The Src family of protein tyrosine kinases: regulation and functions

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SUMMARY

Most of the nine members of the Src family of tyrosine kinases are restricted in their expression, often to cells of the haematopoietic lineage, while some, particularly Src, Fyn and Yes, are more ubiquitously expressed. We have been studying the functions of Src, Fyn and Yes in fibroblasts. We have shown that stimulation of quiescent fibroblasts with platelet-derived growth factor (PDGF) causes Src, Fyn and Yes to become activated, and to associate transiently with the PDGF receptor. To address the role of Src, Fyn and Yes in the response to PDGF, we have used a dominant negative approach, in which cells were engineered to express catalytically inactive forms of Src kinases. These cells were unable to enter S phase in response to PDGF, and we therefore conclude that Src family tyrosine kinases are required in order for the PDGF receptor to transmit a mitogenic signal.

It has previously been shown that the kinase activity of Src is negatively regulated by phosphorylation of tyr 527 in its carboxy-terminal tail. A kinase, Csk, that phosphorylates tyr 527 has recently been identified. We expressed Src in yeast to test the model that phosphorylation of tyr 527 represses activity by promoting intramolecular association between the tail and the SH2 domain. Inducible expression of Src in S. pombe caused cell death. Co-expression of Csk counteracted this effect. Src proteins mutated in the SH2 domain were as lethal as wild-type Src, but were insensitive to Csk. We interpret these results in favour of an SH2 domain : phosphorylated tail interaction repressing Src activity. However, we have also found that Src molecules containing mutations in the SH3 domain are not regulated by Csk, suggesting that the SH3 domain also functions in the intramolecular regulation of Src activity.

Key words: protein tyrosine kinase, Src, PDGF receptor, Csk, S. pombe

INTRODUCTION

The Src family of protein tyrosine kinases comprises at least nine members: Src, Yes, Yrk, Fyn, Fgr, Hck, Lyn, Lck and Blk (for review see Cooper, 1990 and Courtneidge, 1993). All share a characteristic topology (see Fig. 4), including a myristylation sequence at the amino terminus required for their association with membranes, Src homology (SH) domains 3 and 2, a catalytic domain, and regulatory carboxy-terminal tail sequences. The only region of great difference is the so-called unique domain, in the first 50-80 amino acids, which as the name implies, is unique to each member of the family. Most of the Src family kinases are relatively restricted in their tissue expression, being found predominantly in haematopoetic cells. However at least three, Src, Fyn and Yes, are more ubiquitously expressed. In particular, these three are expressed in fibroblasts, which is our model system for studying the normal functions of these kinases. Some members of the Src family were first described as the oncogenes of avian and mammalian retroviruses, while others were identified by screening appropriate cDNA libraries. However, even those identified by the latter method can be converted into oncogenes by appropriate mutagenesis. This has led to the generally accepted idea that the Src family kinases function in signal transduction pathways that control cell growth, even though these kinases are not expressed on the cell surface. The identification of factors able to activate Src family kinases has been slow. The most progress has been made in the haematopoietic field, where it is now clear that Src family kinases form complexes with several transmembrane receptors which themselves lack intrinsic tyrosine kinase activity, and are activated by ligand binding to the associated transmembrane protein (reviewed by Bolen, 1991; Bolen et al., 1992). The activation of the Src family kinases in fibroblasts is less well understood, although one growth factor has been reported to stimulate Src, Fyn and Yes activity: this is PDGF (Gould and Hunter, 1988; Kypri et al., 1990; Ralston and Bishop, 1985), whose receptor is itself a protein tyrosine kinase.

