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Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions

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The protein tyrosine kinase c-Src is negatively regulated by phosphorylation of Tyr527 in its C-terminal tail. The repressed state is achieved through intramolecular interactions involving the phosphorylated tail, the Src homology 2 (SH2) domain and the SH3 domain. Both the SH2 and SH3 domains have also been shown to mediate the intermolecular interaction of Src with several proteins. To test which amino acids of the Src SH3 domain are important for these interactions, and whether the intra- and intermolecular associations involve the same residues, we carried out a detailed mutational analysis of the presumptive interaction surface. All mutations of conserved hydrophobic residues had an effect on both inter- and intramolecular interactions of the Src SH3 domain, although not all amino acids were equally important. Chimeric molecules in which the Src SH3 domain was replaced with those of spectrin or Lck showed derepressed kinase activity, whereas a chimera containing the Fyn SH3 domain was fully regulated. Since spectrin and Lck SH3 domains share the conserved hydrophobic residues characteristic of SH3 domains, other amino acids must be important for specificity. Mutational analysis of non- or semi-conserved residues in the RT and n-Src loops showed that some of these were also involved in inter- and intramolecular interactions. Stable transfection of selected SH3 domain mutants into NIH-3T3 cells showed that despite elevated levels of phosphotyrosine, the cells were morphologically normal, indicating that the SH3 domain was required for efficient transformation of NIH-3T3 cells by Src.

Key words: Csk/protein–protein interaction/*Schizosaccharomyces pombe*/SH3 domain/Src

Introduction

The Src homology 3 (SH3) domain consists of ~60 amino acids and has been recognized in several proteins (Musacchio *et al.*, 1992a). Many of these, for example phospholipase Cγ (Stahl *et al.*, 1988), GRB2 (Lowenstein *et al.*, 1992) and the phosphatidylinositol 3-kinase (PI 3-K; Otsu *et al.*, 1991), are known to be involved in signal transduction processes. Several others, for example spectrin and cortactin, are cytoskeletal proteins (Lehto *et al.*, 1988; Dubreuil *et al.*, 1989; Wasenius *et al.*, 1989; Wu and Parsons, 1993). The SH3 domain is conserved throughout evolution from yeast to mammals. Different lines of evidence suggested that SH3 domains would be involved in protein–protein interactions. Indeed, in recent years several proteins with the capacity to associate with SH3 domains *in vitro* and *in vivo* have been described (Cicchetti *et al.*, 1992; Gout *et al.*, 1993; Fumagalli *et al.*, 1994; Pleiman *et al.*, 1994; Rotin *et al.*, 1994; Taylor and Shalloway, 1994).

The first evidence for the nature of SH3 domain interactions with other proteins came from the work of Baltimore and colleagues. Screening of a cDNA expression library with the SH3 domain of the protein tyrosine kinase Abl led to the identification of two proteins: 3BP1 and 3BP2 (Cicchetti *et al.*, 1992). The binding sites on these proteins were mapped to short proline-rich motifs (Ren *et al.*, 1993). It has been shown subsequently that proline-rich motifs in other proteins also mediate their interaction with SH3 domains, e.g. the interaction of GRB2 with the guanine nucleotide exchange protein Sos (Egan *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Rotin *et al.*, 1994). While it is now clear that proline residues are involved in the SH3 domain interaction, what exactly determines the specificity of binding of any given SH3 domain awaits further investigation.

To date, the structures of several SH3 domains have been solved (Musacchio *et al.*, 1992b; Yu *et al.*, 1992; Booker *et al.*, 1993; Kohda *et al.*, 1993; Noble *et al.*, 1993; Borchert *et al.*, 1994): all show a very similar fold despite the low degree of conservation of primary sequence. The structure consists of two three-stranded antiparallel β-sheets packed together at approximately right angles, with the conserved residues clustered together on one side of the molecule (Figure 1). It has been proposed that it is this surface that interacts with ligands (Musacchio *et al.*, 1992b; Yu *et al.*, 1992; Noble *et al.*, 1993; Lim and Richards, 1994). Indeed, recent crystal and NMR structures of SH3 domains complexed with proline-rich peptides (Musacchio *et al.*, 1994; Yu *et al.*, 1994) confirm that this hydrophobic patch comprises the core of the ligand binding surface. The contribution of residues in the loops flanking the hydrophobic patch, however, is not clear from these studies with short peptides.

Protein tyrosine kinases of the Src family have one SH3 and one SH2 domain N-terminal to the catalytic sequences, as well as a short C-terminal tail containing a regulatory tyrosine phosphorylation site (Koegl and Courtneidge, 1992). Conversion of this tyrosine (Y527) into phenylalanine increases kinase activity and is sufficient to convert proto-oncogene to oncogene (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987). The tyrosine

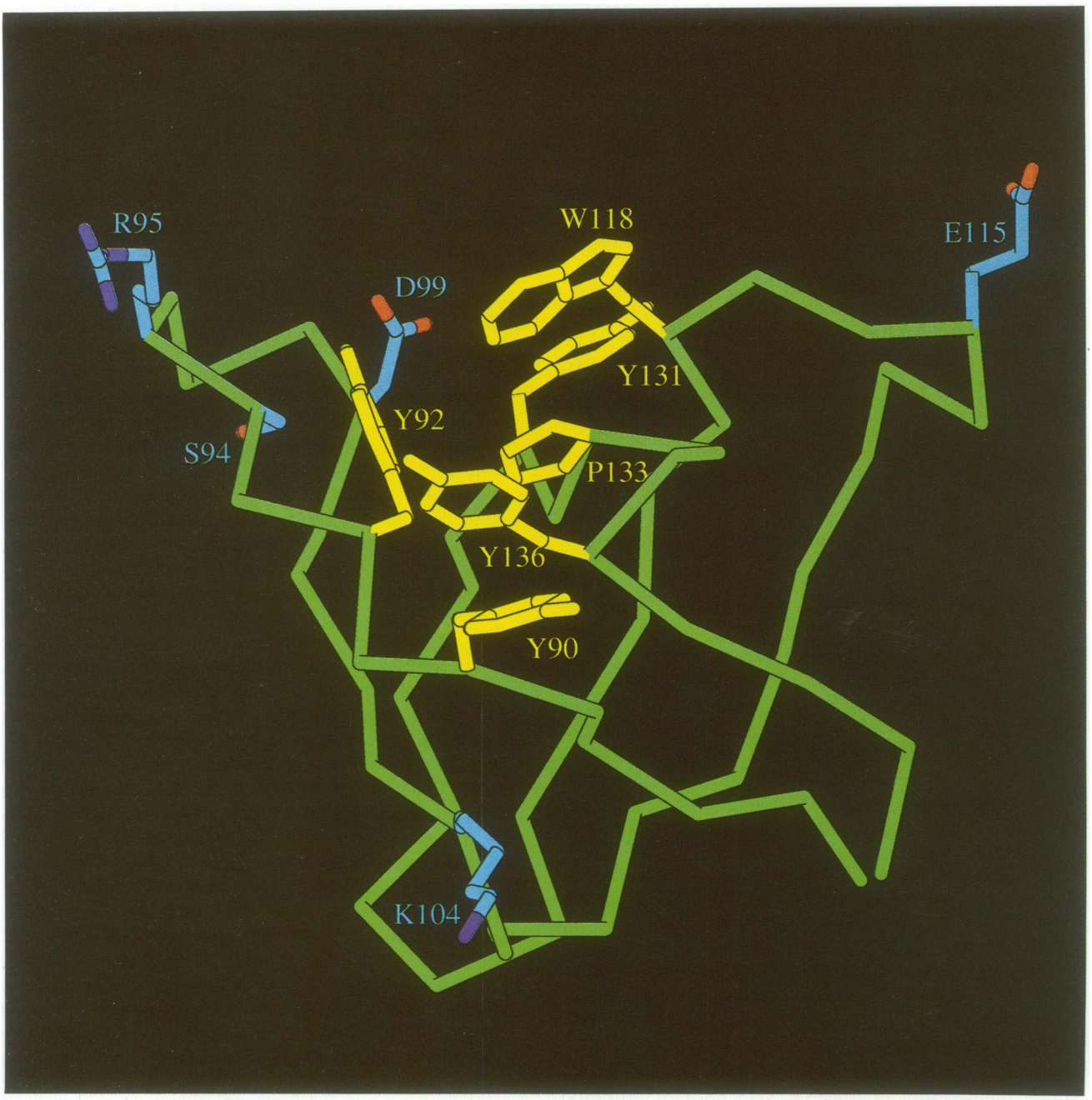

Fig. 1. Cα trace of the Src SH3 domain. The Cα trace (in green) was drawn from the coordinates of the NMR Src SH3 structure (Brookhaven Protein Database accession code 1SRL; Yu *et al.*, 1992) using XOBJECTS (M.E.M.Noble, Oxford University, UK, unpublished results). Side chains of residues mutated in this study are shown in yellow for the hydrophobic patch and blue for residues of the loops (oxygen atoms in red and nitrogen atoms in dark blue).

