The SH3 domain of Src can downregulate its kinase activity in the absence of the SH2 domain-pY527 interaction

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Abstract

The contact between the SH2 domain and the C-terminal tail of c-Src inhibits its kinase activity via a complex network of interactions, including the SH3 domain. We examined the role of the SH3 domain in v-Src, where the C-terminal tail is mutated and unbound. We used the v-Src variants Prague C (PRC) and Schmidt-Ruppin A (SRA), which are of low and high kinase activities, respectively, to measure phosphorylation in vitro by immunoprecipitated kinases produced in Saccharomyces cerevisiae. Swapping the regulatory domains between SRA and PRC revealed that N117D, I96T, and V124L mutations in the n-src- and RT-loops of the SH3 domain of PRC are responsible for the low kinase activity of PRC. Moreover, introducing D117N, R95W, T96I, and L124V into activated c-Src(Y527F) caused a 2.5-fold increase in its activity. The mutations in the CD linker KP249,250DG and L255A, which were shown to activate c-Src, had no effect on the activity of the “SH2-activated” Src kinases. Together our data suggest that in the “SH2-activated” forms of Src, the SH3 domain continues to influence the kinase activity via the direct contacts of the n-src- and RT-loops with the kinase N-terminal lobe.

The protooncogenic c-Src can be permanently activated by frame-shift mutation in the C-terminus, which eliminates the tail's binding by the SH2 domain and gives rise to the oncoprotein v-Src [3]. This type of activation was originally discovered to be the underlying principle of the ability of Rous Sarcoma Virus (RSV) to transform cells. We refer in the text to “SH2-activated” or “SH2-inhibited” Src, when the C-terminal tail binding to the SH2 domain is abolished or in effect, respectively. The most widely used v-Src variant, derived from the Schmidt-Ruppin A strain of RSV (SRA; [4]), is 5–25 times more active in vitro than SH2-inhibited c-Src. We found that v-Src derived from the low oncogenic Prague C strain of RSV (PRC; [5]) is ~5 times less active than SRA and is thus closer to c-Src, despite the lack of the inhibitory interaction between its SH2 domain and the C-terminus [6]. We were able to ascribe the differences in kinase activity between PRC and SRA to amino acid substitutions in their regulatory domains.

The crystal structures of chicken c-Src, human c-Src [7], and the related Hck kinase [8], all in the SH2-inhibited conformation and without the unique domain, allowed to outline the framework of regulatory interactions among the SH3, SH2, and catalytic domains of c-Src and the linker regions of c-Src translates regulatory inputs into catalytic and binding activities (for review see [2]).

Abbreviations: PTP, protein tyrosine phosphatase; SH, Src homology; RSV, Rous sarcoma virus; SRA, v-Src derived from Schmidt-Ruppin A strain of RSV; PRC, v-Src derived from Prague C strain of RSV; FAK, focal adhesion kinase; CD, catalytic domain; SRM, kinase inactive mutant of v-Src derived from Schmidt-Ruppin A.

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interactions within the c-Src molecule. The kinase activity is downregulated by distortions of the catalytic site, which occur through binding of the SH3 domain to the SH2--kinase domain linker and to the kinase domain itself. Interactions also exist between the linker and the kinase N-terminal lobe. The binding of the SH3 domain is believed to be stabilized by the SH2 domain, which is in turn tethered in its position by ligating the C-terminal tail. The binding of the C-terminal tail to the SH2 domain is of moderate affinity when compared to other SH2-binding peptides or proteins [9]. Likewise, the sequence of the SH3-binding CD linker is only partially conforming to the consensus for SH3 ligands and the interaction could be influenced by the presence of proteins with SH3 binding sites, such as FAK or synapsin [10,11]. Such interactions were reported to increase the activity of c-Src [10,11] and are implicated in targeting the protein to its proper substrates [12].

