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COUPLING KINASE ACTIVATION TO SUBSTRATE RECOGNITION IN SRC-FAMILY TYROSINE KINASES

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1. ABSTRACT

Signal transduction molecules translate extracellular inputs into their corresponding intracellular responses. Given the complexity and number of signaling pathways present in the eukaryotic cell, it is not surprising that the functions of signaling molecules are often tightly regulated. Autoinhibition is a prevalent mechanism for governing the function of signaling molecules. The relationship between the viral, oncogenic form of Src (v-Src) and the corresponding cellular proto-oncogene (c-Src) highlights the importance of inhibitory intramolecular interactions. Src provides an example of the dramatic cellular consequences arising from the loss of autoregulation.

2. INTRODUCTION

In the early 1900s Peyton Rous observed that extracts from chicken tumors could induce tumors of the same type when injected into other chickens (1). The agent responsible was later identified as a retrovirus and named Rous Sarcoma virus. A single gene product, v-Src, endowed the Rous Sarcoma virus with the ability to promote tumors in animals, giving rise to the term oncogene (2). Further investigation into the origin of v-Src revealed that the oncogene was a modified version of a normal cellular gene, c-Src (3). Subsequent work focused on how a protein originating from the cellular genome could be responsible for directing cellular transformation. A key observation was that Src can catalyze the phosphorylation of proteins on tyrosine residues (4). c-Src was shown to have lower levels of tyrosine kinase activity than its viral counterpart, v-Src (5,6). The sequences of c-Src and v-Src diverge at several positions, particularly in the C-terminal region of the molecule (7). More recent work has revealed that this region is crucial for autoinhibition and proper regulation of the enzymatic activity of c-Src. By escaping the intramolecular regulation to which c-Src is normally subjected, v-Src is constitutively active and thus capable of promoting tumor formation in animals. These findings initiated two decades of intense research into the regulation and normal biological function of Src as an approach to understand the biochemical basis of cancer.

Tyrosine kinases influence an extensive array of signaling pathways in the cell (8). Tyrosine kinases occur as membrane-bound receptors, or as non-receptor cytoplasmic proteins (9). Receptor tyrosine kinases are composed of an extracellular ligand-binding domain, a transmembrane region, and an intracellular tyrosine kinase domain. They span the plasma membrane once and signal by ligand-mediated dimerization, or ligand-mediated activation of a preexisting dimer (10,11). In contrast, non-receptor tyrosine kinases can be membrane associated, cytoplasmic, or nuclear. Their subcellular localization, however, is not static; many members can move between different cellular compartments as part of their biological function. There are presently 8 different classes of non-receptor tyrosine kinases, grouped in families according to sequence and structural similarity: Src, Csk, Fak, Syk, Jak, Fps, Frk, Tec, and Abl.

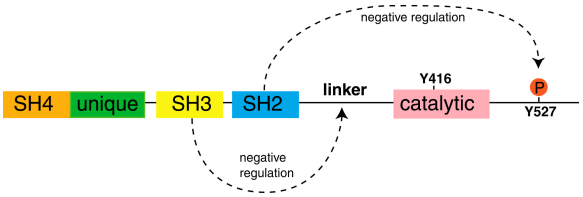

Figure 1. Structural organization of Src family tyrosine kinases. The two inhibitory intramolecular interactions are shown by dotted lines. Phosphorylation at Tyr416 in the catalytic domain activates Src, while phosphorylation at Tyr527 in the C-terminal tail is inhibitory.

A characteristic feature of non-receptor tyrosine kinases is that they possess additional domains or protein modules in addition to the catalytic domain. (In fact, to date no tyrosine kinase has been found to exist as an isolated catalytic domain). These accessory domains are often involved in mediating protein-protein interactions, and they are also found in many unrelated signaling molecules (12). While it is generally established that accessory domains play a positive role in subcellular localization and substrate recognition, in the case of Src kinases they have the additional task of maintaining the catalytic domain in an inactive conformation. As discussed below, both processes combine to ensure maximum fidelity and efficiency during signalling cascades.

