The EMBO Journal vol.12 no.7 pp.2625-2634, 1993

Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src

Giulio Superti-Furga[1], Stefano Fumagalli[1], Manfred Koegl, Sara A.Courtneidge[2] and Giulio Draetta

Differentiation Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, 6900 Heidelberg, Germany

[1]Should both be considered first authors
[2]Corresponding author

Communicated by S.A.Courtneidge

The protein tyrosine kinase c-Src is negatively regulated by phosphorylation of Tyr527 in its carboxy-terminal tail. A kinase that phosphorylates Tyr527, called Csk, has recently been identified. We expressed c-Src in yeast to test the role of the SH2 and SH3 domains of Src in the negative regulation exerted by Tyr527 phosphorylation. Inducible expression of c-Src in *Schizosaccharomyces pombe* caused cell death. Co-expression of Csk counteracted this effect. Src proteins mutated in either the SH2 or SH3 domain were as lethal as wild type c-Src, but were insensitive to Csk, even though they were substrates for Csk *in vivo*. Peptide binding experiments revealed that Src proteins with mutant SH3 domains adopted a conformation in which the SH2 domain was not interacting with the tail. These data support the model of an SH2 domain-phosphorylated tail interaction repressing c-Src activity, but expand it to include a role for the SH3 domain. We propose that the SH3 domain contributes to the maintenance of the folded, inactive configuration of the Src molecule by stabilizing the SH2 domain-phosphorylated tail interaction. Moreover, the system we describe here allows for further study of the regulation of tyrosine kinases in a neutral background and in an organism amenable to genetic analysis.

Key words: Csk/SH2 domain/SH3 domain/Src/tyrosine kinase

Introduction

The c-Src protein tyrosine kinase comprises several distinct functional domains (reviewed in Pawson, 1988; Koegl and Courtneidge, 1992). The amino-terminal half of the molecule, which does not have catalytic functions, contains a myristylation domain, a unique domain and Src homology domains 3 and 2 (SH3 and SH2). Unlike the myristylation and unique domains, which have different degrees of conservation but are characteristic for the Src family of tyrosine kinases, the SH3 and SH2 domains are also present in several other proteins not related to tyrosine kinases (reviewed in Koch *et al.*, 1991; Pawson and Gish, 1992; Mayer and Baltimore, 1993) and are essentially modules directing protein-protein interactions. The carboxy-terminal half of the Src protein contains the catalytic domain followed by a short stretch of amino acids known as the tail. Tyr527, one of the major *in vivo* phosphorylation sites of c-Src, is located within these carboxy-terminal sequences. Phosphorylation of Tyr527 has a strong negative effect on the activity of the kinase and therefore is an important regulatory site (reviewed in Hunter, 1987; Koegl and Courtneidge, 1992). Indeed, mutagenesis of Tyr527 to phenylalanine (Y527F) increases kinase activity and is sufficient to convert proto-oncogene to oncogene.

Substantial evidence suggests that phosphorylation of Tyr527 *in vivo* is unlikely to occur predominantly by autophosphorylation. Indeed, in cells in which the endogenous c-*src* gene has been disrupted by homologous recombination, a re-introduced kinase negative version of c-Src is fully phosphorylated on Tyr527 (Thomas *et al.*, 1991). A Tyr527 kinase, termed Csk, has been identified and cloned (Okada and Nakagawa, 1989; Nada *et al.*, 1991). This protein is related (~45% identity) to c-Src, having both Src homology (SH) regions 2 and 3, and a catalytic domain, but lacks sequences amino-terminal to the SH3 domain, as well as carboxy-terminal regulatory sequences (Nada *et al.*, 1991; Partanen *et al.*, 1991). Csk phosphorylates c-Src on Tyr527 when the two molecules are co-expressed in *Saccharomyces cerevisiae* (Nada *et al.*, 1991). The *in vitro* phosphorylation of c-Src, Lyn, Fyn and Lck by Csk at their carboxy-terminal tails also correlates with a repression of the activity of these enzymes *in vitro* (Okada *et al.*, 1991; Bergman *et al.*, 1992).

It is now well-established that SH2 domains bind phosphotyrosine-containing proteins (reviewed in Koch *et al.*, 1991; Pawson and Gish, 1992), the specificity of the interaction being dictated both by the phosphorylation of the tyrosine as well as by the surrounding sequences (Anderson *et al.*, 1990; Matsuda *et al.*, 1990; Mayer and Hanafusa, 1990; Mayer *et al.*, 1991; Fantl *et al.*, 1992). The crystal structure of the v-Src SH2 domain complexed with a phosphorylated peptide shows that it can interact with phosphotyrosine (Waksman *et al.*, 1992). The SH2 domain appears to be roughly hemispherical in shape with the phosphotyrosine recognition site in a deep pocket in the middle of the flat surface. Highly conserved residues in the FLVRESE motif, the central importance of which had been previously recognized by genetic evidence (Hirai and Varmus, 1990; Clark *et al.*, 1992; Mayer *et al.*, 1992), are found in this pocket and involved in binding the phosphotyrosine (Waksman *et al.*, 1992). In particular, the arginine at position 175 makes contact with the phosphate group on the tyrosine residue.

Some small mutations in the SH2 region of Src augment its kinase activity and confer transformation competence even in the presence of Tyr527, whereas other mutations reduce the transforming ability of activated alleles of c-*src* (Hirai and Varmus, 1990; O'Brien *et al.*, 1990), suggesting that the domain has both positive and negative effects on Src activity, and that perhaps these mutations alter the spectrum of proteins to which Src can bind. However, complete

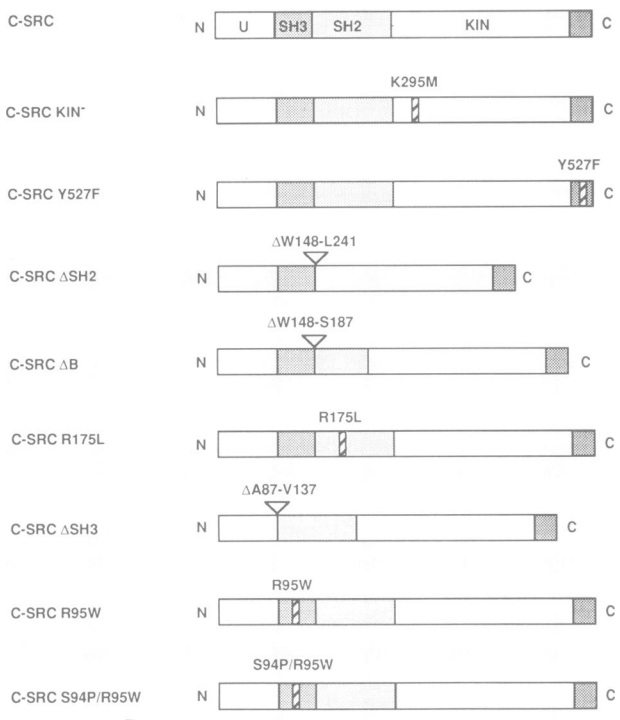

Fig. 1. Constructs used in this study. All the mutants are derivatives of pRSP-c-Src, which contains the 533 amino acid wild type chicken c-Src molecule (see Materials and methods). The domain structure of c-Src is illustrated. The abbreviations are: U, unique domain, amino acids 15–86; SH3, Src homology 3 domain, amino acids 87–137; SH2, Src homology 2 domain, amino acids 148–241; KIN, kinase domain, amino acids 260–516; N, amino-terminus; C, carboxy-terminus. The point mutations are drawn as narrow hatched boxes and carry the identity of the mutated residue on top. The deletions are marked by a triangle with the deleted residues shown above.