The catalytic activity of the Src family tyrosine kinases is tightly regulated in vivo. One of the most important regulatory sites is the carboxy-terminal tail, within which is found a tyrosine residue (in Src tyr 527; Cooper et al., 1986). When phosphorylated at this site, Src is inactive; dephosphorylation results in activation of its intrinsic kinase activity (Courtneidge, 1985). The importance of this regulatory site
is underscored by the observation that replacement of tyr 527 with phenylalanine is sufficient to convert proto-oncogene to oncogene (Cartwright et al., 1987; Knisicik and Shalloway, 1987; Piwnica-Worms et al., 1987; Reynolds et al., 1987). The phosphorylation of tyr 527 is not predominantly an autophosphorylation reaction (Thomas et al., 1991). Several years ago, an enzyme called Csk for e-Src kinase, was shown to phosphorylate Src on tyr 527, and reduce its intrinsic kinase activity in vitro (Okada and Nakagawa, 1989). More recent evidence suggests that Csk may phosphorylate all members of the Src family (Bergman et al., 1992; Okada et al., 1991). Subsequent cloning of the cDNA encoding Csk indicated that it had strong similarities to Src itself, with SH2, SH3 and catalytic domains arranged with similar topography (Nada et al., 1991). However, it lacks myristylation and unique domain sequences. Furthermore, its carboxy-terminal tail contains no tyrosine residues. Whether tyr 527 kinases exist other than Csk is unclear. Moreover, correct regulation of Src requires not just the phosphorylation of tyr 527, but also the integrity of other domains in the molecule, particularly the SH2 and SH3 domains (Hirai and Varmus, 1990a; Hirai and Varmus, 1990b; Kanner et al., 1991; Kato et al., 1986; O'Brien et al., 1990; Potts et al., 1988; Seidel-Dugan et al., 1992).

**SIGNAL TRANSDUCTION INVOLVING SRC FAMILY TYROSINE KINASES**

The cellular response to PDGF is complex (reviewed by Heldin and Westermark, 1990; Williams, 1989). It is initiated when binding of PDGF to its cognate receptor results in receptor dimerization (Heldin et al., 1989). The close apposition of the catalytic domains of the dimerized receptor then allows transphosphorylation of multiple tyrosine residues throughout the intracellular domain of the receptor. These phosphorylated tyrosine residues in turn form the binding sites for several other proteins (see Table 1), including phospholipase C (Kumjian et al., 1989; Meisenhelder et al., 1988; Morrison et al., 1990; Wahl et al., 1989), rasGAP (Kaplan et al., 1990; Kazlauskas et al., 1990; Molley et al., 1989), the phosphatidylinositol 3-kinase (PI 3-K) (Auger et al., 1989; Kaplan et al., 1987; Kazlauskas and Cooper, 1990), SH2 and SH3 domain containing “adaptor” proteins such as GRB2 (Lowenstein et al., 1992) and Nck (Hu et al., 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992), tyrosine kinases of the Src family (Src, Fyn and Yes) (Krypta et al., 1990), and a phosphorytropic-specific phosphatase, Syt (Feng et al., 1993; Vogel et al., 1993). Each of these proteins contains one or more SH2 domains that are responsible for mediating its interaction with the receptor. Several of the proteins are activated concomitantly with association with the receptor, most become phosphorylated on tyrosine residues, and several change their location from the cytoplasm to the membrane.

In the case of the Src family kinases, PDGF stimulates the intrinsic kinase activity of Src, Fyn and Yes 2- to 5-fold (Gould and Hunter, 1988; Krypta et al., 1990; Ralston and Bishop, 1985). Only those Src family kinases that associate with the PDGF receptor (which at 5 minutes after growth factor addition comprises some 5% of the total) become activated (Krypta et al., 1990). The receptor-associated Src, Fyn and Yes become phosphorylated on several tyrosine residues in their amino-terminal halves. This phosphorylation is catalysed by the PDGF receptor (Twanmley et al., 1992); however the consequences of these phosphorylations on the activity of Src, Fyn and Yes is not yet clear. The association between the Src family kinases and the PDGF receptor is mediated by the SH2 domains of Src, Fyn and Yes (Twanmley et al., 1992) binding to one of two phosphorylated tyrosine residues (tyr 579 and tyr 581) in the juxtamembrane region of the PDGF receptor (Mori et al., 1993). Expression of catalytically active Fyn to high levels in NIH-3T3 cells results in the PDGF receptor associating predominantly with Fyn, and to a much lower extent than normal with Src and Yes, suggesting that all three kinases bind to the same sites on the PDGF receptor (Krypta et al., 1990), because of the high degree of similarity of their SH2 domains.