kinase Csk (Okada and Nakagawa, 1989; Nada *et al.*, 1991; Partanen *et al.*, 1991) is able to phosphorylate Src on Tyr527 and this results in a reduction of Src kinase activity (Okada *et al.*, 1991; Bergman *et al.*, 1992). Several pieces of evidence support the model that the kinase activity of Src is regulated by the intramolecular interaction of the tail with the SH2 domain (Cooper and King, 1986; MacAuley and Cooper, 1989; Roussel *et al.*, 1991; Superti-Furga *et al.*, 1993). Dephosphorylation of the tail, or the binding of the SH2 domain to phosphopeptides with higher affinity than the tail, de-represses kinase activity. Using yeast expression systems to study Src regulation, we and others have shown recently that the efficient regulation of Src by tail phosphorylation also requires the SH3 domain (Murphy *et al.*, 1993; Okada *et al.*, 1993; Superti-Furga *et al.*, 1993). We have further shown that in Src molecules lacking the SH3 domain, the SH2 domain was not able to associate with the tail (Superti-Furga *et al.*, 1993). Thus it seems that the SH3 domain of Src is required for an intramolecular interaction that stabilizes the binding of the SH2 domain with the phosphorylated tail.

Several proteins have been described that are able to bind to the SH3 domain of Src *in vitro* and *in vivo* (Liu *et al.*, 1993b; Prasad *et al.*, 1993; Vogel and Fujita, 1993; Weng *et al.*, 1993, 1994; Fumagalli *et al.*, 1994; Kapeller *et al.*, 1994; Pleiman *et al.*, 1994). The regulatory subunit

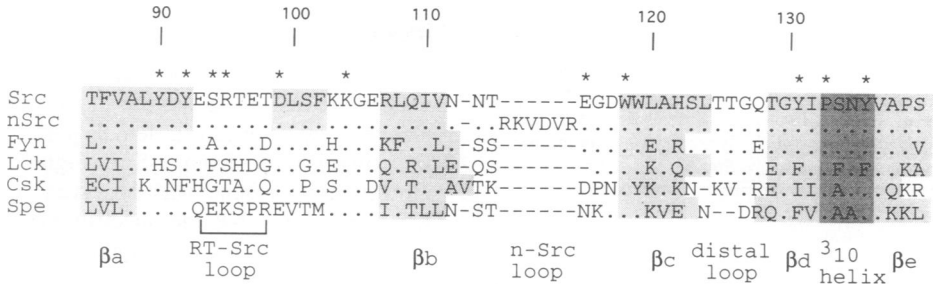

Fig. 2. Sequence comparison of SH3 domains used in this study. The numbering above the alignment refers to chicken c-Src. Regions of β-strand are shaded in light grey and regions of 3_{10}-helix in dark grey. Secondary structure assignments were taken from Borchert *et al.* (1994), Eck *et al.* (1994), Musacchio *et al.* (1992b), Noble *et al.* (1993) and Yu *et al.* (1992). Mutated residues are indicated by an asterisk. Sequences are from chicken c-Src (Takeya and Hanafusa, 1983), chicken n-Src (Levy *et al.*, 1987), human Fyn (Semba *et al.*, 1986), murine Lck (Marth *et al.*, 1986), human Csk (Partanen *et al.*, 1991) and chicken brain α-spectrin (Wasenius *et al.*, 1989).

of PI 3-K, p85, has been shown by several groups to bind to the SH3 domain of members of the Src family of tyrosine kinases *in vitro*. Recently, Pleiman *et al.* (1994) reported that this binding led to an increase in the specific activity of PI 3-K. Another protein able to associate with the Src SH3 domain is Sam68 (Courtneidge and Fumagalli, 1994; Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994). It has been shown that Sam68 is associated with and phosphorylated by c-Src derived from mitotic but not from asynchronous extracts, suggesting that Sam68 might be an important substrate for mitotically activated Src.

In this study we investigated which amino acids in the Src SH3 domain were important for Src regulation *in vivo*, tested heterologous SH3 domains for their ability to substitute for this function, and compared the binding potential of the mutated SH3 domains with known SH3 binding proteins *in vitro*.

Results

To facilitate the analysis of the individual role of residues of the Src SH3 domain in inter- and intramolecular interactions, we first used the PCR to create new restriction sites N- and C-terminal of the Src SH3 domain. This procedure only involved changes at the nucleotide level, and left the protein sequence unchanged. All point mutations in the SH3 domain of Src were made by PCR and introduced into the yeast expression vector pRSPsrcND using this cassette system.

The conserved hydrophobic residues of the Src SH3 domain are important for Src regulation

Molecular structures of several SH3 domains have revealed that the residues conserved among all SH3 domains form a cluster on one side of the molecule (Musacchio *et al.*, 1992b; Yu *et al.*, 1992; Booker *et al.*, 1993; Kohda *et al.*, 1993; Noble *et al.*, 1993). It has been predicted that these residues represent the core of the ligand binding surface. We wanted to know whether these same conserved residues were involved in the intramolecular interactions necessary for the correct regulation of Src. In our first series of mutants, the six residues of the hydrophobic patch were mutated individually (shown in yellow in Figure 1, and indicated by asterisks in Figure 2). The conserved proline residue at position 133 in c-Src was mutated to leucine, since the same mutation in the SH3 domain of the *Caenorhabditis elegans* protein Sem-5 had been shown to render it non-functional, with the consequence of impairing vulval development (Clark *et al.*, 1992). The other five aromatic residues were changed individually to alanine to replace bulky aromatic residues with small hydrophobic residues. These mutations were thought not to influence the overall stability of the SH3 domain since all aromatic side chains point into the solvent and are not involved in any contacts to main-chain atoms in the rest of the molecule.

We have reported previously that the inducible expression of c-Src in the fission yeast causes growth arrest of the cells (Superti-Furga *et al.*, 1993). The *nmt1* promoter of *Schizosaccharomyces pombe* used for this purpose is strongly induced by thiamine deprivation (Maundrell, 1990). Src expressed from this promoter starts to accumulate at ~12 h after induction and reaches maximal levels by 20 h. The expression of Src alleles with point mutations of the hydrophobic residues in the SH3 domain in the same fashion resulted in growth arrest 20–25 h after induction (Table I), indicating that the mutations introduced into the SH3 domain did not affect the ability of Src to cause lethality in *S.pombe*. We have shown previously (Superti-Furga *et al.*, 1993) that the expression of Csk, under the control of the constitutive *adh1* promoter of *S.pombe* (Russell and Hall, 1983; McLeod *et al.*, 1987), counteracted the phenotype caused by wild-type (wt) Src expression (Figure 3). We therefore tested the SH3 mutants in this assay to assess their ability to be regulated by C-terminal phosphorylation. We have observed that the growth curve measurements are accurate enough to detect subtle changes in the activity of Src. Each mutant was therefore scored as +++ (wt rescue, Figure 3A), ++ (incomplete rescue, Figure 3B), + (weak rescue, Figure 3C) or − (no rescue, Figure 3D). None of the six proteins with mutations in the hydrophobic patch were rescued as well as wt Src, as judged by these growth curves (Table I). Four mutants (Y92A, W118A, P133L and Y136A) showed no rescue when co-expressed with Csk, whereas two mutants showed a weak (Y90A) or incomplete (Y131A) rescue. Immunoblotting experiments showed that equivalent levels of mutant c-Src proteins and Csk were expressed (data not shown).