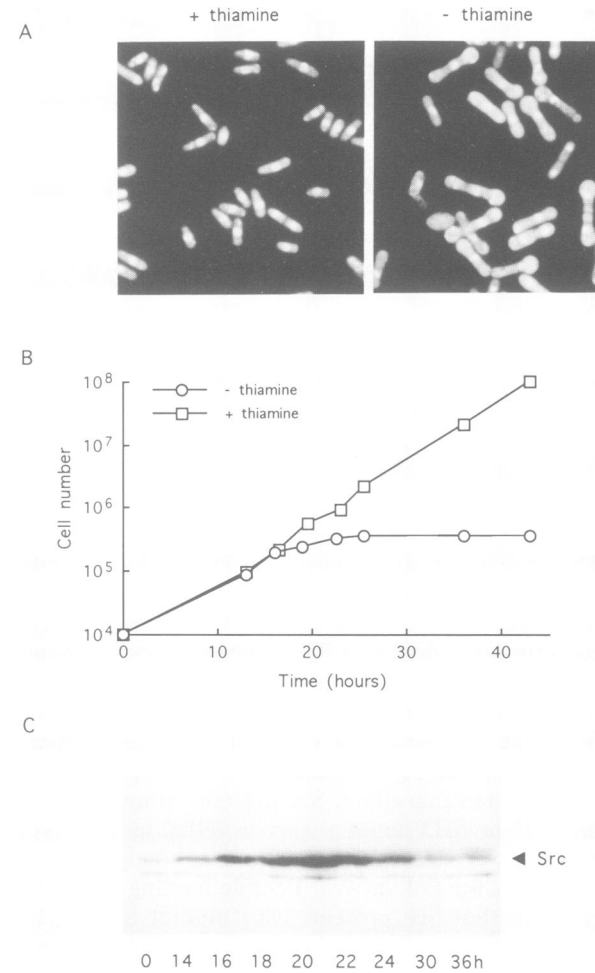

Fig. 2. Expression of c-Src in *S.pombe*. (**A**) Phenotype of *S.pombe* cells (SP200) transformed with pRSP-c-Src grown in minimal medium (PMA) under repressed (+ thiamine) conditions or after 24 h of induction (− thiamine). (**B**) Growth curve of SP200 cells transformed with pRSP-c-Src. At different time points after induction, a sample was taken and cell number determined. (**C**) Immunoblot of lysates prepared from SP200 cells expressing c-Src grown for different times in the absence of thiamine. The position of Src is marked. The lower band was also seen in extracts from non-transformed SP200 cells (not shown).

deletion of the SH2 domain activates the transforming potential of Src, demonstrating that it is not required for Src function and that its principle effect when present is negatively to regulate the activity of Src (Seidel-Dugan *et al.*, 1992). A model has been proposed that accommodates the requirement for the integrity of both the SH2 domain and Tyr527 (Figure 10E). Phosphorylated Tyr527 interacts intramolecularly with the SH2 domain to generate a conformation that has little kinase activity, and that prevents the SH2 domain from interacting with other proteins (Matsuda *et al.*, 1990). Dephosphorylation of Tyr527 (or mutation of either it or the SH2 domain) allows c-Src to adopt an active conformation. Much circumstantial evidence favours this model. For example, an antibody that recognizes the carboxy-terminus of Src augments the kinase activity of c-Src *in vitro* (Cooper and King, 1986), suggesting that the antibody relieves an inhibitory function of the carboxy-terminus. Furthermore, a peptide modelled on the carboxy-terminus of c-Src and containing phosphorylated Tyr527 binds wild type Src poorly and Y527F well *in vitro* (Roussel *et al.*, 1991) but only if Src has an intact SH2 domain.

The SH3 domain is also thought to interact with other proteins and although few such binding proteins have been described to date (Cicchetti *et al.*, 1992), it has been suggested that hydrophobic residues in the SH3 domain interact with proline-rich sequences in the target protein (Cicchetti *et al.*, 1992; Ren *et al.*, 1993). Mutations in the SH3 domain of the Src family tyrosine kinases c-Src (Kato *et al.*, 1986; Potts *et al.*, 1988; Seidel-Dugan *et al.*, 1992) and Lck (Reynolds *et al.*, 1992; Veillette *et al.*, 1992), lead to an increase in activity of these enzymes and in some cases partially augment their transformation potential. In the context of activated versions of these molecules (Src Y527F and Lck Y505F), however, positive, neutral and negative effects of SH3 mutations have been reported (Hirai and Varmus, 1990; Kanner *et al.*, 1991; Reynolds *et al.*, 1992; Veillette *et al.*, 1992), such that the influence of the SH3 domain on regulation by phosphorylation of the carboxy-terminal tyrosine could not be established. Several possibilities have been put forward to explain the negative effect of SH3 domains. These include binding to an inhibitor (Pawson, 1988; Cooper, 1990; Reynolds *et al.*, 1992), interaction with the catalytic domain (Kato *et al.*, 1986; Grandori, 1989) and interference with the carboxy-terminal tail–SH2 domain interaction (Reynolds *et al.*, 1992).

The interpretation of the effects of SH2 and SH3 mutants in mammalian cells has been hampered by the fact that both regions may mediate association with proteins able to affect transformation potential both positively and negatively. Src

Table I. Effect of tyrosine kinase expression in *S.pombe*

Kinase	Growth inhibition	Rescue by CSK
Src	+	+
Src K295M	−	
Src Y527F	+	−
Fyn	+	+
Fyn K299M	−	
Src ΔSH2	+	−
Src ΔB	+	−
Src R175L	+	−
Src ΔSH3	+	−
Src R95W	+	(+)
Src S94P/R95W	+	−
Csk	−	

SP200 cells that were transformed with the tyrosine kinases shown and under the control of the *nmt1* promoter were induced for expression by removing thiamine from the medium. All strains displayed either typical growth arrest (+) as exemplified by SP200 cells expressing wild type c-Src (see Figure 1) or normal growth (−). No intermediate behaviour was observed. Regarding the rescuing action of Csk co-expression, either growth arrest was fully suppressed (+) or not affected at all (−). Only in the case of the R95W Src did we observe incomplete rescue by Csk [(+)] (see also Figure 6D).

expressed in *S.cerevisiae* has been found to have only a low stoichiometry of phosphorylation on Tyr527 and is highly active in *in vitro* kinase assays (Cooper and Runge, 1987). We have exploited the absence of the Tyr527 kinase in yeast to study the regulation of c-Src and test the validity of the current inactivation models.

Results

Expression of c-Src is lethal to S.pombe

We expressed chicken c-Src under the control of the thiamine-repressible *nmt1* promoter (Maundrell, 1990) in the yeast *S.pombe* and studied the cells at various times after induction of the promoter by removal of thiamine. We observed that cells elongated and acquired a typical bone-like or dumbbell shape (Figure 2A) and stopped growing (Figure 2B). This phenotype became more severe until at ~20 h after induction cells started to die, as judged by lack of refractility and viability assays. At 20 h after induction 50% of the cells were able to grow when plated on medium containing thiamine and by 24 h this value had dropped to 5% (data not shown). Flow cytometric analysis of cells 24 h after induction revealed that 95% of the cells had a 2N DNA content, indicating that most of the cells had passed through S phase before division had ceased. Microscopic inspection after staining for chromosomal DNA with DAPI showed that the majority of cells displayed condensed chromosomes (Figure 2A and data not shown), suggesting that the cells may have been arrested at mitosis. Until now, however, we have found no evidence for elevated levels of H1 histone kinase activity in these cells (data not shown). Concomitant with the appearance of this phenotype, we observed the accumulation of c-Src protein (Figure 2C). In the presence of thiamine, under repressed conditions, the promoter has a measurable basal level of transcription (Maundrell, 1990; Basi *et al.*, 1993) and indeed, low levels of Src protein were present before induction. These low levels, however, had no detectable effects on *S.pombe* and the doubling time in the presence of thiamine was identical

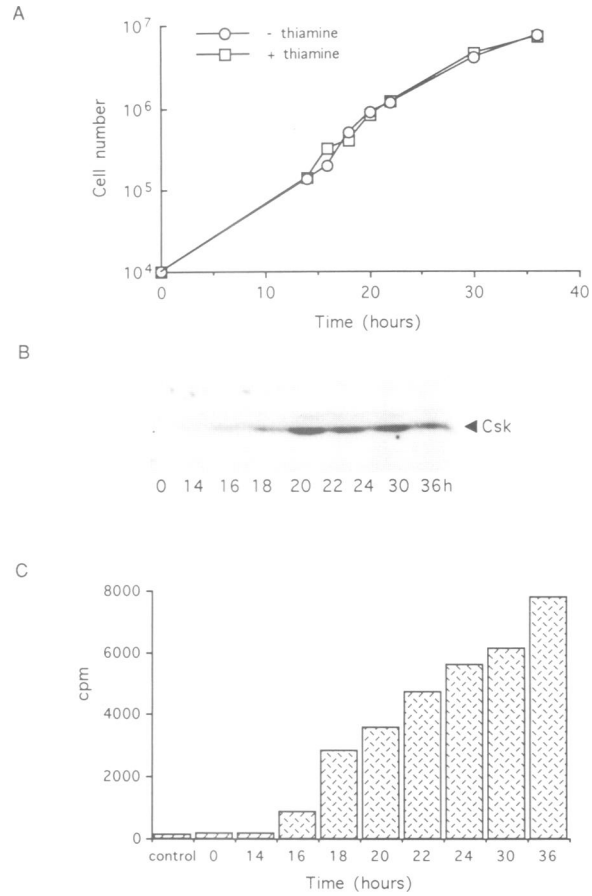

Fig. 3. Expression of Csk in *S.pombe*. (**A**) Growth curve of SP200 cells transformed with pRSP-Csk. Cells were grown in the presence or absence of thiamine as described in Materials and methods. (**B**) Immunoblot of lysates prepared from *S.pombe* cells expressing Csk (p50). Csk was detected from cell lysates using an antibody specific for the carboxy-terminal 10 amino acids of Csk (M.Koegl and S.A.Courtneidge, unpublished). (**C**) Csk kinase assay. Extracts derived from untransformed SP200 cells (control) and from cells transformed with pRSP-Csk were immunoprecipitated with anti-Csk antibodies and the kinase activity of immunoprecipitates was measured.

to that of the parental strain. A phenotype identical to the one caused by expression of Src was observed when the closely related tyrosine kinase Fyn was expressed (Table I), suggesting that the ability to cause lethality to *S.pombe* was common to two and possibly all members of the Src family of tyrosine kinases. Fyn and c-Src proteins mutated in sequences required for ATP binding did not affect the growth of *S.pombe* (Table I), indicating that a functional catalytic domain was essential for the observed phenotype. Interestingly, expression of the Src mutant Y527F (see Figure 1), in which the regulatory Tyr527 in the carboxy-terminus had been mutated to phenylalanine, which cannot be phosphorylated, conferred a phenotype that was indistinguishable from wild type Src. Therefore Src did not appear to be regulated in *S.pombe* cells, suggesting the absence of a kinase able to phosphorylate Tyr527 in this organism (see also Figure 8; Nada *et al.*, 1991).

