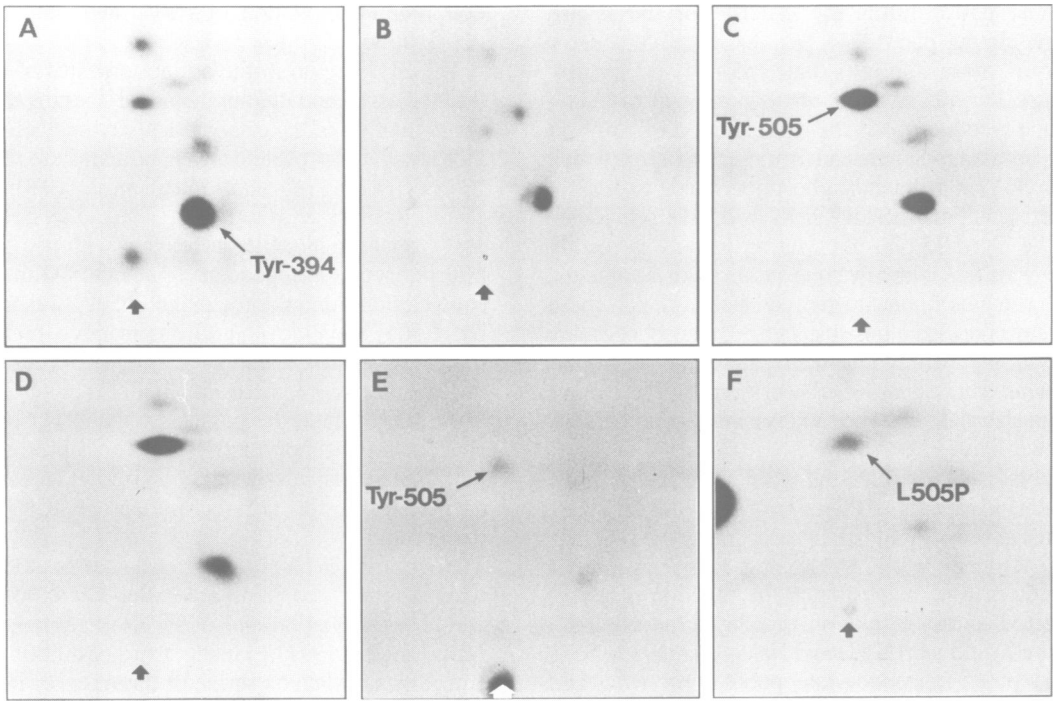


**Fig. 3.** p50$^{csk}$ phosphorylates p56$^{lck}$ on Tyr-505. Tryptic peptide maps of (**A**) *in vitro* autophosphorylated recombinant p56$^{lck}$, (**B**) p56$^{lck}$ treated with an immunoprecipitate obtained with preimmune serum, (**C**) and (**D**). p56$^{lck}$ treated with immunoprecipitated p50$^{csk}$ (two separate experiments are shown), (**E**) *in vivo* $^{32}$P-labelled p56$^{lck}$ and (**F**). Tryptic digestion of L505P phosphorylated by immunoprecipitated p50$^{csk}$. Sample origin is marked by an arrow, the anode was at left. Exposure times: 1–7 days.

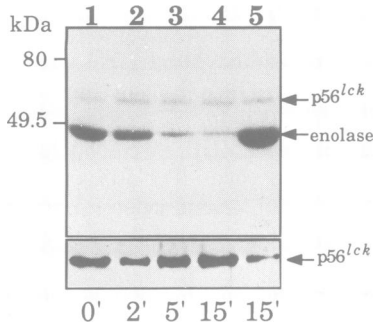


**Fig. 4.** p50$^{csk}$ suppresses the PTK activity of p56$^{lck}$. **Upper panel**, catalytic activity of recombinant p56$^{lck}$ incubated with immunoprecipitated p50$^{csk}$ and ATP for 0 (lane 1), 2 min (lane 2), 5 min (lane 3), 15 min (lane 4) or with a preimmune serum (control) precipitate for 15 min (lane 5). Exposure time, 6 h. **Lower panel**, immunoblot showing the amount of p56$^{lck}$ in the gel in the upper panel.