We have shown previously that the activity of Src in yeast results in the phosphorylation of many endogenous proteins (Superti-Furga *et al.*, 1993; Figure 4A) and that this correlated with the growth arrest of the cells. This phosphorylation was suppressed if Csk was co-expressed

Table I. Properties of mutant SH3 domains

Mutation	Kills *S.pombe*?[a]	Intramolecular interactions[b]	Intermolecular interactions[c]	
		Regulation by Csk	Binding to Sam68	Binding to p85
c-Src	yes	+++	+++	+++
Y90A	yes	+	−	−
Y92A	yes	−	−	−
W118A	yes	−	−	−
Y131A	yes	++	+	+
P133L	yes	−	−	−
Y136A	yes	−	−	−
S94P/R95W[d]	yes	+	+++	++++
R95W[d]	yes	+++	+++	++++
D99K	yes	−	+	−
K104E	yes	+++	+++	+++
E115K	yes	+++	+++	+
nSrc SH3	yes	+	−	+
Src(SpecSH3)	yes	−		
Src(LckSH3)	yes	−		
Src(FynSH3)	yes	+++		

[a]The indicated Src alleles were expressed under inducing conditions (− thiamine) in *S.pombe*. This resulted in a lethal phenotype of the cells, indicating that the mutations did not affect kinase activity of Src.
[b]Csk and mutant Src alleles were co-expressed in *S.pombe* to assess their ability to be regulated by C-terminal phosphorylation. Growth curve measurements as in Figure 3 were used to detect subtle changes in the activity of Src. Each mutant was therefore scored as +++ (wt rescue), ++ (incomplete rescue), + (weak rescue) or − (no rescue).
[c]Intermolecular interactions between the mutant GST−SH3 fusions and Sam68 or p85 were tested as described in the legend to Figure 7. Binding was scored as ++++ (better than wt binding), +++ (wt), ++ (intermediate binding), + (weak binding) or − (no binding).
[d]Results of expression of these mutants in *S.pombe* have been published previously (Superti-Furga *et al.*, 1993).

(Figure 4). To test whether this correlation held true with the mutants, extracts were made of *S.pombe* cells expressing the mutants in the presence and absence of Csk. The proteins were resolved by SDS−PAGE and immunoblotted with antibodies specific for phosphotyrosine. As shown in Figure 4, there was a good correlation between the ability of the SH3 mutants to cause growth arrest in *S.pombe* in the presence of Csk and their ability to phosphorylate host proteins. For example, mutant SrcY131A, which was the only one rescued to near wild-type levels, showed a significant reduction in phosphorylation of host proteins in the presence of Csk, whereas phosphorylation in cells expressing the mutations SrcY92A, SrcW118A, SrcP133L or SrcY136A was not reduced. Altogether, these data indicate that the residues forming the hydrophobic patch are important for the ability of Src to be regulated by Csk.

SH3 domain residues outside of the conserved region are important for Src regulation

To test whether the conserved residues of the hydrophobic region were sufficient for the regulation of Src, we replaced the Src SH3 domain with that of the unrelated cytoskeletal protein α-spectrin. Comparing the spectrin and Src SH3 domains, five of the six residues that comprise the hydrophobic patch are identical, and Y131 of c-Src is conservatively substituted with the aromatic residue phenylalanine (Figure 2). Overall, the SH3 domain of spectrin has 35% identity to c-Src. The chimeric c-Src molecule, Src(SpecSH3), was introduced into *S.pombe* under the control of the *nmt1* promoter. The Src(SpecSH3) protein was expressed to a similar degree as c-Src (data not shown) and induced a lethal phenotype in a manner indistinguishable from c-Src (Figure 5A and Table I). However, no rescue was observed by co-expression of Csk, indicating that the spectrin SH3 domain could not substitute for the Src SH3 domain in the regulation of Src activity. This was also confirmed by the phosphotyrosine analysis of *S.pombe* proteins in the presence or absence of Csk (Figure 6). Therefore, the hydrophobic residues, though necessary, were not sufficient to endow the spectrin SH3 domain with the required specificity.

The regulation of the protein tyrosine kinases Lck and Fyn, which are members of the Src family, is very similar to Src. Both are phosphorylated on a tyrosine residue in the tail by Csk, resulting in down-regulation of kinase activity. The SH3 domains of Lck and Fyn have 54 and 79% identity, respectively, to c-Src (Figure 2). Src(LckSH3) and Src(FynSH3) were expressed in fission yeast as above. Both induced a lethal phenotype in a manner indistinguishable from c-Src (Figure 5B and C, and Table I). Whereas a Src molecule containing the SH3 domain of Fyn was rescued as well as wt Src when co-expressed with Csk, the Src chimera with the Lck SH3 domain continued to show a lethal phenotype in the presence of Csk (Figure 5 and Table I). A comparison of the phosphotyrosine profiles from cells expressing these chimeras confirmed that only the SH3 domain of Fyn allowed a repression of kinase activity in the presence of Csk (Figure 6). Despite the facts that both Fyn and Lck are members of the Src family of protein tyrosine kinases, and both are regulated by Csk, their SH3 domains differed in their ability to substitute for the Src SH3 domain in intramolecular regulation of Src.

The RT loop and the n-Src loop influence the intramolecular interaction of the Src SH3 domain

To test if SH3 domain residues other than the conserved residues of the hydrophobic patch also influenced the regulation of Src by Csk, we mutated charged amino acids in the loops bordering the hydrophobic patch (D99 and E115; Figure 1). We also mutated a charged residue (K104; Figure 1), that lies opposite the proposed ligand binding surface, to test whether other surface regions of the SH3 domain were involved in the regulation of Src. We had shown previously that a double mutation in the RT loop (S94P/R95W) affected the ability of Src to adopt the inactive conformation after phosphorylation of Y527 by Csk (Superti-Furga *et al.*, 1993). The semi-conserved D99 and the non-conserved E115 were changed to lysines, and K104 was replaced with glutamic acid. Additionally, we tested the ability of Csk to regulate the neuronal form of Src (n-Src), which is a splicing variant characterized by a six amino acid insert in the n-Src loop of the SH3 domain (Levy *et al.*, 1987). None of the mutations affected the ability of c-Src to cause growth arrest when expressed in *S.pombe* (Figure 4B and Table I). The SrcD99K was not rescued by co-expression with Csk, as judged by growth curves and phosphotyrosine immunoblotting (Table I and Figure 4B), indicating the importance of the RT loop for the intramolecular interaction of the SH3 domain.