Expression of Csk causes no phenotype in S.pombe

We then tested the effects of expression of the human c-Src kinase, Csk (a kind gift of M.Bergman and K.Alitalo), in *S.pombe*. Csk expressed from the thiamine-repressible *nmt1* promoter had no detectable effect on the growth of *S.pombe*

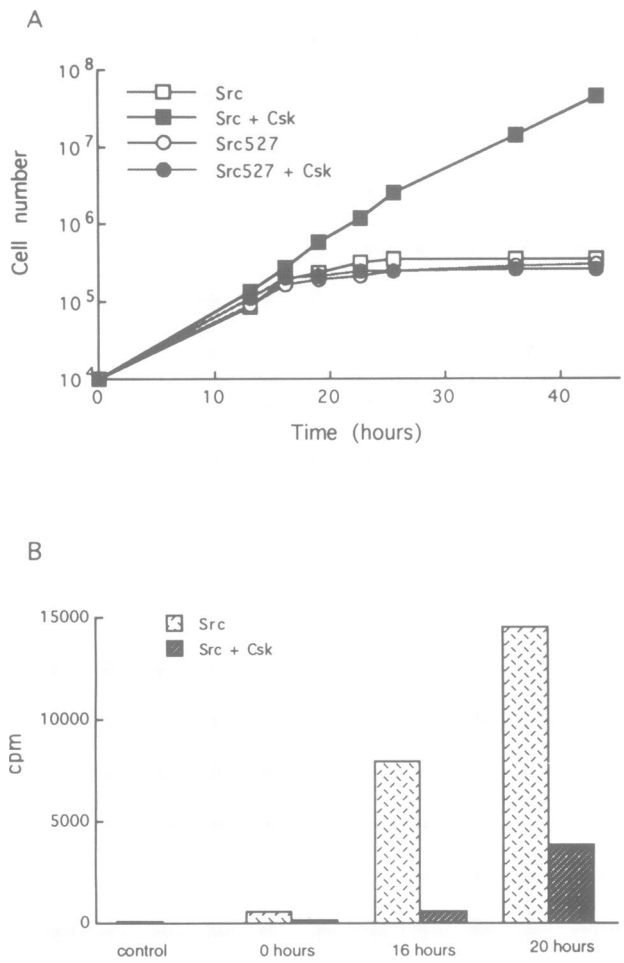

Fig. 4. Co-expression of Src and Csk in *S.pombe*. (**A**) Growth curve of SP200 cells expressing Src and Csk. Cells were transformed with either pRSP-c-Src or pRSP-Src527 (Y527F) as well as with either the control vector pAU or with pAU-Csk, and grown in the presence or absence of thiamine. (**B**) Src kinase assay. Extracts derived from untransformed SP200 cells and from cells in which c-Src was induced, both in the absence and in the presence of Csk, were assayed for c-Src kinase activity using enolase as exogenous substrate as described (Kypta *et al.*, 1990).

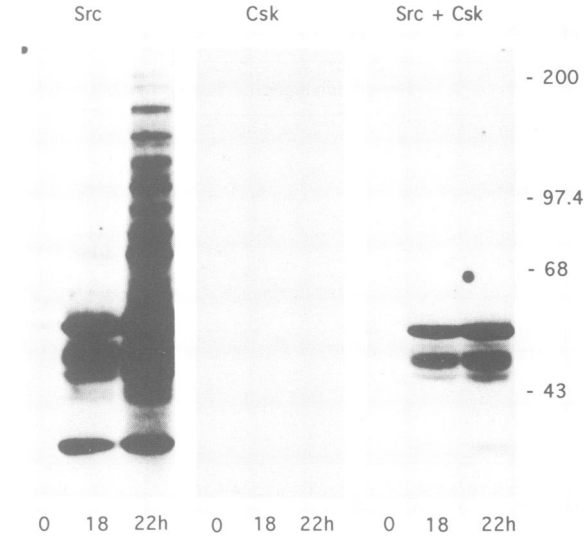

Fig. 5. Analysis of phosphotyrosine-containing proteins. SP200 cells transformed with either pRSP-c-Src and pAU (control vector) (left-hand panel), pRSP-Csk and pAU (middle panel), or pRSP-c-Src and pAU-Csk (right-hand panel), were induced by removal of thiamine from the medium for the indicated times. For each sample 75 μg of extract were resolved by SDS−PAGE and probed with antibodies specific for phosphotyrosine. Migration of molecular markers is shown on the right. The autoradiographs were exposed for identical time periods. No bands were visible in the Csk panel even after much longer exposure.

(Figure 3A). Synthesis of the Csk protein, however, was efficiently induced in these cells (Figure 3B). To test whether Csk recovered from the expressing strain was active, we immunoprecipitated the protein from cells that had been induced with thiamine for different times and performed kinase assays using poly(Glu−Tyr) as an exogenous substrate (Figure 3C). Kinase activity was found to parallel the induction profile of the protein, indicating that an active tyrosine kinase was being made in these cells.

Csk expression counteracts the Src-induced phenotype

Csk has been shown to phosphorylate Src when the two proteins are co-expressed in the yeast *S.cerevisiae* (Nada *et al.*, 1991). We tested whether co-expression of Csk and c-Src in *S.pombe* would affect the kinase activity of either protein and counteract the lethal phenotype caused by c-Src expression. We used a different constitutive promoter to express Csk, in order to minimize possible effects of promoter interference. For this purpose, Csk was expressed from the *adh1* promoter of *S.pombe* (Russell and Hall, 1983). [At the fully induced stage, the *nmt1* promoter is ~5 times stronger than the *adh* promoter (Basi *et al.*, 1993).] As expected, cells that expressed Csk constitutively were able to grow. However, when c-Src expression was induced in these cells, no growth inhibition was observed (Figure 4A) and the cells were indistinguishable from wild type upon microscopic inspection. Thus, constitutive expression of Csk was able to counteract fully the c-Src-induced phenotype. To test whether Csk was acting through phosphorylation of Tyr527 of Src, we co-expressed Csk and a Y527F allele of c-Src (Figure 4A). Csk was unable to prevent lethality of Y527F Src, suggesting that phosphorylation of this site by Csk was required for rescue to occur.

We analysed the repression of c-Src by Csk in more detail. First, we determined that co-expression of the Csk and c-Src proteins did not alter the expression level of either (data not shown). We next immunoprecipitated c-Src at different time points after induction from Csk-expressing cells and from cells carrying the parental vector as control. These immunoprecipitates were tested in a kinase assay using enolase, which is not a substrate for Csk (Okada and Nakagawa, 1988b; our unpublished observations), to measure c-Src activity (Figure 4B). A low level of activity was detected in cells expressing c-Src at time 0, but this basal activity was not detected in cells that co-expressed Csk. Indeed Src kinase activity was always lower in extracts derived from Csk-expressing cells than in equivalent extracts from cells that did not express Csk, even when the levels of Src expression had reached their maximum. Thus, the rescue of the Src-induced phenotype by Csk was accompanied by a reduction in Src kinase activity.