SH2-inhibited c-Src could be brought to the SH2-activated form by dephosphorylating Y527 by tyrosine phosphatases [13], by strong SH2 domain ligands [14], or constitutively by Y527F mutation [15]. The modeling of nanosecond time-scale motions of SH3 and SH2 domains of c-Src and Hck, using the closed inactive structure as a template, suggested that the domains are coupled dynamically when the pY527–SH2 domain interaction was in place [16]. When the tail was dephosphorylated, the motions of the two domains in Hck became uncorrelated and the fluctuations of the SH3 domains, with respect to the N-terminal lobe, increased [16], however, neither domain was detached substantially from the rest of the molecule, at least during the short time of the simulation (4 ns). The structure of activated c-Src being not available, the displacement of the SH3 and SH2 domains and also the position of the unique domain (!), can only be hypothesized.

We present data suggesting that the SH3 domain continues to exert negative regulatory effect on the kinase activity even in the absence of the pY527–SH2 domain interaction and that this effect is not mediated via the CD linker binding, but, rather, through a direct contact between the SH3 and kinase domains.

Materials and methods

Cloning and mutagenesis of src variants. The chicken c-src and SH2-activated c-src mutant (Y527F) were obtained from Prof. J. Svoboda (Inst. Molecular Genetics, Prague). The PRC variant of v-src was from pATV8 ([17]; ATCC), the SRA variant and the kinase-null mutant (SRM; K295M) were kindly provided by Prof. G.S. Martin (University of California, USA). The source v-src DNAs were PCR-amplified (5'-gtcggatcc tgggtagtag caagagcaagc-3' and 5'-gccaagctt tgtcagcga-3'), inserted between BamHI and EcoRI sites of pBluescript II KS(+) (Stratagene, USA), and verified by sequencing. For the expression in Saccharomyces cerevisiae the fragments were recloned into pYES2 (Invitrogen, USA).

We constructed chimeric src genes by exchanging restriction fragments among the v-src variants PRC and SRA and c-src (Y527F). Chimeric genes PRC(SRA) and SRA(PRC) were prepared by the replacement of BamHI/BsaHI fragment of PRC, corresponding to the unique domain of the molecule (amino acids 1–82), with the BamHI/ BsaHI fragment of SRA and vice versa. Chimeras PRC(SRA) and SRA(PRC) were obtained by reciprocal exchange of BsaHI/Esp31 fragment (amino acids 82–178). Chimera c-src(F+3SRA) was obtained by the replacement of the BsaHI/Esp31 fragment of c-src (Y527F) with the corresponding fragment from SRA. Chimera SRA(F2PRC) was prepared by exchanging Esp31/MluI fragment of SRA with the Esp31/MluI fragment of PRC (amino acids 178–278).

The CD linker mutations L255A (A) and KP249.250DG (DG) were generated in the template molecules using the Altered Sites II in vitro Mutagenesis System according to the manufacturer’s instructions (Promega, USA). Primers 5'-tccctgggttgtg cactcagcga-3' and 5'-caacgctcgagctgccgacg-3' were used for generating the mutations (DG) and (A), respectively. All chimeras and their mutated versions were verified by DNA sequencing (AGOWA, Germany).

His-tagged v-src variants [18] were cloned from pDXA-HC into pYES2 using BamHI and NotI sites. C-terminal Src kinase ORF obtained in pYpAB23BXN (gift of Prof. D.O. Morgan, University of California, USA), was cloned into pRS313 (Invitrogen, USA) as BamHI fragment. The substrate peptide pI0AR ([19]) is a 92 amino acid fragment of Sin, which was mutated in its SH3 binding motif (R304A). Its DNA was kindly provided by Prof. K. Alexandropoulos (New York University, New York) in Escherichia coli expression vector pGEX-2T (APB, USA). The plasmids were transformed into S. cerevisiae strain EGY48 by standard Li-acetate method, and into E. coli by electroporation.