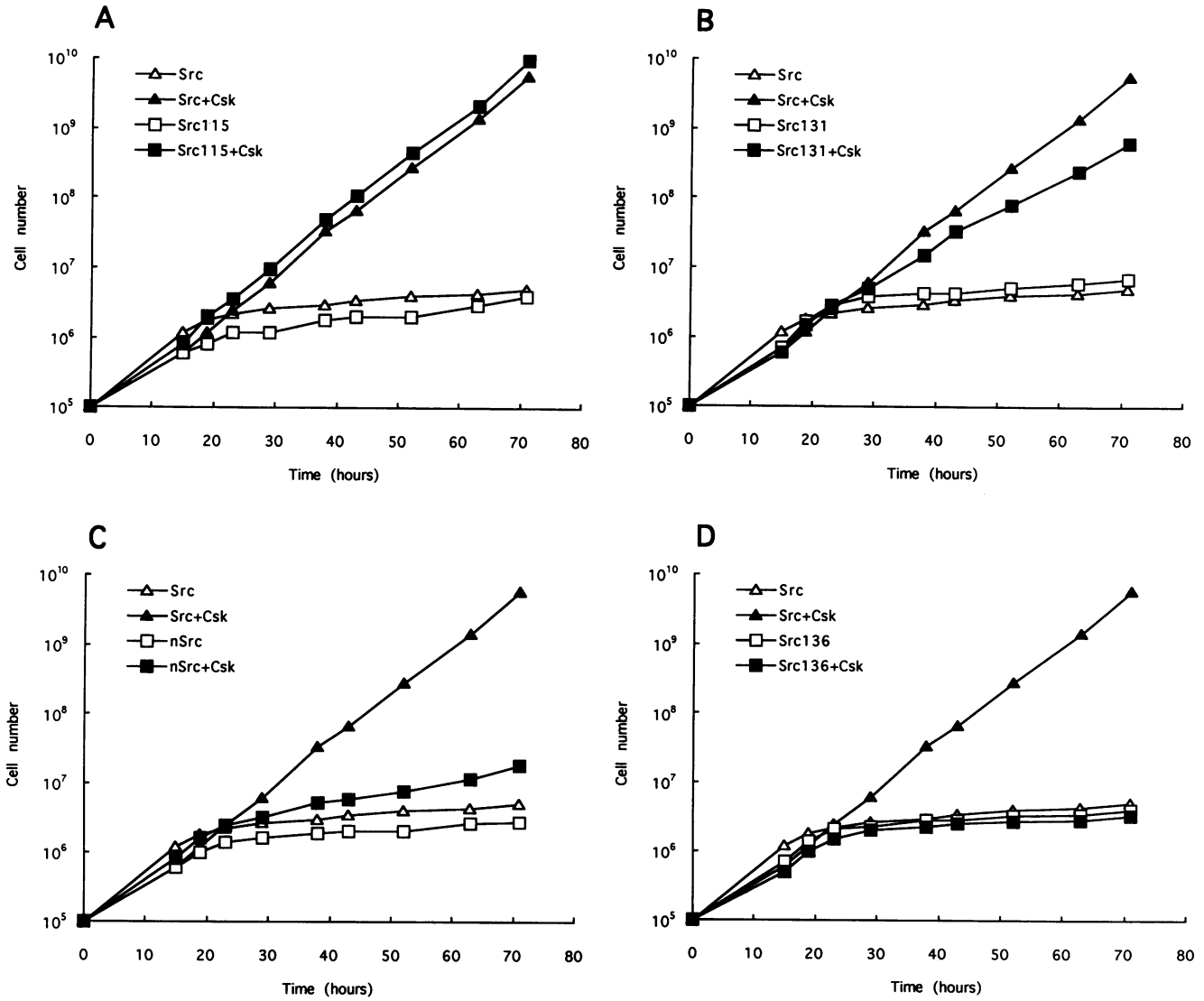

Fig. 3. Growth curves of *S.pombe* cells expressing various Src alleles in the presence or absence of Csk. SP200 cells were transformed with either pAU (control) or pAU-Csk and various Src alleles. Src expression was induced by thiamine deprivation. At different time points, samples were taken and the cell number determined. The strain indicated by the open triangle was transformed by pRSPc-Src/pAU; the strain indicated by the filled triangle was transformed by pRSPc-Src/pAU-Csk. (**A**) Strain indicated by the open squares was transformed with pRSPc-SrcE115K/pAU; filled squares, pRSPc-SrcE115K/pAU-Csk. (**B**) Strain indicated by the open squares was transformed with pRSPc-SrcY131A/pAU; filled squares, pRSPc-SrcY131A/pAU-Csk. (**C**) Strain indicated by the open squares was transformed with pRSPn-Src/pAU; filled squares, pRSPn-Src/pAU-Csk. (**D**) Strain indicated by the open squares was transformed with pRSPc-SrcY136A/pAU; filled squares, pRSPc-SrcY136A/pAU-Csk.

In contrast, co-expression of SrcE115K with Csk resulted in a wt phenotype of the *S.pombe* cells (Table I and Figure 4). A major alteration of the n-Src loop, however, in the form of the six amino acid insertion of neuronal Src was only weakly rescued by Csk, as shown in the growth curve in Figure 3C and the immunoblots in Figure 4B. These results show that both loops flanking the hydrophobic patch could affect intramolecular SH3 domain function. The SrcK104E was fully rescued by C-terminal phosphorylation, indicating that this part of the SH3 domain does not participate in intramolecular contacts that regulate Src activity.

Intra- and intermolecular associations of the Src SH3 domain use the same binding surface

We next wanted to test whether the c-Src SH3 domain used the same ligand binding surface in both inter- and intramolecular interactions. To do this we expressed all mutated SH3 domains in *Escherichia coli* as GST fusion proteins (using the pGEX-2T expression vector), and used these fusion proteins to test if the mutants could bind to known c-Src SH3 binding proteins *in vitro*. The first protein we tested was Sam68 (Courtneidge and Fumagalli, 1994), a mitotic substrate for c-Src which we and others had shown previously to be associated with the SH3 domain of Src (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994). Equivalent amounts of individual mutant SH3–GST fusion proteins were bound to glutathione agarose and incubated with mitotic lysates of NIH-3T3 cells that were transformed by an activated (Y527F) allele of Src. After several washes, bound proteins were eluted and separated by SDS–PAGE and immunoblotted with a monoclonal antibody against phosphotyrosine (Figure 7A). Only one band with an apparent molecular

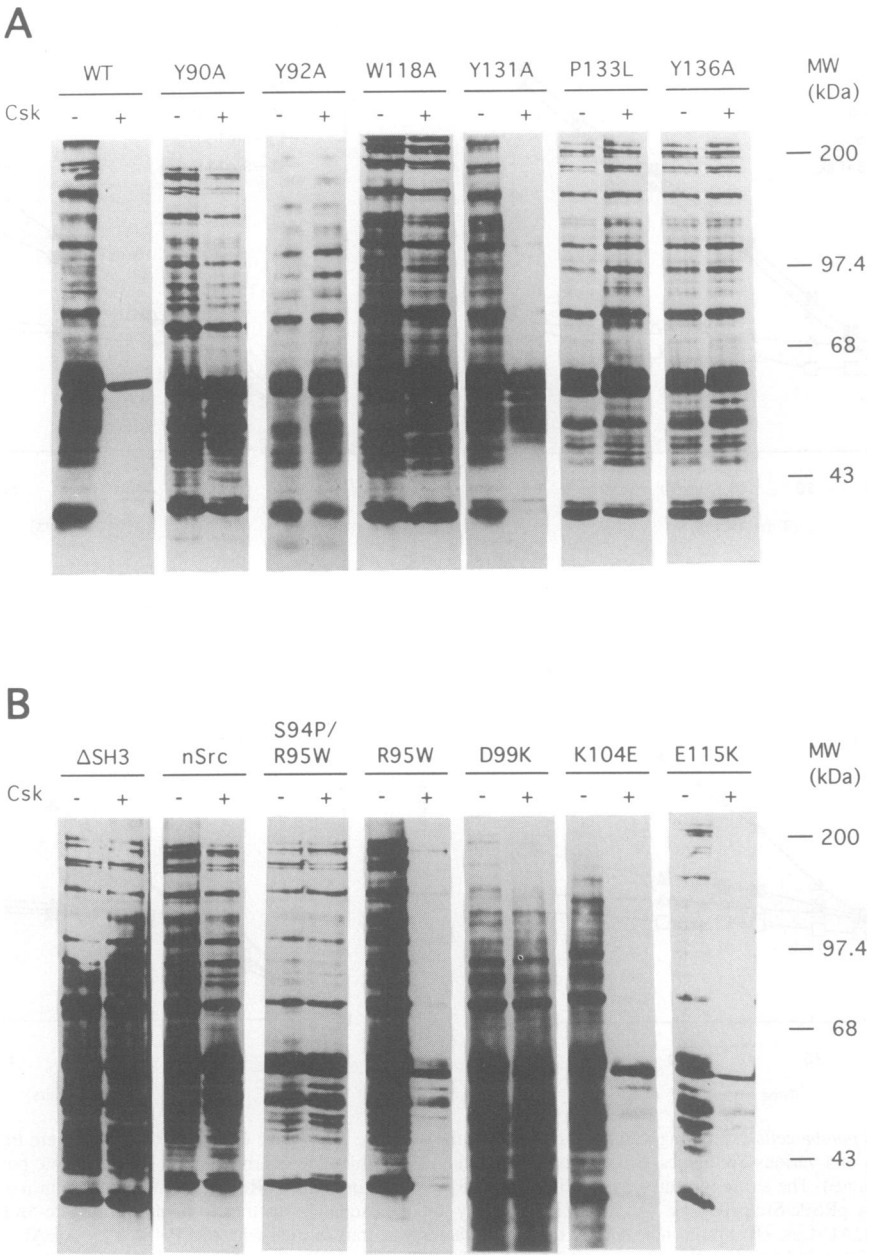

Fig. 4. Phosphotyrosine-containing proteins induced by expression of the various Src SH3 mutants in the absence or presence of Csk. Extracts of SP200 cells transformed with either pAU (control) or pAU-Csk and the various Src mutants were analysed for *in vivo* kinase activity by immunoblotting with anti-phosphotyrosine antibody. Cells were harvested between 17 and 25 h of induction. However, for any given mutant expressed with and without Csk, the lysates were made from cells taken at the same time after promoter induction. (**A**) Mutations of the conserved hydrophobic residues. (**B**) Mutations in the accompanying loops. The results for the Src mutants S94P/R95W and R95W have been shown previously and have been added for comparison.

weight of 68 kDa was detected using wt Src SH3–GST fusion protein. The identity of the band as Sam68 was confirmed with specific antibodies (data not shown). Using the mutant SH3 domains, we found a good correlation between the ability to participate in the regulation of Src and to interact with Sam68. The five mutations/insertions (Y92A, W118A, P133L, Y136A and n-Src) that were only weakly rescued or not rescued at all did not associate detectably with Sam68. The three mutations (R95W, K104E and E115K) that did not affect Src regulation displayed Sam68 binding that was indistinguishable from wt Src. Interestingly, two of the mutants (D99K and Y131A) that displayed a phenotype of incomplete rescue also bound weakly to Sam68, emphasizing the good correlation between these two different types of interaction. However, there were some minor deviations from this correlation. For example, the Y90A mutation, which showed a weak rescue by Csk in co-expression experiments in *S.pombe*, showed no binding to Sam68. The most interesting exception, however, was the SrcS94P/R95W double mutant that was able to bind Sam68 well. This is in contrast to the finding that this allele was only weakly rescued by Csk co-expression in yeast, as reported previously (Superti-Furga *et al.*, 1993).