To test the ability of Src and Csk to phosphorylate endogenous proteins and thus measure the activity of c-Src and Csk *in vivo*, we probed blots of total cellular proteins with antibodies against phosphotyrosine (Figure 5). No phosphotyrosine-containing proteins were detected in wild

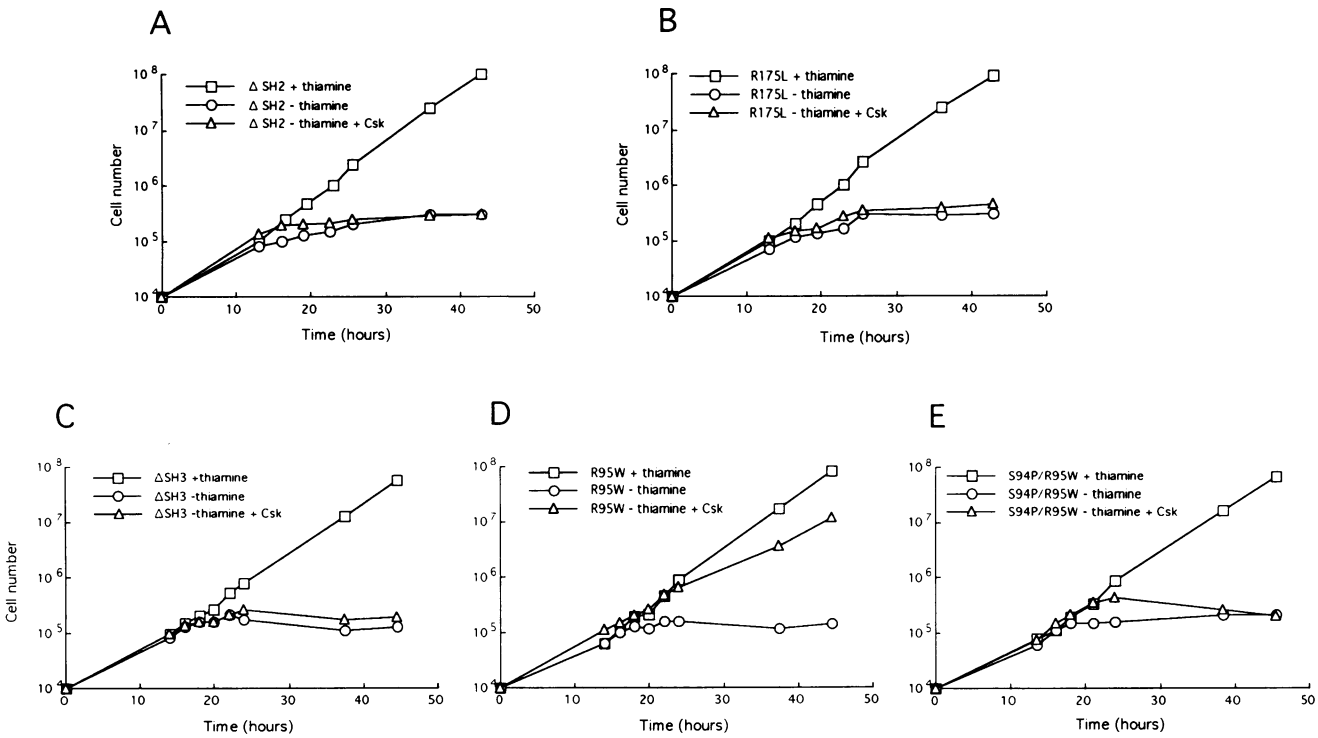

Fig. 6. Growth curves of *S.pombe* cells co-expressing Csk and various Src mutants. SP200 cells transformed with either pAU (control vector) or pAU-Csk and any of the various Src mutants were grown under repressed (+ thiamine) or induced (− thiamine) conditions. At different time points, samples were taken and the cell number determined by counting under the microscope. (**A**) ΔSH2 Src and Csk; (**B**) R175L Src and Csk; (**C**) ΔSH3 Src and Csk; (**D**) R95W Src and Csk; (**E**) S94P/R95W Src and Csk.

type *S.pombe* cells in either exponential or stationary phase, even after prolonged exposure of the autoradiograph. We attribute this to the fact that the detection of yeast proteins phosphorylated on tyrosine (e.g. cdc2) requires the immunoprecipitation of these proteins from at least 20 times more extract than we used in these analyses (Lundgren *et al.*, 1991; our unpublished observations). Upon induction of c-Src expression, a large number of phosphotyrosine-containing proteins was observed. In contrast, no such phosphotyrosine-reactive bands appeared concomitant with the induction of Csk expression from the same promoter, even after long induction times and prolonged exposure of the autoradiograph. This result is in agreement with the difference between the ability of the two proteins to cause growth arrest and lethality when expressed in *S.pombe*. We also tested cells co-expressing c-Src and Csk. In these cells we observed a marked reduction in the accumulation of phosphotyrosine-containing proteins of all sizes, except for two major bands of ~60 and 50 kDa. Depletion with anti-Src antibodies revealed that these bands corresponded to c-Src and a degradation product thereof (data not shown). Taken together, these results indicate that c-Src phosphorylated a number of proteins in yeast cells, whereas Csk had a much narrower substrate specificity, with its only substrate being c-Src itself.

c-Src SH2 domain mutants are insensitive to Csk action

To test the model that downregulation of Src activity occurs via an intramolecular interaction between the tail and the SH2 domain (Figure 10E), we have created three c-Src mutants (Figure 1): in the first, the whole SH2 domain was deleted (ΔSH2, Trp148 to Leu241; Seidel-Dugan *et al.*, 1992); in the second, the B box was deleted (ΔB, Trp148 to Ser187; Seidel-Dugan *et al.*, 1992); and in the third, the arginine at position 175 was changed to leucine (R175L; Hirai and Varmus, 1990). When introduced into *S.pombe* under the control of the *nmt1* promoter, all of these c-Src mutants induced the lethal phenotype in a manner indistinguishable from wild type c-Src (Figure 6A and B, and Table I), demonstrating that the SH2 domain was not required for c-Src to cause growth arrest. Interestingly, an intact SH2 domain was also not required to target c-Src to substrates, since neither qualitative nor quantitative differences in phosphotyrosine-reactive bands were detected between cells expressing the mutants and wild type Src (Figures 7A and 5). We then asked whether Csk was able to rescue the lethal phenotype caused by these mutants (Figure 6). In no case did co-expression of Csk rescue the phenotype, in contrast to the ability of Csk to rescue wild type c-Src (Figure 4A). Immunoblotting experiments showed that equivalent levels of mutant c-Src proteins (Figure 7C) and Csk (data not shown) were expressed. In keeping with this lack of rescue, the introduction of Csk into cells expressing the SH2 mutants had little effect on the phosphorylation of endogenous proteins (Figure 7A). A slight downregulation of R175L Src by Csk, as judged by the abundance of tyrosine-phosphorylated proteins, was detected, perhaps reflecting a residual affinity of the mutated SH2 domain for phosphorylated Tyr527 or residual regulation by the SH3 domain (see below). However, this inhibition of Src activity by Csk was undetectable at the phenotype level (Figure 6B).

c-Src SH3 domain mutants show different sensitivities to Csk

To study the properties of the SH3 domain in the *S.pombe* system, we created three Src SH3 mutants (Figure 1): a