Protein purification and kinase assays. S. cerevisiae cells expressing Src proteins were used to prepare native extracts as previously described [6]. Src proteins were immunoprecipitated with anti-v-Src mAb327 monoclonal antibody [6]. Alternatively, His-tagged Src was purified using the TALON Metal Affinity Resin and a standard protocol (Clontech, USA). Src protein levels were quantified using mAb327 and densitometry scanning. Kinase activities were measured as incorporation of 32P into a synthetic substrate ([32P]ATP, 3000 Ci/mmol; APB, USA), and into a synthetic peptide substrate RKLPPRPRR (Biosource International, USA; 77–85). The chicken c-src (uPRC2) was expressed in S. cerevisiae strain EGY48 (gift of Prof. D.O. Morgan, University of California, USA), was cloned into pRS313 (Invitrogen, USA) as BamHI fragment. The substrate peptide pI0AR ([19]) is a 92 amino acid fragment of Sin, which was mutated in its SH3 binding motif (R304A). Its DNA was kindly provided by Prof. K. Alexandropoulos (New York University, New York) in Escherichia coli expression vector pGEX-2T (APB, USA). The plasmids were transformed into S. cerevisiae strain EGY48 by standard Li-acetate method, and into E. coli by electroporation.

Results

The SH3 domain of PRC is responsible for its low kinase activity

In our previous publication [6] we compared the kinase activities of SRA and PRC variants of v-Src, which
differ in the efficiency with which they induce oncogenic transformation [21]. Normalized kinase activity of the low oncogenic PRC was found to be only 20% of the activity of SRA. The swapping experiments showed that the low activity of PRC is due to the inhibitory effect of its N-terminal regulatory part.

To localize the region, responsible for the downregulation of the activity of PRC, we prepared a set of chimeric proteins by swapping individual regulatory domains between the PRC and SRA molecules (Fig. 1). The chimeric proteins were produced in S. cerevisiae EGY48 strain, immunoprecipitated, and tested for kinase activity with (EAY)₆ polypeptide as substrate. In preliminary experiments, we confirmed that measurements of either immunopurified or chelate chromatography purified PRC gave identical results; the same was true for SRA. The difference between PRC and SRA was thus independent of method of preparation. The chimeras, generated by swapping of the unique or SH2 domains, had activities equal to (PRC(uSRA)) or slightly higher than (SRA(uPRC) and SRA(2PRC)) their parental molecules (Fig. 2A). The data on these chimeras demonstrated that the substitutions in the unique or SH2 domains are not responsible for the inhibitory effect of the regulatory part of PRC. Remarkably, however, SRA(3PRC), which contains the SH3 domain from PRC, has almost as low kinase activity as PRC (25 ± 2%; Fig. 2A) and the activity of PRC(3SRA) was elevated to the level of SRA (111 ± 17%). We verified these findings using two other substrates, the recombinant p10aR protein, which is derived from an authentic c-Src target [19], and a 13-mer peptide containing a tyrosine kinase substrate consensus (not shown). The observation that the SH3 domain of PRC downregulated the phosphotransferase activity of the SRA catalytic domain allowed us to make implications about the role of the differing amino acids in v-Src regulation (see Fig. 2B and below).

Introducing the SRA specific mutations in the SH3 domain of c-Src(Y527F) increased its kinase activity

The SH3 domain of PRC is identical, except for one residue, to the SH3 domain of c-Src(Y527F) (c-Src(F); see Fig. 1). The effects of activating mutations (e.g., those present in SRA) within the SH3 domain of c-Src are documented [22] but no data exist on the SH2-activated c-Src(F). We thus tested whether the introduction of the SH3 domain of SRA could influence the activity of c-Src(F). Indeed, c-Src(F+3SRA) was 2.5 times more active than c-Src(F), which made it a more active kinase than SRA itself. The same activating effect was confirmed using the two other Src substrates, tyrosine kinase specific peptide and p10aR (not shown). The substitutions in the C-terminus between c-Src(F) and SRA could possibly explain this difference.