Why does the PDGF receptor associate with so many proteins? Does each protein have a specific function, or is there redundancy? The response to PDGF is complex, involving not just stimulation of DNA synthesis, but also changes in cytoskeletal organization and cell shape, and responses to other growth factors; possibly each protein participates in one of these responses. However, most investigators score for one criterion as a measure for receptor integrity: induction of DNA synthesis. There are two approaches that can be taken to identify the function of each of the proteins that associate with the PDGF receptor. First, knowing the binding site for the protein of interest, one can create a receptor mutated in this site, and assess its function after introducing it into an appropriate cell type lacking the wild-type receptor (mutant receptor approach). Second, one can introduce into cells a form of the protein of interest which is catalytically inactive but still has the ability to associate with the receptor, so that it is present in excess over the active form of the protein, and then measure the ability of the cells to respond to ligand (the so called “dominant negative” approach). The disadvantage of the mutant receptor approach is that the receptor may not function normally in a heterologous environment. The disadvantage of the dominant negative approach is that it may not be possible to express catalytically inactive proteins to high enough levels in stable cells, and a transient expression system may therefore be required. In either case, for the

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**Table 1. Proteins that have been shown to associate via their SH2 domains with the activated PDGF receptor in vivo**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Size</th>
<th>Binding site on PDGF receptor</th>
</tr>
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<tbody>
<tr>
<td>phospholipase Cγ</td>
<td>150 kD</td>
<td>tyr 1021 (and tyr 1009)*</td>
</tr>
<tr>
<td>RasGAP</td>
<td>120 kD</td>
<td>tyr 771</td>
</tr>
<tr>
<td>PI 3-K</td>
<td>110 and 85 kD</td>
<td>tyr 740 and tyr 751</td>
</tr>
<tr>
<td>Syt</td>
<td>65 kD</td>
<td>tyr 1009</td>
</tr>
<tr>
<td>Src, Fyn and Yes</td>
<td>60 kD</td>
<td>tyr 579 and tyr 581</td>
</tr>
<tr>
<td>Nck</td>
<td>45 kD</td>
<td>?</td>
</tr>
<tr>
<td>GRB2</td>
<td>25 kD</td>
<td>?</td>
</tr>
</tbody>
</table>

*One study reports that this is a weak binding site for phospholipase Cγ, while another did not detect binding of phospholipase Cγ in this site.

†Also known as SH-PTP2 and PTP1D.
experiments to be interpretable, each protein must have a distinct binding site on the receptor. This indeed seems to be the case for the majority of the proteins whose binding site is known. Phospholipase Cγ associates with tyr 1021 (Rönning et al., 1992; Valius et al., 1993), PI3 K with tyr 740 and tyr 751 (Fanti et al., 1992; Kashishian et al., 1992), GAP with tyr 771 (Fanti et al., 1992; Kashishian et al., 1992), the Src family kinases with tyr 579 and tyr 581 (Murt et al., 1993), and Syp with tyr 1009 (Kazlauskas et al., 1993) (note however that in one report phospholipase Cγ had weak affinity for tyr 1009, the binding site for Syp, as well as strong binding to tyr 1021).

We have used the dominant negative approach to investigate whether the association of the PDGF receptor with Src, Fyn and Yes is required for the initiation of DNA synthesis. We used a transient expression system, in which quiescent NIH-3T3 cells were microinjected with plasmids capable of expressing the proteins of interest. Previous work has shown that this allows the rapid, high level expression of proteins (Sorrentino et al., 1990). The catalytically inactive proteins we chose were Src and Fyn molecules bearing point mutations in their ATP binding sites, expressed from the SV40 early region promoter/enhancer.

A typical experiment is shown in Fig. 1, in which a plasmid encoding catalytically inactive Src was injected into cells 6 hours prior to PDGF stimulation, and cells were fixed and stained for Src expression as well as bromodeoxyuridine incorporation 18 hours after PDGF addition (for full details of these experiments, see Twamley-Stein et al., 1993). Fig. 1A shows immunostaining for Src, and the expressing cells are marked with arrows. In B, the same cells were stained using antibodies specific for bromodeoxyuridine, to visualise those cells in or after S phase. It can be seen that those cells that were expressing kinase-inactive Src had not entered S phase, whereas several of the non-microinjected surrounding cells had responded to the PDGF. Quantitative analysis of several similar experiments led to the conclusion that the expression of catalytically inactive Src inhibited PDGF-induced entry into S phase by approximately 90%. A catalytically inactive form of Fyn also inhibited DNA synthesis (Table 2). However, the catalytically active forms of Src and Fyn, when expressed to similarly high levels, had no such inhibitory effect (Table 2), ruling out the possibility that high levels of expression of any protein was inhibitory. It has been shown that each SH2 domain has a preference for phosphotyrosine within a given peptide sequence (Fanti et al., 1992; Songyang et al., 1993), although SH2 domains do also have a measurable, but much lower, affinity for phosphotyrosine alone (Mayer et al., 1991). We were therefore concerned that an SH2 domain-containing protein, when expressed to high levels, might compete with other, less than optimal binding sites on the PDGF receptor. However, this did not seem to be the case, since the catalytically active forms of Src and Fyn did