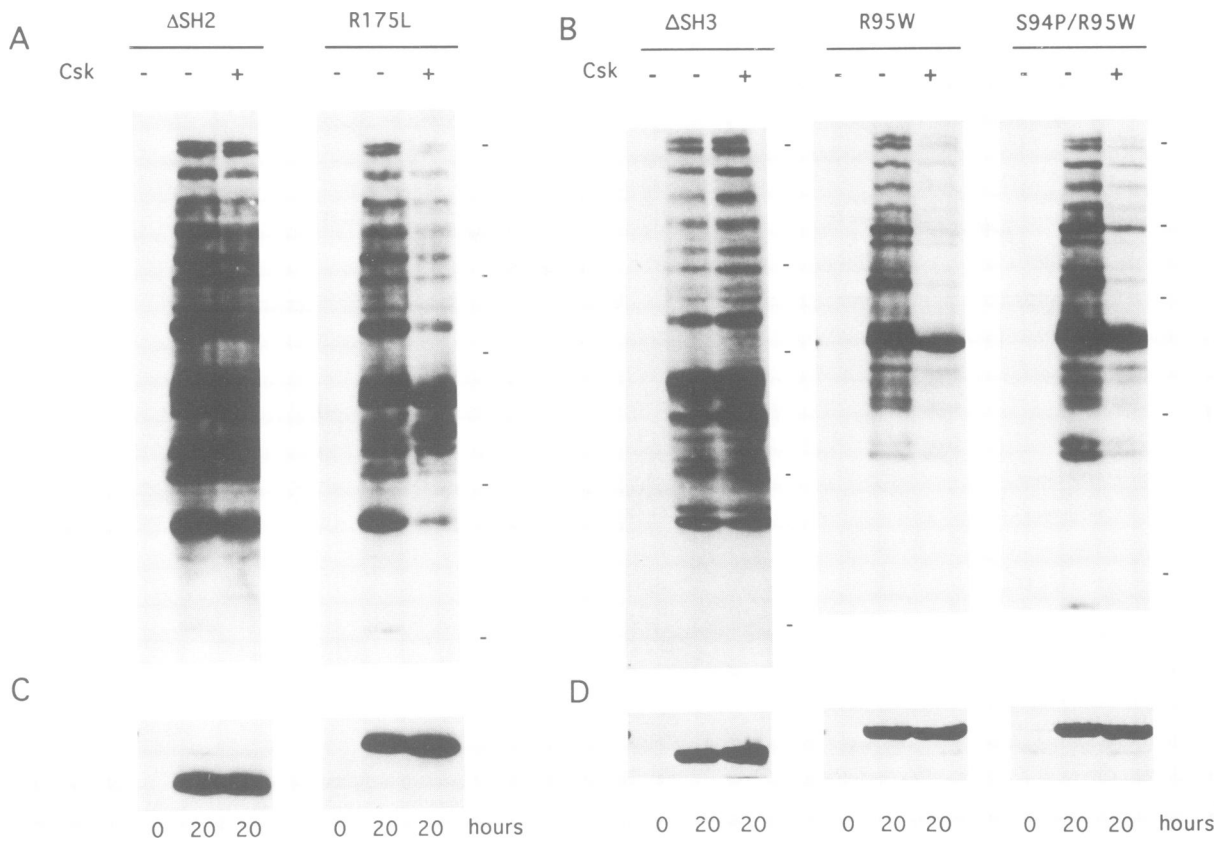

Fig. 7. Analysis of phosphotyrosine-containing proteins induced by expression of the various Src mutants in the absence or presence of Csk. Extracts of SP200 cells transformed with either pAU (control vector) or pAU-Csk and any of the various Src mutants before or after 20 h of induction were analysed for *in vivo* kinase activity by immunoblotting with anti-phosphotyrosine antibody. The expression levels of the Src mutants were monitored by probing parallel blots with anti-Src antibodies and expression of Csk tested by probing with anti-Csk antibodies (not shown). The positions of molecular weight standards are indicated at the right side of the panels, and correspond (from top to bottom) to a molecular weight of 200, 97.4, 68, 43 and 25.7 kDa. (**A**) SH2 mutants, anti-phosphotyrosine; (**B**) SH3 mutants, anti-phosphotyrosine; (**C**) SH2 mutants, anti-Src; (**D**) SH3 mutants, anti-Src.

deletion of the entire SH3 domain (ΔSH3, Ala87 to Val137), a point mutation in a residue frequently mutated in different RSV strains (reviewed in Parsons and Weber, 1989), and found to be sufficient to activate c-Src (Kato *et al.*, 1986; Potts *et al.*, 1988) (R95W), as well as a two amino acid mutant in the same location (S94P/R95W) that was created by chance. Inducible expression of these mutants revealed that an intact SH3 domain was not required for the Src kinase to cause lethality in *S.pombe* (Figure 6C, D and E). The ability of these mutants to phosphorylate endogenous proteins was also indistinguishable from wild type Src (Figures 7B and 5). However, Csk was unable to counteract the lethality caused by expression of the ΔSH3 and S94P/R95W mutants, as judged both by the growth behaviour (Figure 6C and E) and by the phosphorylation of endogenous proteins (Figure 7B). On the other hand, the SH3 mutant carrying the point mutation at position 95 (R95W) was partially rescued by Csk co-expression (Figure 6D). While these cells appeared to have only slightly abnormal cell shapes (not shown), their doubling time at 30°C in minimal medium was ~4 h, compared with 2.5–3 h for a fully rescued strain. This intermediate phenotype was also reflected in the activity of the protein as measured by the phosphorylation of endogenous proteins (Figure 7B). The reduction in anti-phosphotyrosine-reactive bands was not as dramatic as for wild type Src (compare Figure 7B with Figure 5), but in contrast to the levels displayed by the R175L mutant (Figure 7A), was compatible with growth. Possibly these mutants, in the presence of Csk, define a threshold level of Src activity that inhibits growth.

The Src SH2 and SH3 mutants are substrates of Csk

The lack of rescue by Csk of the Src alleles carrying mutations in SH2 or SH3 domains could have been due to failure of the mutated domains to convey the inhibitory effect of Tyr527 phosphorylation or to the failure of Csk to utilize these Src mutants as substrates. To distinguish between these two possibilities cells expressing the mutant proteins in the presence and absence of Csk were labelled with [^{32}P]orthophosphate, c-Src was immunoprecipitated, cleaved with CNBr and its fragments separated on an acrylamide gel. Autophosphorylated c-Src and c-Src phosphorylated *in vitro* by Csk were used as controls to show the 10 kDa fragment containing Tyr416 and the 4 kDa fragment containing Tyr527 (Figure 8). Co-expression of Csk and wild type Src in *S.pombe* led to a strong phosphorylation of Tyr527 of c-Src, and a weaker phosphorylation of Tyr416, in keeping with previously published results. The c-Src mutants carrying deletions of either the SH2 (ΔSH2) or SH3 (ΔSH3) domains were also phosphorylated on Tyr527 to the same extent as wild type. From this we conclude that the failure of Csk to counteract the lethal phenotype caused by overexpression of Src SH2 and SH3 mutants in *S.pombe*, as well as the failure to

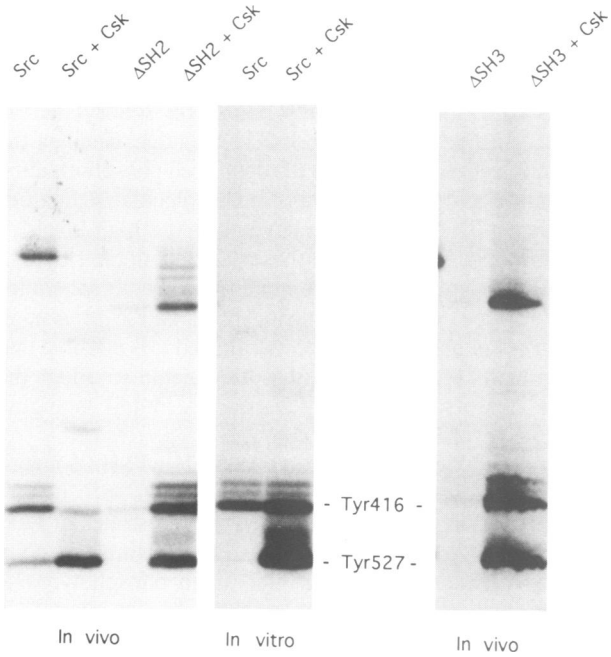

Fig. 8. CNBr mapping of *in vivo* and *in vitro* labelled Src proteins. Lysates from [^{32}P]orthophosphate-labelled cells were immunoprecipitated with anti-Src antibodies and subjected to cleavage by CNBr followed by separation of the peptides by polyacrylamide gel electrophoresis. Src labelled by Csk produced in insect cells was used as control. Prestained molecular weight markers were run in parallel (not shown). Migration of the peptides containing Tyr416 and Tyr527 is indicated.

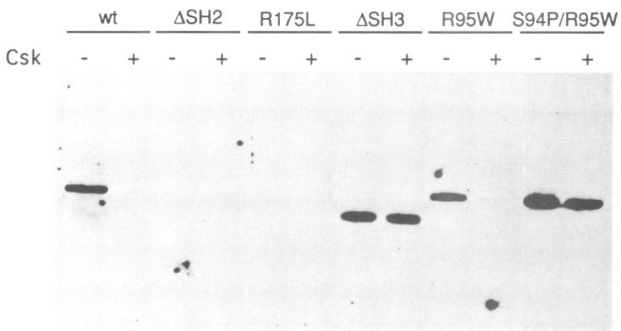

Fig. 9. Binding of Src molecules to a phosphotyrosine-containing peptide. Extracts from *S.pombe* cells expressing the constructs indicated, in the presence and absence of Csk, were incubated with a phosphotyrosine-containing peptide and bound Src molecules identified by immunoblotting as described in Materials and methods.

suppress the kinase activity of these proteins, was not due to the inability of Csk to phosphorylate them.