June *et al.*, 1990a,b). p56$^{lck}$ is likely to participate in these reactions, particularly when the CD4 or CD8 glycoproteins are involved. Addition of interleukin 2 also causes activation of p56$^{lck}$ molecules associated with the high affinity interleukin 2 receptor (Hatakeyama *et al.*, 1991), and consequently phosphorylation of tyrosine on a number of cellular proteins (Mills *et al.*, 1990). The proper function of p56$^{lck}$ in signal transduction or modulation obviously requires efficient regulation of its catalytic activity. Phosphorylation of the negative regulatory site, Tyr-505, by another kinase is implicated in the suppression of the catalytic and potentially transforming activities of p56$^{lck}$ (Amrein and Sefton, 1988; Marth *et al.*, 1988). We have studied a cytosolic kinase, p50$^{csk}$, that specifically phosphorylates Tyr-505 of p56$^{lck}$ and thereby downregulates its catalytic activity. Thus, p50$^{csk}$ fulfils the criteria for the kinase negatively regulating p56$^{lck}$.

While our data show that p50$^{csk}$ efficiently phosphorylates Tyr-505 of p56$^{lck}$ *in vitro*, they do not allow us to conclude whether this is the true function of p50$^{csk}$ *in vivo*. Using an assay (Schuh and Brugge, 1988) in which Tyr-505 of p56$^{lck}$ is efficiently phosphorylated '*in situ*' in detergent-permeabilized LSTRA cells, we have previously found that the Tyr-505 phosphorylating activity is readily washed out by buffers with low concentrations of detergents, while p56$^{lck}$ remains bound to the cell pellet. From these extracts we have purified a possible Tyr-505 phosphorylating ~49 kDa PTK by affinity chromatography on the L505P peptide (T.Mustelin, unpublished work). p50$^{csk}$ is similar to this PTK in size and properties and is expressed in LSTRA cells as shown here.

However, we cannot exlude the possibility that multiple distinct 49–50 kDa soluble PTKs may exist. In addition, the ubiquitous expression of *csk* in cells and tissues (Okada *et al.*, 1991; Partanen *et al.*, 1991), particularly in cells of haematopoietic origin, suggests that the function of p50$^{csk}$ is not restricted to the regulation of p56$^{lck}$. Indeed, both human (Figure 2) and rat (Okada and Nakagawa, 1989; Nada *et al.*, 1991) p50$^{csk}$ phosphorylate pp60$^{c\text{-}src}$ *in vitro* and since all *src* family kinases have an analogous tyrosine residue in a highly conserved context in their carboxyl-termini, it seems likely that p50$^{csk}$ phosphorylates them all. In agreement with this notion, it was recently reported that rat p50$^{csk}$ can phosphorylate and suppress the *src* family kinases p56$^{lyn}$ and p59$^{fyn}$ (Okada *et al.*, 1991), which have been shown to be physically associated with the B cell antigen receptor (Yamanishi *et al.*, 1991) and the T cell antigen receptor–CD3 complex (Samelson *et al.*, 1990; Gassmann *et al.*, 1992) respectively. If this is also the case *in vivo*, p50$^{csk}$ (or a putative *csk* family) is responsible for down-regulation of the catalytic activity of multiple potentially transforming kinases. Thus, p50$^{csk}$ should be viewed as a potential tumor suppressor or 'master kinase', whose activity is crucial for the normal function of cells.

Our data support the concept that the catalytic and potentially transforming activity of p56$^{lck}$ is regulated by the balanced action of two enzymes displaying opposite activities, the cytosolic PTK p50$^{csk}$ phosphorylating the regulatory Tyr-505 of p56$^{lck}$ and the membrane-bound phosphotyrosine phosphatase CD45 dephosphorylating at this site. The former down regulates the activity of p56$^{lck}$ (and presumably other *src* family kinases), while the latter causes activation (Mustelin *et al.*, 1989; Mustelin and Altman, 1990). A more detailed knowledge of the properties and regulation of p50$^{csk}$ will be important for the understanding of the physiological function of p56$^{lck}$ in T cells.