Table 2. Dominant negative forms of Src family tyrosine kinases inhibit the response to PDGF

<table>
<thead>
<tr>
<th>Plasmid microinjected encoded</th>
<th>Inhibition of PDGF-induced DNA synthesis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinase-inactive Src</td>
<td>+</td>
</tr>
<tr>
<td>Kinase-active Src</td>
<td>−</td>
</tr>
<tr>
<td>Kinase-inactive Fyn</td>
<td>+</td>
</tr>
<tr>
<td>Kinase-active Fyn</td>
<td>−</td>
</tr>
<tr>
<td>Kinase-inactive Fyn + Ras</td>
<td>−</td>
</tr>
<tr>
<td>FynSH3AK*</td>
<td>+</td>
</tr>
<tr>
<td>FynSH2AK*</td>
<td>−</td>
</tr>
</tbody>
</table>

*The FynSH3AK construct has the unique and SH2 domains of Fyn fused to β-galactosidase. The FynSH2AK construct has the unique and SH3 domains of Fyn fused to β-galactosidase. Therefore, neither construct contains the catalytic domain of Fyn, which we have previously shown is not necessary to associate with the activated PDGF receptor. For more details on these constructs, see (Twamley et al., 1992).

†PDGF-induced DNA synthesis was measured by determining the number of cells incorporating bromodeoxyuridine 18-24 hours after PDGF addition. Inhibition was determined by comparing the number on non-microinjected cells entering S phase with microinjected cells from the same experiment. +, greater than 80% inhibition; −, less than 10% inhibition.

Fig. 1. Kinase-inactive Src prevents PDGF-induced DNA synthesis. Cells were microinjected with a plasmid encoding a kinase-inactive form of Src (Lys 295 Met) and incubated for 6 hours to allow transcription and translation to take place. Then PDGF and bromodeoxyuridine (BrdU) were added and the cells incubated at 37°C for a further 18 hours. The cells were then fixed and stained with antibodies specific for Src family kinases (cytoplasmic fluorescence in A) and BrdU (nuclear fluorescence in B). The cells expressing kinase-inactive Src are marked with arrows.
not inhibit PDGF-induced DNA synthesis when expressed to the same levels as the inhibitory, catalytically inactive forms of the proteins. A final concern was that the cells expressing catalytically inactive forms of Src and Fyn were simply not viable, and therefore unable to respond to any signals. To test this we co-microinjected quiescent NIH-3T3 cells with plasmids encoding kinase-inactive Fyn and a constitutively active form of Ras. In this case, cells were able to enter S phase (Table 2), demonstrating that the cells were alive, and able to enter the cell cycle when provided with an appropriate signal that kinase-inactive Fyn does not antagonize.

We next asked which sequences in the Src family tyrosine kinases were required for the inhibitory effect to be manifested. To this end, we took advantage of constructs that we had used to map the requirements for Fyn binding to the PDGF receptor (Twanmley et al., 1992). In these chimeras, the catalytic domain of Fyn was replaced by a portion of β-galactosidase; full binding to the PDGF receptor was retained. Further mutagenesis generated the two constructs that we used in these experiments, FynΔSH2AK and FynΔSH3AK, which lack the SH2 domains and SH3 domains respectively. Both constructs retain the myristylation and unique domains of Fyn, and so are still targeted to the membrane. We have previously shown that only FynΔSH3AK is able to bind to the activated PDGF receptor in vivo (Twanmley et al., 1992). When plasmids encoding these chimeric proteins were microinjected into cells, and the response to PDGF measured, we found that only the construct with the SH2 domain was able to inhibit DNA synthesis; the other had no inhibitory effect (Table 2). We conclude that PDGF receptors that are unable to bind to wild-type Src, Fyn and Yes (because the binding site has been saturated with an SH2 domain-containing, catalytically inactive protein) are unable to respond to PDGF by synthesizing DNA. These experiments do not tell us about the requirement for the other proteins that bind to the PDGF receptor; and indeed it seems unlikely that binding of Src, Fyn and Yes alone is sufficient to stimulate DNA synthesis.