The mammalian Src kinase family is composed of 8 members: Src, Yes, Fgr, Hck, Fyn, Lck, Lyn and Blk (13,14). Src, Fyn, and Yes are expressed in most tissues, whereas the expression pattern of the other Src family members is mostly restricted to cells of hematopoietic origin (14). Src kinases participate in a variety of signaling pathways that control a diverse spectrum of biological activities: adhesion, spreading, migration, focal adhesion formation/disassembly, lamellipodia extension, cell cycle progression, apoptosis, gene transcription, and cell differentiation (14,15). These Src-mediated signal transduction events are initiated by the following receptors: antigen recognition receptors, integrins, cadherins, growth factor receptors, G-protein-coupled receptors, and cytokine receptors. More recently, Src kinases have emerged as modulators of gap junction activity, and certain types of voltage and ligand gated channels (14).

Src kinase activity is elevated in cells stimulated with mitogenic factors such as PDGF, CSF1, EGF, and FGF (16). These observations, together with the role of v-Src in tumor formation, led to the suggestion that unregulated Src kinases may play a critical role in the genesis and progression of human cancers. In fact, accumulating biochemical evidence indicates that in over 50% of human tumors, c-Src activity is altered (17). Changes in c-Src activity are chiefly due to overexpression of the Src gene product or to posttranslational events. A c-Src mutation that results in a truncated, activated form of Src has been identified in a highly metastatic form of colon cancer (18). A growing number of studies implicate c-Src in the development of three of the most prevalent types of cancers in adults: colon, breast and lung (17). Thus, considerable interest exists in understanding the various mechanisms behind Src activation as a basis for the development of Src kinase inhibitors as future anti-cancer therapies.

3. DOMAINS OF SRC KINASES

Like most cytoplasmic tyrosine kinases, Src kinases are organized into a set of modular domains. Several of the domains have sequence homology to related domains in other signaling molecules (e.g. Src Homology domains 2 and 3). Others are classified together on the basis of a common function (e.g. SH4 domains confer membrane localization). All Src kinases share roughly 60% amino acid identity and possess the same basic modular architecture (from N- to C-terminus): SH4, unique domain, SH3, SH2, linker region, catalytic domain (SH1), and a C-terminal tail which is critical for negative regulation of the kinase (Figure 1). Src kinases range between 57 – 62 kDa in molecular weight. Amino acids important for the regulation and activity of the protein are conventionally numbered according to those in chicken c-Src (which contains 533 amino acids).

3.1. SH4 Domain

A myristoyl group moiety is covalently attached to the N-terminus of Src kinases. Myristoylation is necessary for membrane localization but not for kinase activity (19). Association of Src with the plasma and intracellular membranes is essential for the biological function of Src; non-myristoylated mutants of v-Src are unable to transform cells (20). Myristoylation alone is not sufficient to anchor the kinases to the plasma membrane. Src and Blk have a cluster of basic residues in the SH4 region that contributes to membrane association (21-23). Other Src family members, which lack a cluster of basic residues, possess palmitoylation sites at cysteines near the N-terminus (19). This dual lipid modification, or the lipid-polybasic cluster combination, stabilizes the association of Src kinases with the plasma membrane and possibly other intracellular membranous compartments. The reduction of dimensionality that occurs upon transfer from a three-dimensional space to a two-dimensional plane (such as the plasma membrane) results in changes in the effective concentration of the kinase and increased proximity to membrane-bound substrates (21,22). The intracellular localization of Src changes when various signaling pathways are activated, and these changes are important for the proper biological function of Src (13-15,24).