A peptide binding assay distinguishes different conformations of Src

The above data derived from the SH3 domain mutants suggest that either the SH2 domain−tail interaction occurs in the absence of an SH3 domain, yet the molecule is nevertheless still active, or that in the absence of an SH3 domain, the tail and the SH2 domain cannot interact. In order to distinguish between these two possibilities, we took advantage of a phosphopeptide binding assay first described by Roussel *et al.* (1991). A phosphopeptide that we had previously shown to have high affinity for activated Src (S.A.Courtneidge, unpublished observations) was coupled to a resin, *S.pombe* extracts from cells containing the various

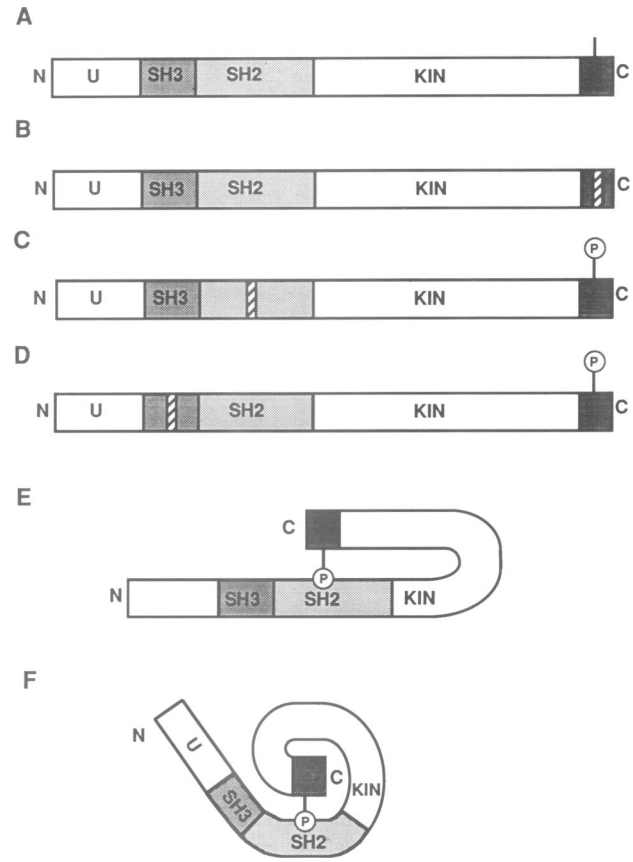

Fig. 10. Models for Src regulation. (A−D), active configurations; (E and F), inactive configurations. (**A**) Unphosphorylated c-Src. The molecule is active and the SH2 and SH3 domains are free to interact with other proteins. (**B**) c-Src molecules mutated at Tyr527 or at residues important for phosphorylation by Csk are unphosphorylated and therefore active. (**C**) c-Src molecules bearing mutations in the SH2 domain are active even when phosphorylated at Tyr527. (**D**) c-Src molecules bearing mutations in the SH3 domain are active even when phosphorylated at Tyr527. (**E**) The interaction of the phosphorylated Tyr527 with the SH2 domain locks the molecule in a conformation that has low kinase activity and in which the SH2 domain is inaccessible to other proteins. (**F**) Phosphorylated Tyr527 interacts with the SH2 domain as in E. The SH3 domain interacts intramolecularly, perhaps with a region near the tail, thus stabilizing the folded, inactive conformation. Both SH2 and SH3 domains are engaged and therefore unable to interact with other proteins.

Src mutants were passed over it and bound Src proteins subsequently identified by immunoblotting (Figure 9). Wild type Src bound to the resin (Figure 9), but not to a similar resin containing non-phosphorylated peptide (data not shown). However, Src derived from cells that contained Csk was unable to bind, demonstrating that the assay distinguished between activated Src molecules and those whose SH2 domains were interacting with tail sequences and therefore unable to bind to the phosphopeptide. We next tested the mutant Src molecules in this assay. As expected, neither the ΔSH2 mutant nor the R175L mutant were able to bind to the resin. However, both the ΔSH3 and S94P/R95W mutants bound to the resin, regardless of whether they were derived from cells expressing Src alone or Src and Csk. The ΔSH3 mutant protein did not bind the control resin with the unphosphorylated peptide (not shown). We interpret these data in favour of a model in which the integrity of the SH3 domain is required for the SH2 domain to interact with the tail of Src.

Discussion

In this study we have found that high level expression of c-Src caused a very characteristic, lethal phenotype in *S.pombe*. A Src molecule with a Tyr to Phe mutation at residue 527 was no more efficient than wild type Src in causing the lethal phenotype, suggesting that *S.pombe* cells do not contain an endogenous Tyr527 kinase. This system therefore, with its neutral background, seemed ideal to study the regulation of Src activity. Indeed, we have shown that Csk, an enzyme previously shown to phosphorylate c-Src *in vitro* and in *S.cerevisiae*, was fully able to regulate c-Src in *S.pombe* and could rescue the lethal phenotype. Our findings are in keeping with recently published experiments (Sabe *et al.*, 1992), in which it was shown that overexpression of Csk in rat fibroblasts transformed by the concerted action of the v-*crk* and c-*src* genes resulted in a suppression of the transformed phenotype, coincident with a slight reduction in kinase activity of Src recovered from these cells. Sabe *et al.* (1992), however, did not test the requirement for SH2 and SH3 domains for Csk action. Indeed, the experiment may not be feasible if intact SH2 and SH3 domains are required for Src/Crk-mediated transformation.

Csk was previously reported to have a narrow substrate specificity *in vitro* (Okada and Nakagawa, 1988b) and we now extend these findings to an *in vivo* system. This is in sharp contrast to the large number of phosphotyrosine-containing proteins observed when c-Src was expressed in *S.pombe*. The structural organization of the two proteins is very similar; the main differences being the lack of a unique domain at the amino-terminus of Csk and the lack of both major tyrosine phosphorylation sites in the carboxy-terminal half of the protein. The dramatic difference in phenotypes that the expression of the two tyrosine kinases caused most likely reflects the difference in substrate specificity of these two enzymes. The system described here provides a powerful assay for the identification of the structural determinants of these substrate specificities.

Phosphorylation of the tail of Src *in vivo* (in mammalian cells) requires few of the residues preceding the Tyr527 to be conserved (MacAuley and Cooper, 1988, 1990; Kussick and Cooper, 1992), however, the spacing from the end of the catalytic domain to Tyr527 appears to be critical (Harvey *et al.*, 1989; Cobb *et al.*, 1991), indicating that the tertiary structure of Src may be important for efficient substrate recognition by Csk. In agreement with this, peptides modelled on the Tyr527 sequence, heat-inactivated c-Src (Okada and Nakagawa, 1988a) and heat-inactivated Fyn (M.Koegl, unpublished results) are all poor substrates of Csk. We have shown that neither the SH2 nor the SH3 domain of Src was required for recognition by Csk, although it is interesting that when these mutant proteins were co-expressed with Csk, they were phosphorylated on both Tyr416 and Tyr527. Either two distinct Src populations were present in these cells or the altered protein structure of these mutants allowed the two tyrosines to be phosphorylated in the same molecule. Since no downregulation of the activity of the mutants by Csk was observed, as judged by growth of the cells or by phosphorylation of endogenous proteins, we believe the latter explanation is more likely to be correct.

A recent study of c-Src SH2 and SH3 deletion mutants (identical to the ΔSH2, ΔB and ΔSH3 mutants of this study) expressed in chicken embryo fibroblasts showed that these mutations had an activating effect in transformation assays and showed more *in vitro* kinase activity than wild type Src (Seidel-Dugan *et al.*, 1992). In these cells, however, the mutants were found to be only poorly phosphorylated on Tyr527, so that it was not possible to judge whether the increase in activity was due to the decreased phosphorylation of the tail or to the impairment of intramolecular regulatory interactions. The fact that we have observed high levels of phosphorylation of the tails of these deletion mutants by Csk in *S.pombe* suggests that these mutants are better substrates than wild type Src for a tyrosine phosphatase present in fibroblasts but absent in yeast cells. Indeed it is possible that in all mutant Src molecules that are unable to adopt the inactive conformation, the phosphorylated tail is highly accessible to phosphatases.

Src molecules bearing mutations in SH2 and SH3 domains were insensitive to the regulatory effects of Tyr527 phosphorylation, even though they were substrates of Csk. A Src molecule with an SH2 domain containing a point mutation predicted to abolish binding to phosphotyrosine (Waksman *et al.*, 1992) was neither able to bind to a phosphorylated peptide *in vitro*, nor to be regulated by Csk *in vivo*. These experiments provide direct evidence for the interaction of phosphorylated Tyr527 with the SH2 domain and thus strongly argue for the validity of the SH2−phosphorylated tail interaction model of Src inactivation. However, we also found that Src molecules with mutations in the SH3 domain were not susceptible to regulation, even though they too were Csk substrates. We therefore propose an extension of the model to include a contribution of the SH3 domain.

How is the negative effect of the SH3 domain conveyed? One possibility is that an inhibitor of Src interacts with the SH3 domain and contributes to downregulation of the enzyme activity. Such an inhibitor would have to be present in *S.pombe* and since Src-like molecules have not been identified in yeast, to have evolved independently of Src and bind Src fortuitously, or be able to interact with SH3 domains in general. While proteins with SH3 domains are known in the yeasts (Drubin *et al.*, 1990; Hughes *et al.*, 1990; Chenevert *et al.*, 1992) and therefore SH3 binding proteins could also be expressed, it is not easy to see why the interaction of such a protein with Src should be dependent on Tyr527 phosphorylation. In addition, the fact that the ΔSH3 Src mutant appeared to be no more active than wild type Src in *S.pombe* argues against this possibility. Moreover, the peptide binding experiments indicated that the SH3 domain was necessary for the Src molecule to acquire the conformation in which the SH2 domain is engaged by interaction with the phosphorylated tail, which can be most simply explained by an intramolecular interaction.