Similar experiments with catalytically inactive forms of the other associated proteins may also help to determine their requirement in PDGF-mediated signal transduction.

What is the role of Src, Fyn and Yes in the response to PDGF? Our preferred hypothesis is that they phosphorylate critical proteins that are not substrates for the PDGF receptor itself. In our current experiments, we are generating cell lines expressing catalytically inactive Src family kinases under the control of an inducible promoter in order to test this hypothesis.

**THE REGULATION OF SRC ACTIVITY**

We described in the Introduction that the integrity of the tail, the SH2 and the SH3 domain are required for correct regulation of Src. A model that accommodates the requirement for both the SH2 domain and the tail has been proposed (Matsuda et al., 1990; Roussel et al., 1991) (see Fig. 4). In this model, the SH2 domain is postulated to have an affinity for the phosphorylated tail, rendering Src in a conformation in which the catalytic domain is not available to substrates, and the SH2 domain is also not able to interact with other proteins. Dephosphorylation of the tail relieves this inhibi-
tion, thereby activating the intrinsic kinase activity of Src, and allowing it to interact with other proteins. But this model cannot explain why the SH3 domain is necessary for correct regulation of Src. Furthermore, it is difficult to test hypotheses on the function of the Src SH2 and SH3 domains in regulation of Src in a higher eukaryotic tissue culture system. Thus, it has been shown that Src molecules deleted in either the SH2 or SH3 domains and expressed in chicken fibroblasts are indeed highly active and transforming, however these molecules are not phosphorylated at tyr 527 (Scidell-Dugan et al., 1992). The transforming properties bestowed by the SH2 and SH3 domain deletions could therefore be due either to a failure of Csk to recognize and phosphorylate these mutant Src molecules, or to the disruption of other regulatory interactions, with dephosphorylation of tyr 527 occurring secondarily to activation. Furthermore, the analysis of other mutants, for example, point mutations and small deletions in the SH2 and SH3 domains, is complicated by the fact that some sequences may not just be involved in regulation of Src, but also in other functions manifested in vivo, such as association with substrate proteins (reviewed by Koegl and Courtneidge, 1992; Parsons and Weber, 1989).

We sought a simpler, heterologous system in which to study the regulation of Src. The yeasts lack Src family tyrosine kinases, and indeed do not appear to have any proteins containing SH2 domains, and therefore seemed an ideal heterologous system in which to study Src regulation. We have recently shown that Src causes a lethal phenotype when expressed in the fission yeast Schizosaccharomyces pombe (Superti-Furga et al., 1993), and that essentially only the catalytic domain of Src is required to elicit this phenotype (unpublished observations). The ability of Src and mutants of Src to induce this lethal phenotype therefore provides a very good assay system to test the requirement for SH2 and SH3 domain sequences in the regulation of Src.

We expressed Src in S. pombe under the control of the nmt1 promoter, which is capable of driving the expression of high levels of protein when cells are maintained in medium lacking thiamine (Maundrell, 1990). Cells containing the Src-encoding vector placed in medium containing thiamine grew logarithmically. Removal of thiamine from the medium resulted in cessation of growth of the cells, which was coincident with the expression of Src protein (Fig. 2 and Superti-Furga et al., 1993). By 24 hours in thiamine-free medium, most of the cells had died, as judged by their inability to grow when seeded into thiamine-containing medium. Death was presumably caused by the phosphorylation of one or more host proteins on tyrosine. Indeed Src phosphorylated a large number of proteins when expressed in S. pombe (Fig. 3). The catalytic activity of Src was required to elicit the lethal phenotype, however mutants carrying phe instead of tyr at 527 were as effective as wild type in killing, suggesting that there were no endogenous enzymes able to phosphorylate 527, in keeping with the results observed for S. cerevisiae (Cooper and Runge, 1987; Nada et al., 1991). We have also shown that Fyn was as effective as Src in eliciting the lethal phenotype in S. pombe (Superti-Furga et al., 1993).