3.2. Unique domain

Following the SH4 domain is a region of 50-70 amino acids termed the unique domain. The unique domain has the lowest degree of sequence homology among Src members. At present, the function of the unique domain is not completely understood, and no high-resolution structure of a unique domain has been obtained. Ser/Thr and Tyr phosphorylation sites are found in the unique region of some Src-family members. c-Src is phosphorylated by Cdc2 at Thr34, Thr46, and Ser72 during cell division (25,26). Hck can undergo an activating autophosphorylation within its unique region (27). Some evidence indicates that the unique region may be involved

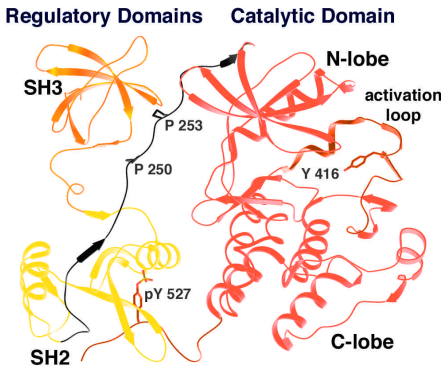

Figure 2. The structure of the inactive form of Hck (52). The catalytic domain is shown in red, the SH3 domain in orange, and the SH2 domain in yellow.

in protein-protein interactions that are specific to each member of the Src family. For example, the unique region of Lck has been shown to associate with the high affinity IgE receptor (28). Similarly, phosphorylation of the NMDA receptor by Src is dependent on the Src unique domain (29).

3.3. SH3 domain

SH3 domains are structurally compact modules of ~50-60 amino acids that are capable of independent folding and function (12,30). SH3 domains are present in many molecules involved in intracellular signaling, either alone or in combination with SH2 or other modular domains (12). As discussed below, SH3 (and SH2) domains have dual functions in Src kinases: they are required for negative regulation of kinase activity, but also play a positive role in proper substrate recognition and/or cellular localization.

SH3 domains recognize proline-rich sequences that adopt a left handed proline type II helix conformation (30). Experiments with combinatorial peptide libraries and phage display libraries have clarified the binding preferences of a variety of SH3 domains (31-34). All SH3 domain ligands contain a core consensus sequence of PXXP, where X denotes any amino acid. Amino acids surrounding the prolines confer additional affinity and specificity for individual SH3 domains (35). In particular, an arginine residue outside the PXXP motif is important for ligand binding to the Src SH3 domain. Crystal structures of isolated SH3 domains bound to polyproline peptides reveal a small, mostly hydrophobic, interaction surface (30,36). As a result, SH3-peptide interactions tend to be weak, with dissociation constants between 1-10 μM (12,30). In intact proteins, interactions outside the PXXP sequence may confer higher binding affinities. This is the case for Nef, an HIV accessory protein containing a PXXP motif. The interaction between Nef and the SH3 domain of the Src family kinase Hck is quite strong (K_d = 0.25 μM) (37). Crystallographic studies of Nef complexed with the SH3 domain of Fyn reveal an additional hydrophobic contact outside the PXXP binding groove (36). Thus, weak binding may be a common feature of SH3-ligand interactions, or alternatively, the weak binding observed may be a result of measurements made using small peptides. Additional biochemical studies reporting binding constants between SH3 domains and native protein ligands are needed to distinguish these possibilities.

One interesting feature of SH3 domains is that they can bind ligands in either orientation: N to C or C to N (38). The more common Class I ligands bind in the N to C orientation whereas Class II ligands bind in the C to N orientation. The SH3 domain of Src is capable of binding both classes of ligands. An arginine either N or C-terminal to the PXXP core pairs with an acidic residue in the SH3 domain, determining the binding orientation of the peptide. This raises the possibility that PXXP-containing substrates of Src can position the catalytic domain of the kinase in close proximity to the phosphorylatable tyrosines.

3.4. SH2 domain

SH2 domains are also compact, modular units that facilitate protein-protein interactions (12,30). The peptide-binding abilities of SH2 domains appear to be independent of the surrounding molecular context. SH2 domains bind tyrosine-phosphorylated peptides and proteins, but not their unphosphorylated counterparts. Binding to tyrosine phosphorylated sites is tighter than SH3-ligand interactions, with measured affinities in the range 0.1-1.0 μM (39). Because binding can be mimicked using short synthetic peptides, SH2-ligand interactions are thought to be independent of the three-dimensional structure of the protein ligand.