The SH3 domains of c-Src(F) and SRA differ in four amino acids, R95W and T96I within the RT-loop, D117N in the n-src-loop, and L124V in the βC–βD loop. The three dimensional structure of c-Src illustrates the role of the RT- and n-src-loops of the SH3 domain in forming the interface between the SH3 and kinase N-lobe [7]. The two domains make direct and extensive contacts through the RT-loop and the β2–β3 loop and through the n-src-loop and R318 of the αC–β4 loop, respectively. The CD linker, which binds the SH3 domain through its KPxxQ motif, is sandwiched between the two domains. The coupling of the SH3 domain, the CD linker, and the kinase N-lobe is believed to be responsible for the downregulation of kinase activity in

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**Fig. 1. Schematic structure of c-Src and the outline of chimeras used in this study.** (A) Domain organization of c-Src. The residues in the SH3 domain and in the linker, which are important for this study, are indicated. (B) The construction of src chimeras. The open, light gray, and dark gray boxes indicate DNA fragments derived from PRC, c-Src(F), and SRA, respectively. The chimera PRC(uSRA) contains, with respect to PRC, amino acid substitutions H16R, D36N, E37K, A43T, N46T, and W62G, all in the unique region. Correspondingly, the chimera SRA(uPRC) represents R16H, N36D, K37E, T43A, T46N, G62W, and D63G in the unique region. The chimera PRC(3SRA) represents T96I, D117N, and L124V in the SH3 domain and K176E, which was introduced into the SH2 domain as part of the swapped fragment. The chimera SRA(3PRC) represents I96T, N117D, V124L, and E176K. The chimera SRA(2PRC) represents T180A, L197P, R205Y, D208Y, S221G, and T242A in the SH2 domain and V271A, which was introduced into the kinase domain as part of the swapped fragment. The chimera cSrc(F+3SRA) represents T96I, D117N, L124V, and R95W in the SH3 domain.
regulated c-Src by forcing the catalytic site into an inactive conformation [23].

We used the chicken c-Src structure (PDB entry 2PTK) as a template and modeled the positions of the mutated residues within the RT- and n-src-loops. The mutation T96I reduced the hydrogen bond network of the RT-loop, which implicates increased flexibility (Fig. 2B). The degree of flexibility of the loop was shown to be important for the binding of the SH3 domain of Hck to its ligands [24]. The substitution D117N of the n-src-loop restricts by half the hydrogen bonding with R318, which borders the crucial C-helix (Fig. 2C). The models show that out of the four activating mutations we introduced into c-Src(F), at least two (T96I and D117N), cause a substantial reduction of electrostatic interactions in the region of direct contacts between the SH3 domain and the kinase N-lobe. The same can be concluded for PRC(3SRA), where the SH3 domain is changed by three amino acid substitutions, T96I, D117N, and L124V. Our results provide evidence that introducing these three mutations into the SH2-activated PRC or c-Src(F) increases in vitro phosphotransferase activities of these molecules.

Fig. 2. The effect of mutations within the SH3 domain on SH2-activated variants of Src. (A) Comparison of kinase activities of SRA, PRC, c-Src(F), and their chimeras. The kinases were produced in S. cerevisiae, immunopurified using mAb 327, and subjected to kinase assay with [γ-32P]ATP and (EAY)n as substrate. Incorporation was normalized with respect to the amount of Src protein, the results from three independent experiments (±SD) are expressed relative to the activity of SRA. The swapped fragment used to prepare PRC(3SRA) also contained the substitution K176E within the SH2 domain (see Fig. 1). The role of the residue 176 in the activation of PRC is, however, unlikely. Both SRA and c-Src(F) have E at 176 and the effect of “(3SRA)” introduction into c-Src(F) on kinase activity is retained. (B) Comparison of the SH3 domain’s RT-loop structures between chicken c-Src (left panel) and a model of SRA (right panel). The WHAT IF program [31] was used to model the structure of c-Src carrying the mutations R95W, T96I, D117N, and L124V as described [32]. Sequence identity between the template 2PTK (chicken c-Src) and the model (SRA v-Src) was 96%. The model quality was checked with WHAT IF structure validation tools [33]. The visualization was performed with the ProlabViewer software (Accelrys, www.accelrys.com). Note the reduction in the extent of the restrictive network of hydrogen bonds in SRA. (C) Comparison of the SH3 domain’s n-src-loop structures between chicken c-Src (left panel) and a model of SRA (right panel). The electrostatic interactions of the n-src-loop-kinase domain interface are reduced in SRA.
Mutations in the CD linker do not influence the kinase activity of SH2-activated Src variants