With which sequences does the SH3 domain interact? An inhibitory interaction of the kinase domain with Tyr90 and Tyr92 in the SH3 domain (a potential pseudosubstrate site) has been proposed (Grandori, 1989). In some instances, these residues are indeed phosphorylated *in vivo* (Espino *et al.*, 1990) and Src molecules in which these tyrosines have been mutated to phenylalanines show an increase in kinase activity. However, such a model does not easily explain the requirement for Tyr527 phosphorylation. We propose that the interaction of the SH2 domain with the phosphorylated tail is stabilized either by binding of the SH3 domain to a

region at the end of the catalytic domain (Figure 10F), or by an SH3 domain−SH2 domain interaction (not shown). Baltimore and colleagues (Cicchetti *et al.*, 1992; Ren *et al.*, 1993) have proposed that SH3 domains recognize proline-rich sequences and we note that residues 482−508 are the most proline-rich region of c-Src (six out of 27 residues) and thus a candidate for an SH3 binding site. In support of this model, it has been shown that alteration of the spacing between the carboxy-terminal tail and the kinase domain at residue 518 induces activation of the enzyme (Cobb *et al.*, 1991). The SH2 and SH3 domains are separated from each other by only a few residues and it may not be possible for both domains to interact with their target sequences if they are not ideally spaced. In support of the second possibility, that the SH3 domain may recognize and stabilize the SH2−tail interaction, is the observation that mutations in the SH3 domain affect the ability of the SH2 domain of Src to interact with the PI-3 kinase (Wages *et al.*, 1992). We will be able to test these models by site-directed mutagenesis of the 482−508 region and by screening for second-site suppressors of SH3 mutants in the presence of Csk.

In summary we have shown that *S.pombe* is an ideal organism in which to study the regulation of heterologous tyrosine kinases. It has a neutral background and appears to be very sensitive to tyrosine kinase activity. Moreover, this model system should represent an excellent assay for the screening and identification of compounds able to inhibit tyrosine kinases or able to interfere with SH2 and SH3 function. Finally, this system provides a means for the genetic identification of other molecules that both positively and negatively regulate c-Src.

Materials and methods

Yeast strains and culture conditions

The yeast strain used in this study is SP200 (*h*$^{-s}$ *leu1.32 ura4 ade210).* Growth conditions were as described in Beach *et al.* (1985) and Moreno *et al.* (1991). To induce the *nmt1* promoter, cells grown to saturation in PMA medium containing 4 μM thiamine were washed three times with PMA lacking thiamine and cultured in PMA either in the presence or in the absence of thiamine. Cells were grown at 30°C in a shaking water bath.

Yeast transformation

Transformation was performed using the lithium acetate method (Moreno *et al.*, 1991) with the following modifications. After addition of DNA, the cells were incubated for 10 min at 30°C prior to addition of 50% PEG. After 40 min incubation at 30°C, the cells were heat-shocked at 42°C for 15 min, washed with 1 ml YEA, resuspended in 1 ml YEA and incubated at 30°C for 1 h in microfuge tubes. Cells were then collected by a short centrifugation and spread directly on PMA plates containing 4 μM thiamine and either leucine, uracil or nothing (Moreno *et al.*, 1991).

DNA constructs

pRSP is a derivative of the *S.pombe* expression vector pRep1 (Maundrell, 1990), which is under the control of the thiamine-repressible promoter of the *nmt1* gene. In pRSP the *Nde*I cloning site was deleted, the *Bam*HI and *Sma*I sites maintained and the unique sites of *Spe*I, *Nru*I, *Xho*I and *Not*I introduced. pAU is a derivative of pART1 (McLeod *et al.*, 1987) in which the *LEU2* selection marker has been substituted with the *ura4*$^{+}$ gene from *S.pombe*. For inducible expression in *S.pombe*, chicken full-length c-*src* cDNA as well as the K295M and Y527F mutants were cloned as *Bam*HI−*Sma*I fragments into pRSP. Full-length human *fyn* cDNA and the K299M mutant version were cloned in the *Bam*HI site of pRSP. Full-length human *csk* cDNA was cloned as a 2033 bp *Sac*I fragment from pSVCyl (a kind gift of M.Bergman and K.Alitalo) through two separate cloning steps designed to add *Bam*HI sites to the *csk* cDNA into pRSP and pAU cut with *Bam*HI. Deletion and point mutations in the chicken c-*src* cDNA were engineered using PCR-based mutagenesis (Higuchi *et al.*, 1988). To minimize the risk of accidental mutations, we have targeted the PCR reactions to a 538 bp fragment spanning from the *Bsa*HI site to the *Mlu*I site. Shortly, amplification was done employing two outside oligos containing the *Bsa*HI site or the *Mlu*I site in combination with internal oligos encompassing the required deletion in a two-step procedure (sequences of the oligos available on request). Reconstruction of the whole *src* cDNA in the inducible pRSP vector was achieved by ligating the mutated *Bsa*HI−*Mlu*I fragments with a fragment spanning the kinase domain and the polyadenylation site in the vector (*Mlu*I−*Sac*I 1700 bp fragment), a fragment containing the amino-terminus of the kinase and part of the 5′ polylinker (*Spe*I−*Bsa*HI 250 bp fragment) and the vector (*Spe*I−*Sac*I ~7900 bp) in four-way ligations followed by electroporation in the *E.coli* DH5α strain. All mutants were checked by sequencing.

Lysates

Lysates were made as follows: the cells were collected by centrifugation at 1500 *g*, followed by one wash with ice-cold PBS containing 0.1 mM sodium orthovanadate. A volume equal to the cell pellet of glass beads and 1 vol of lysis buffer [Lysis buffer: 50 mM Tris pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml TPCK (tosyl phenylalanine chloromethyl ketone), 5μg/ml TLCK (Na-*p*-tosyl-lysine chloromethyl ketone)] were added. The samples were vortexed in the cold-room for 10 min. Cell debris and glass beads were removed by a short centrifugation at full speed in a microfuge. The supernatant was cleared by a second 15 min centrifugation and the protein concentration was measured (Bradford, 1976).

Immunoblots

75 μg of extract were analysed by SDS−PAGE and transferred to a nitrocellulose membrane using the Milliblot-SDE System (Millipore). The filter was immunoblotted with one of the following antibodies: (i) affinity-purified anti-Src monoclonal antibody 2-17 (Microbiological Associates) (1:1500 dilution); (ii) affinity-purified rabbit polyclonal anti-peptide antibodies against the carboxy-terminus of the human Csk kinase [M.Koegl and S.A.Courtneidge, unpublished (1:300 dilution)]; (iii) monoclonal anti-phosphotyrosine antibodies (UBI 1:1500 dilution). Detection was with either ^{125}I-labelled anti-mouse antibody (0.2 μci/ml, Amersham) and autoradiography or by incubation with protein A-coupled with horseradish peroxidase followed by the chemiluminescence ECL (Amersham) procedure.

Kinase assays

Csk was immunoprecipitated using antibodies specific for its carboxy-terminus; the immunoprecipitates were washed three times in RIPA (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxicholate, 1% Triton and 0.1 mM vanadate) and once in TBS (25 mM Tris pH 7.5, 150 mM NaCl and 0.1 mM vanadate), and kinase assays performed for 10 min at 30°C in a buffer containing 20 mM HEPES pH 7.5, 25 μM [^{32}P]ATP, 10 mM $MnCl_2$, 10 mM DTT and 0.1 mg/ml of poly(Glu−Tyr) (1:4). The poly(Glu−Tyr) was resolved by SDS−PAGE and radioactivity incorporated determined by Cerenkov counting. Src was immunoprecipitated using the 2-17 antibody; the washes were performed as described for the Csk immunoprecipitates and Src activity was assayed using acid-denatured enolase as exogenous substrate as described by Kypta *et al.* (1990).

^{32}P *in vivo labelling and CnBr mapping*

For labelling Src proteins expressed from the *nmt1* promoter, cells were washed three times with medium lacking thiamine, grown for 17 h at 30°C and collected by centrifugation. 2×10^8 cells were washed once with pre-warmed dH_20, once with pre-warmed EMMP (Moreno *et al.*, 1991), resuspended in 1.5 ml EMMP containing 50 μM NaH_2PO_4 and tranferred to a 2 ml microfuge tube. 2 mCi/ml [^{32}P]orthophosphate (Amersham) were added, the cells incubated for 40 min at 30°C, then lysed in RIPA buffer. For the *in vitro* labelling, c-Src was immunoprecipitated from NIH3T3 cells overexpressing c-Src and a kinase assay performed in the presence or absence of 1 μg of Csk produced in insect cells (M.Koegl, unpublished). The proteins were resolved on SDS−PAGE, transferred to nitrocellulose and the membrane was exposed for autoradiography. The CNBr cleavage was performed as described (Luo *et al.*, 1990) and the products resolved on a 25% polyacrylamide Tris−borate gel (Schuh and Brugge, 1988).