The specificity of SH2 domains for their ligands has been studied by the use of degenerate peptide libraries (40). Different SH2 domains recognize different sequences in the 3-7 residues following phosphotyrosine in the ligand (these sequences are designated +1, +2, +3, etc). The SH2 domains of Src kinases recognize three amino acids C-terminal to the phosphotyrosine. In particular, the Src, Fyn, Lck, and Fgr SH2 domains select the sequence pYEEI from peptide libraries, with a hydrophobic residue at position +3 being an important determinant (40). Crystal structures of isolated SH2 domains of Src (41,42) and Lck (43) bound to high affinity peptide ligands reveal a two-pocket binding mode. The first pocket accommodates the pY, whereas the second one, which is hydrophobic in nature, accommodates the hydrophobic residue at position +3. Many proteins that bind to Src kinases possess SH2 ligand sequences that conform to these preferences; tyrosine phosphorylation of these proteins directs the assembly of a signalling complex with Src. Examples of proteins shown to interact with the Src SH2 domain *in vivo* include FAK, p130Cas, the p85 subunit of PI 3-Kinase, and p68sam (44-46).

3.5. Catalytic domain

The isolated catalytic domain of Src is functional as a tyrosine kinase but possesses low transforming ability. This reflects the importance of accessory domains of Src for the biological function of the molecule. The three-dimensional structures of the catalytic domains of Src, Hck, and Lck show considerable similarity to structures of other

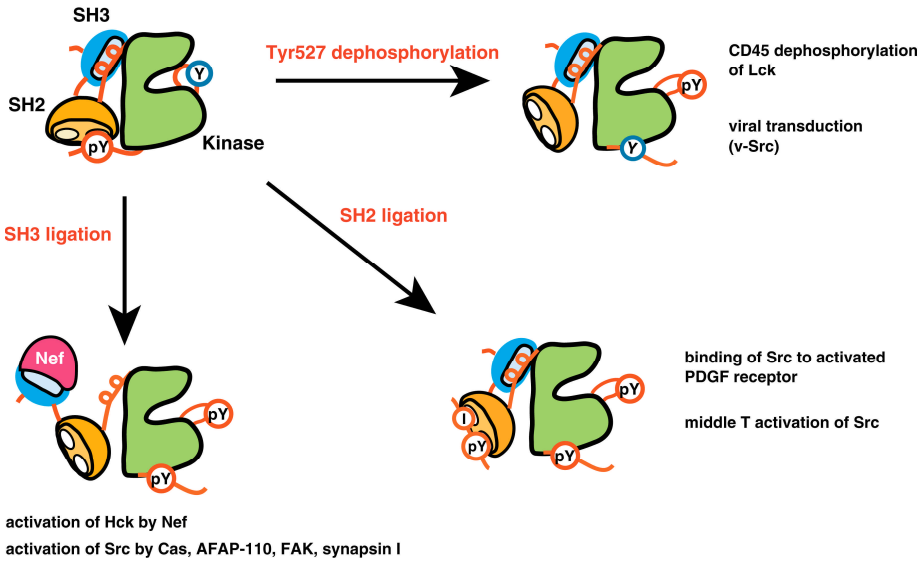

Figure 3. Pathways of Src activation. The inactive form of a Src kinase (upper left) can be activated by at least three mechanisms: Tyr527 dephosphorylation, SH3 ligation, or SH2 ligation. These processes lead to increased phosphorylation of Tyr416 and enhanced catalytic efficiency.

protein kinases, including Ser/Thr kinases (Figure 2) (47-56). Protein kinases possess a smaller N-terminal lobe, largely responsible for binding ATP, and a larger C-terminal lobe that makes most of the contacts with protein or peptide substrate. The activation loop (residues 404-432) lies between the lobes and contains the major autophosphorylation site, Tyr416. When Tyr416 is not phosphorylated, the activation loop is positioned such that access of peptide substrates to the catalytic machinery is blocked. Autophosphorylation produces a conformational change in the activation loop, allowing increased binding of substrates and activation of the kinase.