The majority of known PTKs are transmembrane or membrane-bound proteins. p56$^{lck}$ and other PTKs of the *src* family are bound to the inner surface of the plasma membrane via myristic acid covalently attached to a glycine at position 2 of their amino-termini (Marchildon *et al.*, 1984). p50$^{csk}$ does not contain this myristylation signal or other known membrane-attachment motifs. Indeed, in this respect, p50$^{csk}$ is unusual among PTKs; p56$^{lck}$, e.g. is predominantly membrane-bound and largely cytoskeletal (Louie *et al.*, 1988). The membrane-associated 20% of p50$^{csk}$ may be bound to p56$^{lck}$ or other *src* family kinases. This interaction, however, seems to be too weak to allow coimmunoprecipitation of p50$^{csk}$ and p56$^{lck}$ (unpublished observations).

The SH2 region in the amino-terminus of all *src* family (as well as *fps* and *abl* families) PTKs seems to be important for regulation of the catalytic activity and association of the PTKs with other proteins. SH2 domains are thought to bind PTyr-containing sequences (Koch *et al.*, 1990; Moran *et al.*, 1990; Roussel *et al.*, 1991). Thus, the SH2 domain of p50$^{csk}$ could regulate its interaction with p56$^{lck}$ in a tyrosine phosphorylation-dependent manner. Our experiments showed that immunoprecipitated p50$^{csk}$ was active towards the L505P peptide and kinase-inactive pp60$^{src}$ *in vitro*, indicating that the catalytic activity of *src* family kinases is not absolutely required for p50$^{csk}$ activity. We cannot, however, exclude the possibility that PTyr on p56$^{lck}$ (e.g. at the autophosphorylation site) would promote binding of p50$^{csk}$ and phosphorylation of Tyr-505. This would provide a mechanism for the recruitment of p50$^{csk}$ to the cell membrane.

How, then, is p50$^{csk}$ itself regulated? Instead of a tyrosine residue, p50$^{csk}$ contains a Ser-Ser-Thr motif in the usual autophosphorylation site of PTKs (Partanen *et al.*, 1991; Nada *et al.*, 1991), raising the possibility that it could autophosphorylate on serine or threonine or be regulated by a serine/threonine-specific kinase. Incubation of p50$^{csk}$ with [$\gamma$-$^{32}$P]ATP and $Mn^{2+}$, however, did not lead to detectable autophosphorylation. We have also been unable to detect any phosphorylation of p50$^{csk}$ in cells after [$^{32}$P]orthophosphate labeling, even after treatment of the cells with phorbol esters

or phosphatase inhibitors. Nevertheless, p50$^{csk}$ and three smaller fragments (41,35 and 35 kDa) produced by *in vitro* translation in reticulocyte lysates, reacted with an anti-phosphotyrosine antibody (our unpublished observation). Smaller fragments did not contain phosphotyrosine, mapping the phosphorylated residue(s) to Tyr-156 or Tyr-184 between the SH2 domain and the kinase domain. At present this only shows that p50$^{csk}$ can be phosphorylated on a tyrosine residue(s). The possible relevance of this finding is currently under investigation.

## Materials and methods

### Cells and antibodies

Lymphocytes (~80% T cells) were isolated from the blood of healthy volunteers by gradient centrifugation on Ficoll Isopaque (Pharmacia, Sweden). Human platelets were kindly provided by the Finnish Red Cross Blood Transfusion Center and were stored as cell pellets at −70 °C. The cell lines HEL, K562 and LSTRA were maintained in logarithmic growth in RPMI-1640 supplemented with 5 or 10% heat-inactivated fetal calf serum, L-glutamine and antibiotics. LSTRA cells were grown in the same medium supplemented with 50 μm 2-mercaptoethanol. Cos cells were cultured in DMEM with 7% heat-inactivated fetal calf serum.

The peptides used for immunization were synthesized and kindly provided by Dr G.Evan (Imperial Cancer Research Fund, London, UK). They were conjugated to keyhole limpet hemocyanin using glutaraldehyde. The conjugates were mixed with Freund's complete adjuvant and injected s.c. into rabbits. The rabbits were boosted every third week with the peptide conjugate in incomplete adjuvant.

Affinity-purified anti-PTyr antibodies and a rabbit antiserum against p56$^{lck}$ were kind gifts from Dr A.Altman (La Jolla Institute for Allergy and Immunology, La Jolla, CA). A monoclonal anti-p56$^{lck}$was made as described (Amrein *et al.*, 1992).