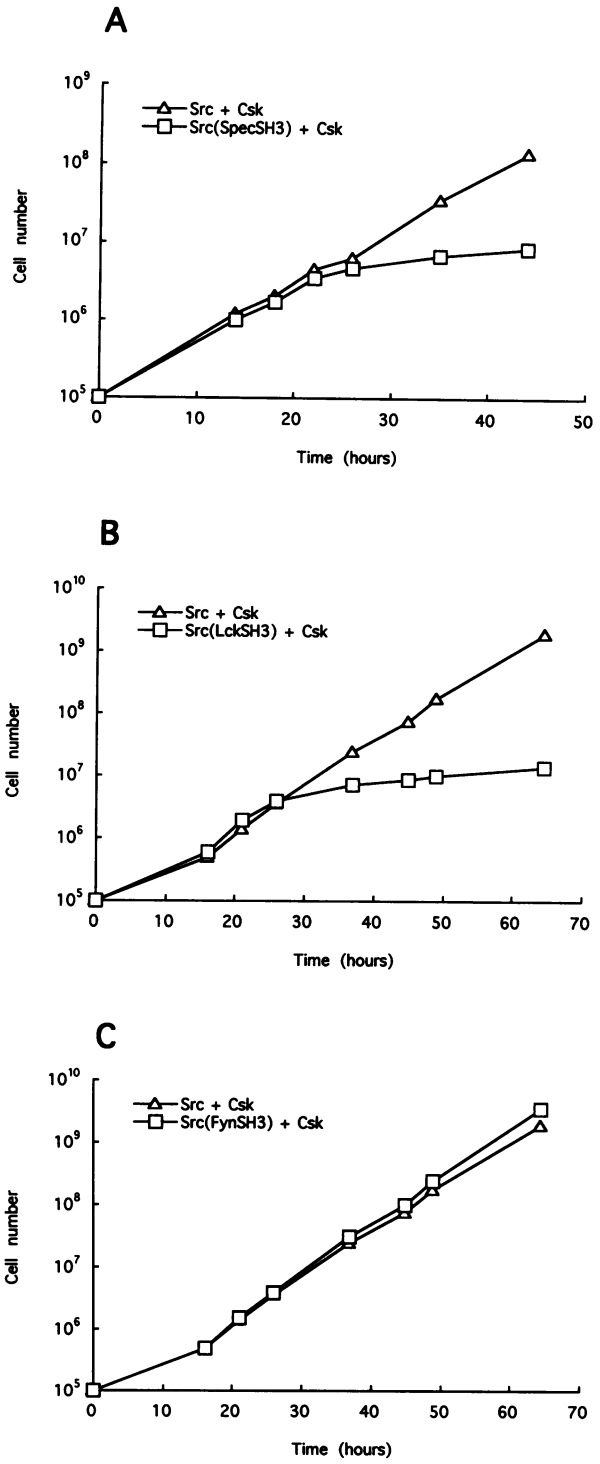

Fig. 5. Growth curves of *S.pombe* cells expressing chimeric Src molecules in the presence of Csk. SP200 cells were transformed with pAU-Csk and the various chimeric Src mutants were grown under induced (− thiamine) conditions. At different time points, samples were taken and the cell number determined. The strain indicated by the open triangles was transformed by pRSPc-Src/pAU-Csk. (**A**) The strain indicated by the open squares was transformed with pRSPc-Src(SpecSH3)/pAU-Csk. (**B**) The strain indicated by the open squares was transformed with pRSPc-Src(LckSH3)/pAU-Csk. (**C**) The strain indicated by the open squares was transformed with pRSPc-Src(FynSH3)/pAU-Csk.

The SH3 mutants were also tested against another Src SH3 binding protein, p85α, the regulatory subunit of PI 3-K. A similar binding pattern (Figure 7B) to that obtained

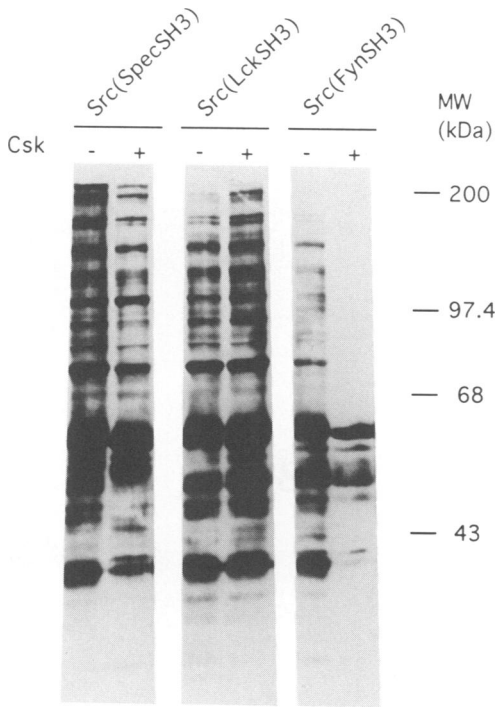

Fig. 6. Phosphotyrosine-containing proteins in *S.pombe* cells expressing chimeric Src mutants in the presence or absence of Csk. SP200 cells were transformed with either pAU (control) or pAU-Csk and the indicated chimeric Src mutants. At 20 h after induction, extracts were prepared and 20 μg of lysates were resolved by SDS−PAGE and probed with antibodies specific for phosphotyrosine.

with Sam68 (Figure 7A) was observed, supporting the idea that different Src SH3 binding proteins interact with the ligand binding surface in a similar manner. However, it is worth noting that some mutants that showed binding to Sam68 equal to wt SH3 (S94P/R95W and R95W) showed an increased ability to bind to p85α. This suggests that these residues affect the specificity of the Src SH3 interactions. To look at this in more detail, we performed a binding assay using [^{35}S]methionine/cysteine-labelled SrcY527F-transformed NIH-3T3 lysates. The overall pattern of ^{35}S-labelled proteins binding to the individual mutants resembled the results obtained from the Sam68 and p85 binding experiments (Figure 7). However, some minor bands in the ^{35}S-labelled extracts showed some variations (e.g. a band of ~220 kDa was associated with S94P/R95W and R95W SH3 domains, but not with wt). This suggests that some of the introduced mutations may have specifically changed the affinity of the Src SH3 domain for particular proteins.

The SH3 domain is necessary for the transformation of NIH-3T3 cells

Some point mutations of the Src SH3 domain abolished the ability of the SH3 domain to bind to any protein *in vitro* or to regulate Src activity in *S.pombe*. We wanted to know if such an allele (SrcP133L) would have elevated kinase activity when expressed in NIH-3T3 cells and whether the loss of binding capacity of the Src SH3 domain affects the transforming ability of a deregulated Src kinase. For this purpose we cloned wt c-Src, SrcY527F, SrcΔSH3 and SrcP133L into the mammalian expression vector pSGT, a derivative of pSG5 where expression is