All Src kinases have a C-terminal extension or 'tail' following the catalytic domain with a conserved Tyr (Tyr527 in Src). When phosphorylated, Tyr527 binds to the SH2 domain. As described below, intramolecular interactions between the phosphorylated tail and the SH2 domain stabilize a conformation with low kinase activity (57). v-Src lacks Tyr527 and is constitutively active.

4. REGULATION OF KINASE ACTIVITY

Numerous *in vitro* and *in vivo* studies over the past two decades suggest that there are multiple ways to activate Src family kinases (Figure 3). The first is by phosphorylation or dephosphorylation of critical tyrosine residues within the molecule. The second is by disruption of repressive intramolecular interactions involving the SH3 and the SH2 domain. Biochemical, genetic, and structural evidence suggests that there is a complex interdependence between both processes.

Src kinases have two principal regulatory tyrosine phosphorylation sites. Phosphorylation within the activation loop at Tyr416 increases activity, whereas phosphorylation of the C-terminal tail at Tyr527 inhibits kinase activity. Other phosphorylation sites resulting in increased kinase activity have also been described (27,58). Cellular exposure to various stimuli, such as growth factors or extracellular matrix components, results in elevated tyrosine kinase activity and in changes in the pattern of tyrosine phosphorylation of Src kinases.

As described above, Tyr416 lies within the activation loop of the catalytic domain. Autophosphorylation at this site expels the loop from the catalytic cleft, facilitating substrate binding (Figure 2). Autophosphorylation of Src kinases at Tyr416 appears to be an intermolecular process. When the activity of downregulated Hck or Src is measured, a lag is observed in substrate phosphorylation that correlates with Tyr416 phosphorylation (59-61). The duration of this lag decreases as the enzyme concentration increases, consistent with an intermolecular process. Preincubation of the enzyme with ATP eliminates the lag in peptide substrate phosphorylation. These observations suggest that, at least *in vitro*, the relative concentration of enzyme can play a role in Src kinase regulation. *In vivo*, the subcellular distribution of Src is not uniform (13). Increased levels of Src are found in specific membrane compartments, such as the plasma and perinuclear membranes, and in focal adhesions (14). Furthermore, Src's subcellular localization is dynamic, changing from different compartments in response to different stimuli (62,63), raising the possibility that the relative concentration of Src in the cell can act as a regulatory mechanism.

Phosphorylation of Tyr527 is carried out by another non-receptor tyrosine kinase, CSK (C-terminal Src Kinase) (64). In resting fibroblasts, Src appears to be constitutively phosphorylated at Tyr527 and thus downregulated (26). Dephosphorylation of Tyr527 *in vivo* (65) or *in vitro* (66) by phosphatases disrupts the SH2-tail

interaction and stimulates kinase activity. For example, the transmembrane tyrosine phosphatase CD45 dephosphorylates Lck at Tyr527 following antigen receptor stimulation, resulting in increased kinase activity (65). Similarly, in v-Src the C-terminal tail is replaced with an unrelated sequence (7). The absence of pTyr527 (coupled with additional mutations throughout the sequence) renders v-Src constitutively active.