Phosphopeptide binding experiments

A peptide corresponding to amino acids 569−584 of the human PDGF receptor, phosphorylated at position 579, which has been shown to have a high affinity for activated Src (S.A.Courtneidge, unpublished observations), was coupled to Acti-gel according to the manufacturer's instructions (Sterogene). 10 μl of beads were incubated with 75 μg of yeast lysate in 250 μl lysis buffer (LB) (containing 150 mM NaCl) for 2 h at 4°C. The

beads were washed three times in LB, the bound proteins eluted with Laemmli buffer, subjected to SDS–PAGE and transferred to nitrocellulose; the blot was incubated with antibody 2-17 and processed as described above.

Acknowledgements

We thank M.Bergman and K.Alitalo for the human Csk cDNA, D.Nalis for phosphopeptide synthesis, M.Stein for technical assistance, G.Smith for help with the flow cytometry and G.Twamley, I.Roussou and G.Basi for reagents and suggestions. G.S.F. is a recipient of an EMBO fellowship, and S.F. of a fellowship from the University of Milan, Italy.

References

Anderson,D., Koch,C.A., Grey,L., Ellis,C., Moran,M.F. and Pawson,T. (1990) *Science*, **250**, 979–982.

Basi,G., Schmid,E. and Maundrell,K. (1993) *Gene*, **123**, 131–136.

Beach,D., Rodgers,L. and Gould,J. (1985) *Curr. Genet.*, **10**, 297–311.

Bergman,M., Mustelin,T., Oetken,C., Partanen,J., Flint,N.A., Amrein,K.E., Autero,M., Burn,P. and Alitalo,K. (1992) *EMBO J.*, **11**, 2919–2924.

Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248.

Chenevert,J., Corrado,K., Bender,A., Pringle,J. and Herskowitz,I. (1992) *Nature*, **356**, 77–79.

Cicchetti,P., Mayer,B.J., Thiel,G. and Baltimore,D. (1992) *Science*, **257**, 803–806.

Clark,S.G., Stern,M.J. and Horvitz,H.R. (1992) *Nature*, **356**, 340–344.

Cobb,B.S., Payne,D.M., Reynolds,A.B. and Parson,J.T. (1991) *Mol. Cell. Biol.*, **11**, 5832–5838.

Cooper,J.A. (1990) In Kemp,B. and Alewood,P.F. (eds), *Peptides and Protein Phosphorylation*. CRC Press, Boca Raton, FL, pp. 85–113.

Cooper,J.A. and King,C.S. (1986) *Mol. Cell. Biol.*, **6**, 4467–4477.

Cooper,J.A. and Runge,K. (1987) *Oncogene Res.*, **1**, 297–310.

Drubin,D.G., Mulholland,J., Zhu,Z. and Botstein,D. (1990) *Nature*, **343**, 288–290.

Espino,P.C., Harvey,R., Schweickhardt,R.L., White,G.A., Smith,A.E. and Cheng,S.H. (1990) *Oncogene*, **5**, 283–293.

Fantl,W.J., Escobedo,J.A., Martin,G.A., Turck,C.W., del Rosario,M., McCormick,F. and Williams,L.T. (1992) *Cell*, **69**, 413–423.

Grandori,C. (1989) *Nature*, **338**, 467.

Harvey,R., Hehir,K.M., Smith,A.E. and Cheng,S.H. (1989) *Mol. Cell. Biol.*, **9**, 3647–3656.

Higuchi,R., Krummel,B. and Saiki,R.K. (1988) *Nucleic Acids Res.*, **16**, 7351–7367.

Hirai,H. and Varmus,H.E. (1990) *Mol. Cell. Biol.*, **10**, 1307–1318.

Hughes,D.A., Fukui,Y. and Yamamoto,M. (1990) *Nature*, **344**, 355–357.

Hunter,T. (1987) *Cell*, **49**, 1–4.

Kanner,S.B., Reynolds,A.B., Wang,H.R., Vines,R.R. and Parsons,J.T. (1991) *Mol. Cell. Biol.*, **10**, 1689–1698.

Kato,J.-Y., Takeya,T., Grandori,C., Iba,H., Levy,J.B. and Hanafusa,H. (1986) *Mol. Cell. Biol.*, **6**, 4155–4160.

Koch,C.A., Anderson,D., Moran,M.F., Ellis,C. and Pawson,T. (1991) *Science*, **252**, 668–674.

Koegl,M. and Courtneidge,S.A. (1992) *Semin. Virol.*, **2**, 375–384.

Kussick,S.J. and Cooper,J.A. (1992) *Oncogene*, **7**, 1577–1586.

Kypta,R.M., Goldberg,Y., Ulug,E.T. and Courtneidge,S.A. (1990) *Cell*, **62**, 481–492.

Lundgren,K., Walworth,N., Booher,R., Dembski,M., Kirschner,M. and Beach,D. (1991) *Cell*, **64**, 1111–1122.

Luo,K., Hurley,T.R. and Sefton,B.M. (1990) *Oncogene*, **5**, 921–923.

MacAuley,A. and Cooper,J.A. (1988) *Mol. Cell. Biol.*, **8**, 3560–3564.

MacAuley,A. and Cooper,J.A. (1990) *New Biol.*, **2**, 828–840.

McLeod,M., Stein,M. and Beach,D. (1987) *EMBO J.*, **6**, 729–736.

Matsuda,M., Mayer,B.J., Fukui,Y. and Hanafusa,H. (1990) *Science*, **248**, 1537–1539.

Maundrell,K. (1990) *J. Biol. Chem.*, **265**, 10857–10864.

Mayer,B.J. and Baltimore,D. (1993) *Trends Cell Biol.*, **3**, 8–13.

Mayer,B.J. and Hanafusa,H. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 2638–2642.

Mayer,B.J., Jackson,P.K. and Baltimore,D. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 627–631.

Mayer,B.J., Jackson,P.K., Van Etten,R.A. and Baltimore,D. (1992) *Mol. Cell. Biol.*, **12**, 609–618.

Moreno,S., Klar,A. and Nurse,P. (1991) In Guthrie,C. and Fink,G. (eds), *Guide to Yeast Genetics and Molecular Biology*, Vol. 194. Academic Press, New York, pp. 795–826.

Nada,S., Okada,M., MacAuley,A., Cooper,J.A. and Nakagawa,H. (1991) *Nature*, **351**, 69–72.

O'Brien,M.C., Fukui,Y. and Hanafusa,H. (1990) *Mol. Cell. Biol.*, **10**, 2855–2862.

Okada,M. and Nakagawa,H. (1988a) *Biochem. Biophys. Res. Commun.*, **2**, 796–802.

Okada,M. and Nakagawa,H. (1988b) *J. Biochem.*, **104**, 297–305.

Okada,M. and Nakagawa,H. (1989) *J. Biol. Chem.*, **264**, 20886–20893.

Okada,M., Nada,S., Yamanashi,Y., Yamamoto,T. and Nakagawa,H. (1991) *J. Biol. Chem.*, **266**, 24249–24252.

Parsons,J.T. and Weber,M.J. (1989) *Curr. Top. Microbiol. Immunol.*, **147**, 79–127.

Partanen,J., Armstrong,E., Bergman,M., Mäkelä,T.P., Hirvonen,H., Huebner,K. and Alitalo,K. (1991) *Oncogene*, **6**, 2013–2018.

Pawson,T. (1988) *Oncogene*, **3**, 491–495.

Pawson,T. and Gish,G.D. (1992) *Cell*, **71**, 359–362.

Potts,W.M., Reynolds,A.B., Lansing,T.J. and Parson,J.T. (1988) *Oncogene Res.*, **3**, 343–355.

Ren,R., Mayer,B.J., Cicchetti,P. and Baltimore,D. (1993) *Science*, **259**, 1157–1161.

Reynolds,P.J., Hurley,T.R. and Sefton,B.M. (1992) *Oncogene*, **7**, 1949–1955.

Roussel,R.R., Brodeur,S.R., Shalloway,D. and Laudano,A.P. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10696–10700.

Russell,P.R. and Hall,B.D. (1983) *J. Biol. Chem.*, **258**, 143–149.

Sabe,H., Okada,M., Nakagawa,H. and Hanafusa,H. (1992) *Mol. Cell. Biol.*, **12**, 4706–4713.

Schuh,S.M. and Brugge,J.S. (1988) *Mol. Cell. Biol.*, **8**, 2465–2471.

Seidel-Dugan,C., Meyer,B.E., Thomas,S.M. and Brugge,J.S. (1992) *Mol. Cell. Biol.*, **12**, 1835–1845.

Thomas,J.E., Soriano,P. and Brugge,J.S. (1991) *Science*, **254**, 568–571.

Veillette,A., Caron,L., Fournel,M. and Pawson,T. (1992) *Oncogene*, **7**, 971–978.

Wages,D.S., Keefer,J., Rall,T.B. and Weber,M.J. (1992) *J. Virol.*, **66**, 1866–1874.

Waksman,G. *et al.* (1992) *Nature*, **358**, 646–653.

Received on February 12, 1993; revised on March 26, 1993