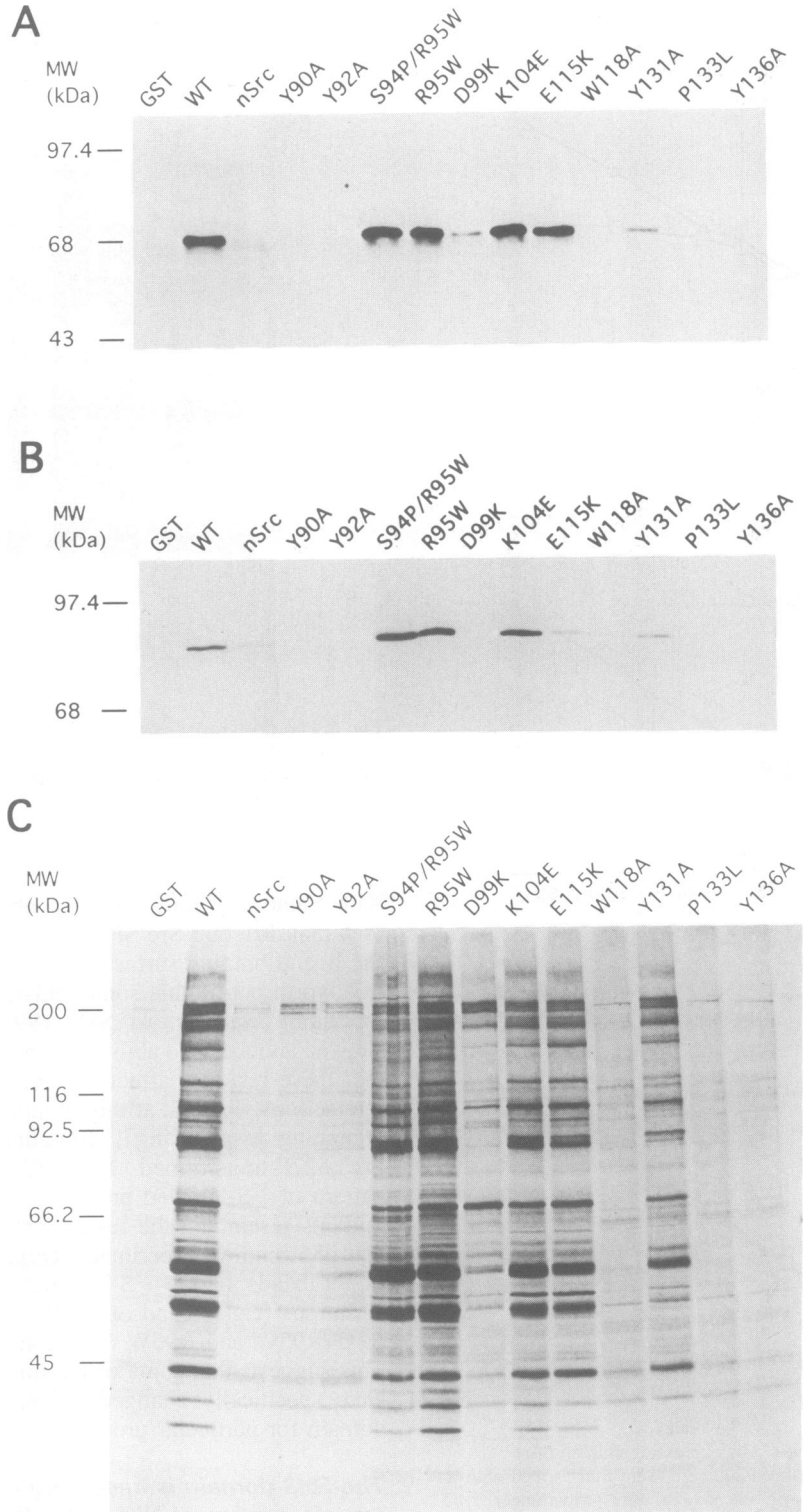

Fig. 7. Intermolecular interactions. GST fusions of various Src SH3 mutants were bound to glutathione beads and the indicated lysates added for 1 h. After several washes in RIPA or LB, bound proteins were eluted with gel loading buffer. (**A**) 50 μg of mitotic lysates from SrcY527F-transformed NIH-3T3 cells were used for each GST fusion construct. Bound protein was detected after blotting with a monoclonal antibody against phosphotyrosine. (**B**) 200 ng of purified p85α from a baculovirus p85α-overexpressing system were used for each GST fusion construct. Bound protein was detected after blotting with a polyclonal antibody against p85α. (**C**) 100 μg of ^{35}S-labelled lysates from SrcY527F-transformed NIH-3T3 cells were used for each GST fusion construct. Bound proteins were detected by autoradiography.

under the control of the SV40 promoter. NIH-3T3 cells were transfected and individual clones isolated. Of all constructs tested, only SrcY527F-transfected cells showed a transformed phenotype as described previously (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987).

Table II. A comparison of phenotypes in *S.pombe* and NIH-3T3 cells

Mutation	Is regulated by Csk in *S.pombe*?[a]	Overexpressed in NIH-3T3 cells[b]		
		Transforming ability?	Specific kinase activity	Phosphotyrosine levels
Src	yes	no	1	normal
Src Y527F[c]	no	yes	6×	high
Src ΔSH3[c]	no	no	10×	high
Src P133L	no	no	8×	high

[a]Csk and mutant Src alleles were co-expressed in *S.pombe* to assess their ability to be regulated by C-terminal phosphorylation.
[b]Cell lines were derived from NIH-3T3 cells transfected with the listed Src constructs.
[c]Results of expression of these mutants in *S.pombe* have been published previously (Superti-Furga *et al.*, 1993).

Neither wt c-Src, SrcΔSH3 nor SrcP133L were able to morphologically transform these cells (Table II; data not shown) at expression levels equal to or higher than SrcY527F (data not shown). To measure the specific kinase activity of these mutant Src molecules, we immunoprecipitated the expressed Src proteins with an antibody specific for chicken Src (EC10) and performed *in vitro* immune complex kinase assays using enolase as exogenous substrate. The Src molecules with deleted or point-mutated SH3 domains had a high intrinsic kinase activity (comparable with SrcY527F), indicating that, as in the yeast cells, these mutants were not regulated. Total lysates of these cells were separated by SDS−PAGE and transferred to nitrocellulose. Probing with a monoclonal antibody against phosphotyrosine showed that cell lines that expressed SrcY527F, SrcΔSH3 and SrcP133L had high levels and similar patterns of tyrosine-phosphorylated proteins, compared with wt c-Src-transfected cells which had only a few phosphorylated proteins (Figure 8A) at similar expression levels to these Src alleles (Figure 8B). These data confirm that the kinase activities of Src molecules with altered SH3 domains were deregulated in NIH-3T3 cells as well as in *S.pombe*. Since these cells were morphologically normal, these results suggest that the SH3 domain of Src was required for the manifestation of the transformed phenotype in NIH-3T3 cells.

Discussion

We have analysed the requirement of individual residues of the Src SH3 ligand binding surface in its participation in intramolecular regulation of Src *in vivo* as well as in intermolecular interactions with known proteins *in vitro*. We and others have shown previously that the SH3 domain contributes to the stability of the repressed conformation of the Src tyrosine kinase (Murphy *et al.*, 1993; Okada *et al.*, 1993; Superti-Furga *et al.*, 1993). Here we have shown that mutations in the core of the ligand binding surface also resulted in a constitutively active form of the enzyme that was not able to achieve the repressed conformation by phosphorylation of the C-terminal tyrosine. We and others (Okada *et al.*, 1993; Superti-Furga *et al.*, 1993) have shown previously that Src molecules lacking the SH3 domain are phosphorylated by Csk as

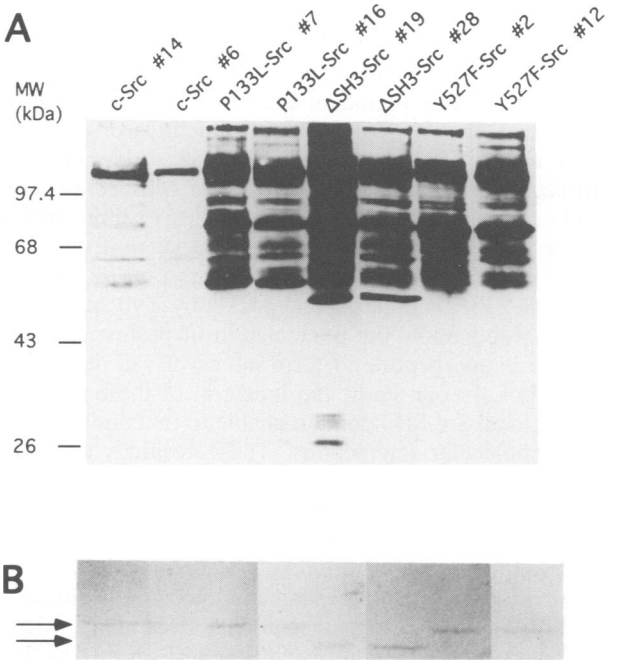

Fig. 8. Phosphotyrosine-containing proteins in extracts of NIH-3T3 cells expressing Src mutants. Cell lines (indicated by #) were derived from NIH-3T3 cells transfected with the indicated Src constructs and total lysates prepared. 20 μg of lysate were loaded in each lane, separated by SDS−PAGE and transferred to nitrocellulose. All lanes were blotted at the same time and have identical exposure times after probing with the antibodies indicated below. (**A**) The blot was probed with an antibody specific for phosphotyrosine. (**B**) The stripped blot was reprobed with an antibody specific for Src. The upper arrow indicates wt Src or point mutants, whereas the lower arrow shows the SrcΔSH3.

efficiently as wt Src. Therefore we do not believe that our current results can be explained by poor substrate recognition. Rather, our data determine the amino acids that contact another region of Src. We are currently trying to identify this contact area in Src. Recently Eck *et al.* (1994) reported the crystal structure of the SH2/SH3 domains of Lck. Their data suggest a possible interaction between the SH2 and SH3 domains *in trans*, although direct evidence for dimer formation of Src family tyrosine kinases has not been reported. We show here that the inter- and intramolecular interactions are affected by the same mutations, suggesting similarity in their binding requirements. SH3 domains are known to bind to sequences rich in prolines (Egan *et al.*, 1993; Li *et al.*, 1993; Ren *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Rotin *et al.*, 1994). Two regions in c-Src contain several proline residues: the unique domain and the end of the catalytic domain. c-Src with a deleted unique domain was still regulated by Csk in *S.pombe* (M.Koegl, S.A.Courtneidge and G.Superti-Furga, manuscript in preparation), and mutations of proline residues in the catalytic domain resulted in proteins that were either unstable or fully regulated by C-terminal phosphorylation (G.Superti-Furga, unpublished results). The chimeric protein Src(LckSH3) was not regulated by Csk, indicating that the appropriate binding region for the Lck SH3 domain may be missing. By adding back other domains of Lck into Src(LckSH3) and co-expressing these with Csk in yeast, we are trying to identify the interaction surface of the Lck SH3 domain.