### Cell transfections and in vitro translation

The *csk* cDNA in a pGEM 3 vector was linearized with *Hin*dIII and transcribed with T7 DNA polymerase (Boehringer, Germany) according to the manufacturer's recommendations in a reaction mixture containing ribonucleotides, a capping analogue (Pharmacia, Sweden), dithiothreitol and RNasin. *In vitro* translation of the *csk* RNA in the presence of [$^{35}$S]methionine was performed in rabbit reticulocyte lysates (Promega). Aliquots of the lysates were analyzed by SDS−PAGE.

Cos cells were transfected with the pSV*csk* plasmid containing the full-length *csk* cDNA in the pSVpoly expression vector (Stacey and Schneike, 1990) using the DEAE−dextran method (McCluthcan and Pagano, 1968). Two days after transfection the Cos cells were labelled for 4 h with 120 μCi/ml [$^{35}$S]methionine, washed in 20 mM Tris−HCl pH 7.2, 150 mM NaCl and lysed in RIPA buffer (10 mM Tris−HCl pH 7.5, 50 mM NaCl 0.5% sodium deoxycholate, 0.5% NP-40, 0.1% SDS, 1 mM PMSF). The lysates were sonicated and centrifuged at 50 000 *g* for 30 min and the supernatants were preabsorbed with preimmune serum and protein A−Sepharose. The supernatants were then incubated with 2 μl of antiserum overnight, followed by adsorption to protein A−Sepharose. Immune complexes were washed three times in RIPA, once in phosphate-buffered saline (PBS) and finally suspended in SDS sample buffer and boiled for 2 min.

### Immunoblots

Cells were lysed in hot SDS−PAGE sample buffer and their DNA was sheared (Voronova *et al.*, 1984). Proteins were resolved on SDS gels and transferred onto nitrocellulose filters. The filters were blocked in 5% non-fat dry milk in Tris-buffered saline and immunoblotted with a 1:1000 dilution of the antisera. The blots were washed and stained with alkaline phosphatase or processed for detection by the ECL method (Amersham, UK).

### Immunoprecipitation of p50$^{csk}$

All steps were carried out at 0−4 °C. Thrombocyte pellets (100 μl) or ~$10^8$ lymphocytes were lysed in 1 ml of 1% NP-40, 20 mM Tris−HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM $Na_3VO_4$, 10 μg/ml leupeptin and aprotinin. The lysates were clarified by centrifugation at 13 000 *g* for 5 min and preadsorbed to agarose-conjugated goat anti-rabbit IgG. p50$^{csk}$ was allowed to bind to 10 μl of specific antiserum for 2 h, and the immune complexes were adsorbed to agarose-conjugated goat anti-rabbit IgG for 1 h. Immune complexes were washed three times in lysis buffer, once in lysis buffer with 0.5 M NaCl, and again twice in lysis buffer before analysis by SDS−PAGE. For phosphorylation assays the immunoprecipitates were washed twice more in 10 mM HEPES pH 7.4, 5 mM $MnCl_2$.

### Phosphorylation of L505P

The phosphorylation of the L505P peptide (CWKERPEDRPTFDFLRS-VLEDFFTATEGQYQPQP) by immunoprecipitated-p50$^{csk}$ was measured by incubating 15 μl of p50$^{csk}$ immunoprecipitate with 20 μl Affigel 10 to which the peptide had been covalently coupled at 5 mg/ml (according to the manufacturer's instructions). The reaction contained 10 mM HEPES pH 7.4, 5 mM $MnCl_2$ and was initiated by addition of 5 μl of 10 μM ATP with 10 μCi [$\gamma$-$^{32}$P]ATP. After the indicated times at 30 °C, the reaction was terminated by addition of 1 ml of 20% SDS, and the agarose beads were washed several times in buffers with high or low pH, high salt and finally in PBS. The radioactivity was counted by liquid scintillation. The counts incorporated into the beads in the presence of a preimmune serum immunoprecipitate were subtracted. For phosphoamino acid analysis the phosphorylated product was digested with proteinase K and the released material was subjected to hydrolysis in 6 M HCl for 2 h. Phosphoamino acids were separated by two-dimensional electrophoresis at pH 1.9 and pH 3.5 as described (Hunter and Sefton, 1980). Phosphorylation of free L505P for analysis by tryptic peptide mapping was carried out in a similar reaction mixture. The phosphorylation peptide was separated from ATP by passage through a Sephadex G-15 column lyophilized and treated as described below.