In regulated c-Src, the interaction between the SH3 domain and the CD linker is believed to be crucial for coupling this domain to the kinase N-lobe, thus maintaining the inactive conformation of the kinase domain itself. We confirmed that c-Src kinase activity in our assay was elevated ~3 times after the addition of 1 mM SH3-binding peptide (not shown). Double mutation KP249,250DG, which destroys the SH3-binding KPxxQ motif, was found previously to activate regulated c-Src [23]. The mutation L255A, which eliminates a hydrophobic side chain buried deeply in the kinase N-lobe, was also shown in c-Src to elevate kinase activity [25].

We introduced these mutations into the SH2-activated variants of Src: PRC, SRA, c-Src(F), PRC(3SRA), and SRA(3PRC). We found that neither of the CD linker mutations significantly influenced the activity of the SH2-activated variants (Fig. 3A). Our data demonstrate that SH2-activated forms of Src are refractory to CD linker mutations, known to increase the kinase activity of regulated c-Src.

Discussion

The protooncogenic kinase c-Src can be activated by displacing the intramolecular interactions of its SH2 and SH3 domains. We addressed the question whether the SH2-activated forms of c-Src, e.g., c-Src(F) and the oncogenic v-Src, could be further activated by mutations disturbing the SH3 domain–kinase domain contacts. We swapped the regions encompassing the SH3 domain in SRA and PRC, the high and low kinase activity variants of v-Src, respectively. The presence of T96I, D117N, and L124V in PRC and similarly, the presence of R95W, T96I, D117N, and L124V in c-Src(F), activated the kinases (Fig. 2A). The importance of these residues for the activity of regulated c-Src was analyzed previously. The mutations R95W and T96I, which modify the RT-loop of the SH3 domain, activated the kinase and produced cells with partially transformed phenotype [22,26]. The mutation D117N, which is positioned within the n-src-loop of the SH3 domain, was predicted to disrupt the interaction of this loop with the catalytic domain [7]. R95W, D117N, and L124V, when introduced together in regulated c-Src, increased its kinase activity to 84% of SRA [26]. SH2-inhibited c-Src could also be activated by SH3-binding epitopes of Sin, FAK, or synapsin [10,11,19]. The effect of SH3-ligands on SH2-activated c-Src or v-Src was not reported, albeit data exist on the activation of SH2-activated Hck [20]. However, the Hck kinase differs in several important aspects from c-Src, namely in that the CD linker binding motif corresponds to a true SH3 binding site [8].