All mutations of residues building the central hydrophobic patch of the ligand binding area had an effect on inter- and intramolecular binding. This reinforces the importance of this region in the binding to proline-rich peptides, as shown by two recent structures of peptides bound to the SH3 domains of PI 3-K, Abl and Fyn (Musacchio *et al.*, 1994; Yu *et al.*, 1994). Musacchio *et al.* (1994) observed the binding of peptides to three major sites on the SH3 domain. The first pocket is formed in the Src SH3 domain by the side chains of Y92, W118, P133 and Y136, and the second pocket by Y90 and Y136. These pockets allow the intercalation of proline residues from the bound peptide to form intensive van der Waals interactions. In our study, the mutation of these residues resulted in an Src SH3 domain unable to function in inter- or intramolecular interactions. These residues are very well conserved among SH3 domains and are likely to be the key residues in SH3 domains that establish binding to proline-rich peptides. However, due to their relatively conserved nature they are unlikely to be major determinants of selectivity in SH3 domain interactions. Interestingly, the Lck SH3 domain contains a histidine residue at the equivalent position of Y90 in Src, suggesting that changes in binding affinities may also reside in this core binding area. The third pocket is formed by residues W118 and Y131 in Src. We found that mutation of Y131 did not affect inter- and intramolecular interactions to the same degree as the other mutations of these hydrophobic residues. This residue is also not totally conserved, suggesting that it has less of a role in general SH3 domain binding, but rather contributes to the specificity of interaction.

We clearly showed that mutations in the RT and n-Src loops which are adjacent to the hydrophobic surface could influence the ability of the Src SH3 domain to participate in inter- and intramolecular associations. Changing D99 in the RT loop to lysine resulted in an almost complete loss of binding of the SH3 domain to Sam68 or p85, and did not allow the inactive conformation of Src to occur. These results are consistent with the finding of Yu *et al.* (1994), who mutated the equivalent position in the SH3 domain of PI 3-K to asparagine and found a 40-fold reduction in the affinity of the SH3 domain for a nine amino acid peptide. The negative charge at this position is a general feature of SH3 domains, and this residue is probably involved in binding to positively charged residues in SH3 domain binding proteins.

Other mutations of non-conserved residues (R95W and S94P/R95W) in the RT loop did not abolish intermolecular binding. On the contrary, in the case of binding to p85 we observed a small but consistent increase in affinity of the SH3 domains containing an R95W mutation. The paradox is best illustrated by the double mutant S94P/R95W, which was unable to form a strong intramolecular interaction but bound with increased efficiency to p85. This might indicate that (i) the intramolecular interaction surface is more extensive than that involved in intermolecular interactions, or (ii) the wt Src SH3 domain is not an efficient binder of p85. The SH3 domain of n-Src is identical to that of c-Src, with the exception of a six amino acid insert in the n-Src loop. This insertion resulted in an SH3 domain that could participate only poorly in the intramolecular association regulating Src kinase activity, was unable to bind to Sam68 and only had a very weak affinity for p85. Indeed, n-Src expressed in chicken embryo fibroblasts showed increased specific kinase activity when compared with the endogenous c-Src (Levy *et al.*, 1987). The results from mutations in the RT and n-Src loops showed that these loops have an important role in determining the affinity of interaction between SH3 domains and interacting proteins, and are likely to play an important role in determining the specificity of interacting proteins for a given SH3 domain. For the intermolecular interaction studies reported here, full-length proteins that have been shown to associate with the Src SH3 *in vitro* and *in vivo* were used. Many SH3 binding proteins contain several sequence motifs that could bind SH3 domains. Binding to SH3 domains may also involve more than one contiguous motif in Sam68 and p85 (Kapeller *et al.*, 1994). If a more complex mode of binding exists, the use of full-length proteins might be a prerequisite to detecting specificity.

The fact that the same residues that were involved in regulating Src activity were also required for the SH3 domain of Src to make intermolecular contacts with other proteins suggests that the intra- and intermolecular interactions are mutually exclusive. The SH3 domain of an Src molecule that has adopted the repressed conformation would not be able to associate with other proteins. Furthermore, the facts that both the SH2 and SH3 domains of Src are required for the repressed state of Src, and that both domains utilize the same ligand binding surface in inter- and intramolecular interactions, suggest an alternative mode of activation of the Src tyrosine kinase independent of phosphatases. Proteins that bind to the SH2 and/or SH3 domains of Src with a higher effective affinity than the intramolecular interactions might be able to activate Src. Indeed, it has been shown that phosphorylated peptides can activate repressed Src in competition experiments (Liu *et al.*, 1993a; G.Alonso, M.Koegl, N.Mazurenko and S.A.Courtneidge, manuscript submitted), and such activation may occur when Src associates with the activated platelet-derived growth factor receptor (Kypta *et al.*, 1990; Twamley *et al.*, 1992; Mori *et al.*, 1993; Twamley-Stein *et al.*, 1993). Activated forms of Src lacking the SH2 or SH3 domain are hypophosphorylated at Y527 *in vivo* (Seidel-Dugan *et al.*, 1992), suggesting that a phosphatase can act on this site once Src is in the active conformation.

NIH-3T3 cells expressing c-Src mutants with a deleted SH3 domain or a P133L mutation showed no morphological differences when compared with cells expressing c-Src. In contrast, NIH-3T3 cells expressing the Y527F allele of c-Src were morphologically transformed as described previously (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Reynolds *et al.*, 1987). Comparison of phosphotyrosine levels in cell lysates, as well as measuring *in vitro* kinase activity, showed that the SH3 domain mutants were as active as the transforming Src. These results seem to be in discrepancy with those obtained by Seidel-Dugan *et al.* (1992) who showed that chicken embryo fibroblasts expressing a Src construct lacking an SH3 domain were morphologically transformed. Two possible explanations could account for this finding. It has been shown previously that Src proteins carrying mutations in the SH2 or SH3

domain can exhibit a host range phenotype upon expression in chicken embryo fibroblasts or NIH-3T3 cells (Verderame *et al.*, 1989; Hirai and Varmus, 1990). Secondly, expression in chicken embryo fibroblasts was driven from a retroviral-based expression vector which probably results in much higher expression levels than the expression we achieved with the SV40 promoter-based expression vector used in this study (Johnson *et al.*, 1985). In our study, at an expression level sufficient for SrcY527F to induce a transformed phenotype in NIH-3T3 cells, a Src mutant with a deleted SH3 domain was non-transforming. The SH3 domain of Src might be required for the correct localization of Src in these cells, or for the selection of substrates whose tyrosine phosphorylation is necessary for transformation. We could not detect differences in the phosphorylation status of the major tyrosine-phosphorylated proteins in cells transfected by SrcY527F or SrcΔSH3 in anti-phosphotyrosine immunoblots of 1-D SDS−PAGE gels. Nevertheless, minor substrates important for transformation may have passed undetected.

Materials and methods

DNA constructs

Chicken c-Src cDNA was expressed under the control of the thiamine-repressible promoter of the *nmt1* gene in the pRSP vector as described previously (Superti-Furga *et al.*, 1993). To facilitate easy cloning of the point mutations, new silent restriction sites upstream (*Nhe*I) and downstream (*Dra*III) of the coding region for the SH3 domain were introduced using the following oligos: *Nhe*I, 5′-CGGGGCGCTAGC-TGGCGGCGTCA-3′ (coding strand), 5′-CGCCAGCTAGCGCCC-CGGCACGCT-3′ (non-coding); and *Dra*III, 5′-GAAGATCACACGTC-GTGAGTCCGAG-3′ (coding), 5′-ACTCACGACGTGTGATCTTCCC-AAAG-3′ (non-coding). The resulting construct was called pRSPsrcND. Human Csk cDNA was expressed under the control of the constitutive *adh1* promoter in the pAU vector, a derivative of pART1 (McLeod *et al.*, 1987; Superti-Furga *et al.*, 1993).