The lethal phenotype we observed may have been caused by the expression of any tyrosine kinase in S. pombe. However, this appears not to be the case, since expression of Csk to the same levels did not affect the growth of the cells. In keeping with this, we observed that Csk was unable to phosphorylate any endogenous S. pombe proteins (Fig. 3), demonstrating its remarkably restricted substrate specificity compared to Src family kinases (Okada and Nakagawa, 1988). We next tested whether Csk was able to regulate Src in this heterologous system. Fig. 2 shows that cells co-expressing Src and Csk were able to grow normally, demonstrating that Csk did regulate Src in an in vivo setting. Indeed, an analysis of tyrosine phosphorylated proteins in cells expressing both Src and Csk showed that the predominant phosphorytrosine-containing proteins were Src and one

![Fig. 3. Tyrosine phosphorylation in S. pombe cells expressing Src and/or Csk. Lysates were made from S. pombe cells grown for the times indicated after the removal of thymine from the medium, resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with antibodies specific for phosphorytrosine. (A) S. pombe cells expressing Src. (B) S. pombe cells expressing Csk. (C) S. pombe cells expressing Src and Csk.](image-url)
of its breakdown products, showing that once phosphorylated by Csk, Src was no longer able to phosphorylate exogenous substrates. The specificity of this inhibition was demonstrated by the observation that Csk was unable to regulate a Src molecule with the rather than tyr at 527 (Superti-Furga et al., 1993).

We could now test whether the SH2 and SH3 domains of Src were required in order for Csk to regulate Src activity. Several SH2 and SH3 domain mutants of Src were generated, and all were able to kill S. pombe with the same efficiency as wild type, demonstrating that the non-catalytic portions of the molecule were not required for the lethal phenotype. All the mutants were also good substrates for Csk (Superti-Furga et al., 1993). Neither a Src molecule lacking the entire SH2 domain (Fig. 3), nor a point mutant in which Arg 175 was replaced with leu (Superti-Furga et al., 1993), were rescued by Csk. Since structural studies on the interaction of an SH2 domain with a tyrosine phosphorylated peptide show that the arg at position 175 makes contact with the phosphate group on the tyrosine residue, these results strongly favour the model in which the SH2 domain of Src interacts intramolecularly with its own phosphorylated tail. However, deletion of the SH3 domain of Src also blocked the ability of Csk to rescue the lethal phenotype, suggesting that regulation of Src is more complicated than previously thought.

The requirement for an intact SH3 domain of Src for correct regulation could be interpreted in two ways: first, in the absence of an SH3 domain, the SH2 domain-phosphorylated tail interaction may still take place, but this molecule is nevertheless still active; second, in the absence of an SH3 domain, the tail and the SH2 domain may not be able to interact. To distinguish between these two possibilities, we took advantage of the fact that an SH2 domain can only interact with one phosphotyrosine-containing sequence at a time. This property forms the basis of an assay (first described by Roussel et al., 1991) in which Src molecules whose SH2 domain is unoccupied will be retained on a phosphopeptide column, whereas those molecules whose SH2 domains are interacting with carboxy-terminal tail sequences will be unable to bind to the column. This assay revealed that the second hypothesis we proposed above is correct, i.e. that the SH3 domain is required for the SH2 domain to interact with the tail of Src. We therefore propose another model to accommodate these new data, shown in Fig. 4, in which both the SH2 and the SH3 domains of Src participate in regulatory intramolecular interactions. We have shown the SH3 domain interacting with sequences near the tail of Src. This speculation was prompted by the observations of others that SH3 domains may interact with proline-rich sequences (Ren et al., 1993), and this region of Src contains several prolines. However at this point it is equally likely that the SH3 domain interacts with other sequences, for example in the SH2 domain. We will be using the S. pombe system described here to test these alternative hypotheses. This system should also be a very powerful way to screen for other molecules able to regulate the activity of Src both positively and negatively, as well as to identify pharmacological compounds able to inhibit Src kinase activity.

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