### Recombinant human p56$^{lck}$

The plasmid pDS/lck was generated by inserting the 1833 bp *Nco*I−*Hin*dIII fragment from PUC12/YT16 [a kind gift from Dr T.W.Mak, University of Toronto, Toronto, Canada; Koga *et al.* (1987)] encoding human p56$^{lck}$ into the bacterial expression vector pDS56/RBSII (Stüber *et al.*, 1990) using standard techniques (Sambrook *et al.*, 1989). Bacteria transformed with pDS/lck were grown, and production of the heterologous protein induced. After harvesting the bacteria, 100 g of bacterial pellet was suspended in 250 ml of 50 mM Tris−HCl pH 8.0, 1 mg/ml lysozyme, 10 mM EDTA, 20 μg/ml PMSF, 0.2 mM $Na_3VO_4$, 5 mM NaF and kept on ice for 15 min. 200 ml 50 mM Tris−HCl pH 8.0, 37.5 mM $MgCl_2$ and 0.5 ml of 10 mg/ml DNase solution were added and the DNA digested for 15 min on ice. The mixture was sonicated and centrifuged in a Sorvall GSI rotor at 9000 r.p.m. for 30 min. The supernatant was passed three times over an anti-p56$^{lck}$ monoclonal antibody column (prepared by coupling monoclonal anti-p56$^{lck}$ IgG at 5 mg/ml to CNBr-activated Sepharose 4B according to the manufacturer's instructions). The column was washed with 20 column volumes of PBS and 5 vol of 10 mM $Na_2HPO_4$, pH 7.2, 0.5 M NaCl, and p56$^{lck}$ was eluted with 40 ml of 25 mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid), pH 10.0. The eluate was neutralized with 1 ml of 1 M HEPES pH 6.8 and concentrated to 2 ml using Millipore UFC4LGC25 spin dialysis tubes according to the manufacturer's instructions. Glycerol (0.5 ml) and 2.5 μl of 1 M dithiothreitol were added and the solution frozen in liquid nitrogen. The solution was estimated to be 20% pure and 1 mg/ml with respect to p56$^{lck}$ as determined by SDS−PAGE.

### In vitro phosphorylation of p56$^{lck}$ by p50$^{csk}$

The reaction contained 1 μg recombinant p56$^{lck}$ and 20 μl of αCskC immunoprecipitate in 10 mM HEPES pH 7.4, 5 mM $MnCl_2$, 3 μM ATP and was initiated by addition of 10 μCi of [$\gamma$-$^{32}$P]ATP. After 5 min at 30 °C the reaction was terminated by boiling in SDS sample buffer. For determination of the catalytic activity of p56$^{lck}$ after treatment with p50$^{csk}$, only unlabelled ATP was added and the two kinases were separated by centrifugation after various times of coincubation at 25 °C. The soluble p56$^{lck}$ was kept on ice until all samples were ready (the presence of ATP during the whole incubation ensured that varying degrees of autophosphorylation of p56$^{lck}$ would not affect the assay). The activity was then assayed in the presence of [$\gamma$-$^{32}$P]ATP using acid-denatured enolase as a substrate (Cooper *et al.*, 1984). Proteins were resolved by SDS−PAGE and detected by autoradiography.

### Preparation of in vivo $^{32}$P-labelled p56$^{lck}$

LSTRA cells were labelled for 4 h with 4 mCi/ml [$^{32}$P]orthophosphate in phosphate-free MEM medium (with 20 mM HEPES), washed in PBS and lysed by boiling for 1 min in 0.2 ml of 0.5% SDS 5 mM Tris−HCl pH 8.0, 2 mM EDTA (Voronova *et al.*, 1984) and then diluted with 0.8 ml of 3% NP-40, 20 mM Tris−HCl pH 8.0, 100 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 10 μg/ml leupeptin and aprotinin. The lysates were centrifuged, preadsorbed to agarose−goat-anti-rabbit Ig and p56$^{lck}$ was immunoprecipitated.

### Tryptic peptide mapping

For tryptic peptide mapping, labelled proteins were resolved by SDS−PAGE, transferred electrophoretically to nitrocellulose filters, located by

autoradiography and excised. The filter pieces were treated with polyvinylpyrrolidone 350 and TPCK-treated trypsin (Worthington Enzymes) as described in detail by Luo *et al.* (1990). The resulting peptides were separated in two dimenisons by electrophoresis at pH 1.9 and ascending chromatography.

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