Point mutations were generated in a two-step PCR procedure using two internal oligos encompassing the point mutation (see below for the oligos) and two oligos upstream and downstream of the Src SH3 domain. In the first set of PCRs the non-coding internal oligo and an external oligo upstream of the SH3 domain were used in one reaction; the coding internal oligo was used together with an oligo downstream of the SH3 domain in the second reaction. The resulting two fragments were mixed in the second step of the PCR mutagenesis together with oligos upstream and downstream of the SH3 domain. The resulting PCR fragment was digested with *Nhe*I and *Dra*III, gel-purified and ligated into pRSPsrcND. The following oligos were used for the point mutations: Y90A, 5′-GTGGCTCTCGCTGACTACGA-3′ (coding), 5′-TCGTAGTCAGCG-AGAGCCAC-3′ (non-coding); Y92A, 5′-TCTATGACGCCGAGTCC-3′ (coding), 5′-GGACTCGGCGTCATAGA-3′ (non-coding); D99K, 5′-CTGAAACGAAGTTGTCCTT-3′ (coding), 5′-AAGGACAACTTCGT-TTCAGC-3′ (non-coding); K104E, 5′-GTCCTTCAAGGAAGGAG-AAC-3′ (coding), 5′-GTTCTCCTTCCTTGAAGGAC-3′ (non-coding); E115K, 5′-ACAACACGAAAGGTGACTGG-3′ (coding), 5′-AGTCAC-CTTTCGTGTTGTTGA-3′ (non-coding); W118A, 5′-GAAGGTGACG-CGTGGCTGGCTCA-3′ (coding), 5′-TGAGCCAGCCACGCGTCAC-CTTC-3′, (non-coding); Y131A, 5′-ACGGGCGCCATCCCCAGTAAC-TATGT-3′ (coding), 5′-ACATAGTTACTGGGGATGGCGCCCGT-3′ (non-coding); P133L, 5′-GCTACATCCTCAGTAACTATGTCGCG-3′ (coding), 5′-CGCGACATAGTTACTGAGGATGTAGC-3′ (non-coding); and Y136A, 5′-CAGTAACGCTGTCGCGCCCTCAGA-3′ (coding), 5′-TCTGAGGGCGCGACAGCGTTACTG-3′ (non-coding).

The SH3 domains of α-spectrin, Lck, human Fyn and n-Src were swapped in a PCR-based approach (details upon request). Oligos were designed (see below) that consisted of half of the desired new SH3 domain and half of neighbouring sequences in Src. The beginning and end of the swaps were carefully selected, taking into account the known X-ray solution structures of these molecules to minimize folding problems. In the case of n-Src, oligos were designed that inserted 18 bp into chicken Src to create the 'neuronal' form of chicken Src. The following oligos were used for the swaps or n-Src: n-Src, 5′-CGAAA-GGTAGATGTTCGTGAAGGTGACTGGTGGCTG-3′ (coding), 5′-ACGAACATCTACCTTTCGCGTGTTGTTGACAATCTGC-3′ (non-coding); Lck, 5′-CGGGGCGCTAGCTGGCGGAGTCACCCTGGTT-ATCGCCCT-3′ (coding, N-terminus), 5′-ATTCCCTTCAACTTCGT-GGCGCCCTCAGACTCCATC-3′ (coding, C-terminus), 5′-GATGG-AGTCTGAGGGCGCCACGAAGTTGAAGGGAAT-3′ (non-coding, C-terminus); α-spectrin, 5′-CGGGGCGCTAGCTGGCGGCGTCACC-ATTTACGTGGGTAAGTAGACTACGATAGCAG-3′ (coding, N-terminus), 5′-CCGAGCAACTATGTGGCGCCCTCA-3′ (coding, C-terminus), 5′-TGAGGGCGCCACATAGTTGCTCGG-3′ (non-coding, C-terminus); Fyn, 5′-CGGGGCGCTAGCTGGCGGAGTGACACTC-TTTGTG-3′ (coding, N-terminus), 5′-ACAGGTTACATTCCCAGTAA-CTATG-3′ (coding, C-terminus), 5′-CATAGTTACTGGGAATGTA-ACCTGT-3′ (non-coding, C-terminus).

The recombinant pGEX-2T plasmids for expression of the SH3 mutations as GST fusions were generated in the following way. A fragment was amplified by PCR from the pRSPsrcND vector containing the desired mutation using oligos from the N- and C-termini of the SH3 domain with the added restriction sites *Eco*RI (5′-TCTGAATTCATTCA-GCCTGGATGGAGTCTG-3′) and *Bam*HI (5′-TGCGGATCCGGCGT-CACCACTTTCGTG-3′). The resulting PCR fragment was digested with *Eco*RI and *Bam*HI, gel-purified and ligated into pGEX-2T. Some mutants were subcloned from the pRSPsrcND vector into the eukaryotic expression vector pSGT. This vector is a derivative of pSG5 (Stratagene) that contains a different polylinker with the following restriction sites: *Eco*RI, *Spe*I, *Bam*HI, *Eco*RV, *Xho*I and *Bgl*II.

All constructs were verified by sequencing over the entire portion obtained by PCR.

Yeast expression

The yeast strain used in this study was SP200 (*h⁻ˢ leu1-32 ura4 ade2-10*). Growth conditions were as described previously (Beach *et al.*, 1985; 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 put into culture in PMA in either the presence or absence of thiamine. Cells were grown at 30°C in a shaking water bath. Transformation was performed using the lithium acetate method with the modification described previously (Superti-Furga *et al.*, 1993).

Lysates were collected by centrifuging the cells at 1500 *g*, followed by a wash in cold PBS/0.1 mM sodium orthovanadate and subsequent lysis with glass beads and lysis buffer as described previously (Superti-Furga *et al.*, 1993). The lysates were cleared with a 15 min spin at full speed in a microfuge and protein concentration measured (Bradford, 1976).

Preparation of lysates

Mitotic and asynchronous lysates were prepared from Y527F Src-transformed NIH-3T3 cells as described previously. [^{35}S]Methionine/cysteine-labelling, lysis of SrcY527F-transformed NIH-3T3 cells and the baculovirus expression of p85α were performed as described previously (Fumagalli *et al.*, 1994; Koegl *et al.*, 1994).

Kinase assays

Src was immunoprecipitated using EC10 (Parson *et al.*, 1984) from NIH-3T3 cell lysates; the immunoprecipitates were washed three times in RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton, 0.1 mM orthovanadate) and once in TBS (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM orthovanadate). One quarter of the immunoprecipitate was used in a kinase assay performed for 10 min at 30°C in a buffer containing 20 mM HEPES, pH 7.5, 25 μM [γ-^{32}P]ATP, 10 mM $MnCl_2$, 10 mM DTT and acid-denatured enolase as exogenous substrate, as described (Kypta *et al.*, 1990). The samples were resolved by SDS−PAGE, and the amount of radioactivity incorporated determined by PhosphorImager. The remaining immunoprecipitate was used to determine the relative amounts of Src expression in immunoblotting.

Intermolecular binding assay

The expression of GST fusion proteins in *E.coli* was induced with IPTG at an OD_{600} of 0.8. Cells were harvested after 3 h shaking at 37°C, centrifuged and lysed in PBS by sonication. The lysate was clarified after adding Triton X-100 at a final concentration of 1% (v/v) by centrifugation at 12 000 *g*. Equimolar amounts of GST fusions were

bound to a slurry (1:20) of Sepharose 4B/glutathione–Sepharose beads (Pharmacia). This was achieved using a 20-fold excess (~80 μg) of GST fusions over the binding capacity of the glutathione–Sepharose beads. The fusions were incubated for 90 min at 4°C on a rotating wheel and washed three times in PBS/1% Triton X-100 to eliminate non-bound *E.coli* or GST fusion proteins. Lysates containing 50 μg mitotic cells, 100 μg ^{35}S-labelled proteins or 200 ng purified p85α were added to GST fusion proteins coupled to beads, adjusted with either RIPA buffer (in the case of Sam68 and p85α) or LB (in the case of the ^{35}S-labelled proteins) to a volume of 250 μl and incubated at 4°C for 60 min on a rotating wheel. Pellets were washed four times in RIPA or LB buffer, as appropriate, and the binding proteins were eluted with electrophoresis sample buffer. For ^{35}S-labelled binding proteins, the gel was dried and exposed for 1–3 days.

Immunoblots

Samples from intermolecular binding assays or 25 μg of total extracts from *S.pombe* cells were resolved by electrophoresis on 9% polyacrylamide gels and transferred to nitrocellulose membranes using the Milliblot-SDE System (Millipore). The filters were incubated with monoclonal anti-phosphotyrosine antibodies (UBI; 1:1500 dilution) or a polyclonal antibody against p85 (Otsu *et al.*, 1991). Detection was by incubation with protein A coupled to horseradish peroxidase followed by ECL chemoluminescence (Amersham).

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