Fig. 3. The effect of CD linker mutations on SH2-activated variants of Src. (A) Kinase activities of Src variants carrying CD linker mutations KP249,250DG (e.g., PRC(DG)) or L255A (e.g., PRC(A)) are shown in comparison with SRA. Kinase assays were performed as described in Fig. 2, the bars represent percentage of SRA activity obtained in three independent experiments (±SD). (B) Detail of the interface between the SH3 domain and the kinase N-lobe of chicken c-Src (2PTK) without, (upper panel), or with, (lower panel), the residues 255–262, which constitute the C-terminal part of the linker. The area corresponding to the linker residues is outlined by dots. Note that the linker does not fully occupy the interface. The view does not include the residues 249–254 of the linker, which are positioned outside of the area where the n-src- and RT-loops contact the kinase domain. The visualization was done with ProlabViewer software.
This is the first demonstration that SH2-activated Src can be made more active by targeting its SH3 domain. We infer that the C-terminal tail uncoupling in c-Src does not fully release the SH3 domain from its inhibitory interactions. Such a feature makes c-Src similar to Hck, where additive activation by SH2 and SH3 ligands was demonstrated [20,27]. The usual way of schematically depicting activated c-Src and other Src-family kinases, the “beads-on-a-string” model [16], may in fact overemphasize the “disassembly” of the four domains from each other. Notably, a deletion within the SH3 domain of a transformation defective v-Src restored its transforming properties and potentiated its kinase activity [28]. The deletion, which eliminated amino acids 89–91, was proximal to the RT loop of the SH3 domain and was likely to distort the interaction between the RT loop and the N-terminal kinase lobe. The SH2 domain of v-Src was also shown to be a site of kinase-inactivating mutations of v-Src. Either F172V or L186F within the region facing the CD linker drastically reduced the kinase activity using (EAY)₃ as substrate [29], an effect which could not be explained by the “beads-on-a-string” model. The significance of the n-src-loop of the SH3 domain was documented in the neuronal splice variant of c-Src, which contained an insertion of 6 amino acids at position 114 and possessed elevated kinase activity [30].

Computer simulations and mutagenesis data support the view that the uncoupling of the SH2 domain from the C-terminal tail affects the whole molecule [16; see above]. The linker region between the SH3 and SH2 domain, which mediates this coupling, was indeed shown to cause c-Src activation in S. pombe when mutated (S142G, A145G, E146G), [16]. Forced transition of the activation loop of the kinase into the active conformation was also simulated, which resulted in a displacement of the N-terminal lobe from the SH3 domain and was communicated through the SH3–SH2 connector to the SH2 domain. The authors argued that the SH2 domain pY527 binding would impede the SH2 domain displacement, providing the coupling between the SH2 domain and kinase activity [16]. Our results, in addition to the previous data [28], call for further research on the actual mobility of the regulatory domains within SH2-activated c-Src. Instead of bringing the molecule into a state of “beads-on-a-string”, the SH2-activation perhaps acts similarly to the T to R transitions of multisubunit complexes. The interdomain contacts are less extensive and impose fewer negative regulatory effects.

Our results also show that, in the SH2-activated Src, the regulatory contacts of SH3 RT- and n-src-loops with the catalytic N-lobe are less dependent on the CD linker. The activity of SRA(3PRC+DG) is at the level of PRC (Fig. 3A), despite the fact that the KP249,250DG mutation is incompatible with the binding of the CD linker to the SH3 domain via the classical mechanism. The KPxQ motif interacts with the SH3 domain-binding cleft slightly outside of the area where the RT- and n-src-loops touch the kinase N-lobe. Noteworthy also, the SH3 domain– kinase N-lobe interface seems not to be fully occupied by the linker (Fig. 3B). We hypothesize that in the SH2-activated state the CD linker conformation is changed with respect to the fully inhibited state. Clearly, in the absence of the SH2 domain-C-terminal tail contact, the role of the RT- and n-src-loop mediated interactions prevails.

We conclude that c-Src(F) and Prague C v-Src are regulated through the interaction between the SH3 and kinase domains. The results also suggest that in the absence of SH2 domain–C-terminal tail binding the prevailing part of the effect is mediated by direct interactions of the SH3 RT- and n-src-loops with the kinase N-lobe. The persistence of the SH3 domain’s inhibitory effect implies the possibility that upon SH2-activation c-Src maintains part, if not most, of its interdomain contacts.

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References


M.F. Verderame, H.E. Varmus, Highly conserved amino acids in the SH2 and catalytic domains of v-src are altered in naturally occurring, transformation-defective alleles, Oncogene 9 (1994) 175–182.