The SH3 and SH2 domains are both involved in stabilizing the catalytic domain in an inactive conformation (Figure 2). Src kinases can be activated by displacing the phosphorylated tail from the SH2 domain, even in the absence of dephosphorylation of Tyr527. For example, stimulation of cells with PDGF results in activation of Src kinases. Activation is mediated by a PDGF receptor autophosphorylation site, which becomes a high affinity binding-site for the SH2 domain of Src. This higher affinity site displaces the C-terminal tail from Src's SH2 domain, relieving its negative regulatory role. The result is an increase in kinase activity (45,46). This mode of regulation is possible because the intramolecular ligands for the SH2 and SH3 domains do not conform to the sequence requirements for high-affinity ligands. For example, the sequence C-terminal to pTyr527 makes it a poor ligand for the Src SH2 domain. However, the entropic favorability of the intramolecular interaction keeps the kinase in a repressed state. The interaction is sufficiently weak so that competing high-affinity SH2 ligands, such as the phosphorylated PDGFR, activate Src family kinases. This idea has been tested in the Src-family kinase Hck by substituting the naturally-occuring tail sequence (pYQQQ) with a high-affinity SH2 domain ligand (pYEEI). This mutant cannot be activated by exogenously added high affinity SH2 domain ligands. In addition, its SH2 domain is tightly bound to the C-terminal tail, and is unavailable for interaction with substrates (67).

Activation by SH3 domain displacement is another mechanism for Src kinase regulation (61,68). Genetic experiments involving deletions and mutations in the SH3 domain first hinted at this possibility (69,70). This was somewhat surprising, since there are no obvious polyproline regions in the Src protein that would conform to SH3-binding consensus sequences. The mode of SH3-mediated repression of catalytic activity remained obscure until the elucidation of the three-dimensional structures of c-Src and Hck in 1997 (55,56). These structures revealed that the linker region between the SH2 domain and the catalytic domain (which contains only one or two prolines in Src and Hck respectively) forms a left-handed PP-II helix that serves as a docking site for the SH3 domain. In addition, residues found in the linker region also contact the catalytic domain, keeping it in a conformation unfavorable for catalysis (Figure 2). SH3 ligands can therefore disrupt the repressive SH3-linker-catalytic interaction. The interaction between the HIV protein Nef and downregulated Hck provides a good example of this mode of activation (60,61). Nef contains a polyproline sequence that binds the Hck SH3 domain with high affinity. The displacement of the SH3 domain from the catalytic core of the kinase releases repressive interactions, stimulates Hck autophosphorylation at Tyr416 (61), and leads to large increases in enzymatic activity. Similarly, binding of the SH3 domain to a polyproline-containing peptide fragment of Sin (68), FAK (71), or to p130Cas (72,73) is sufficient to induce activation of Src kinases.

It was originally postulated that SH2-mediated inhibition of catalytic activity was accomplished by restricting the access of substrates to the active site through direct blockage by the SH2 domain. An unexpected feature of the Src and Hck structures is that the SH2 domain binds to the C-terminal phosphotyrosine on the opposite face of the catalytic domain from the active site (Figure 2). Down-regulation of Src activity by SH2-tail interaction occurs indirectly through amino acid residues in the N-lobe of the kinase catalytic domain. The orientation of helix alphaC in the N-lobe plays an important role in kinase activity. In the inactive form of Src kinases, Glu310 on helix alphaC is pointed out of the active site. In active Src kinases, Glu310 points into the active site and participates in ATP binding. A series of interactions between the SH3 domain and the SH2-kinase linker stabilizes the inactive conformation of helix alphaC. Displacement of the SH3 domain by an SH3 ligand presumably destabilizes these interactions, allowing reorientation of helix alphaC and, consequently, more efficient catalysis (56).

The repressive control of the SH3 domain appears to be dominant over that of the SH2 domain (61). *In vitro*, SH2 domain displacement by SH2 ligands such as pYEEI peptide results in enzymatic activation, yet further activation can be obtained by addition of an SH3 domain ligand. The opposite is not true: addition of SH2 ligands after SH3 domain displacement results in no further activation of the kinase (61). Furthermore, disrupting the SH3 interaction in the pYEEI-Hck mutant (discussed above) overrides the strengthened SH2-tail interaction (67). Consequently, activation of Src kinases by SH3 domain displacement can be regarded as an "all or none" process.

Displacement of SH2 and SH3 domains by exogenous ligands promotes kinase autophosphorylation, resulting in elevated enzymatic activity. There is cross-talk between SH3/SH2 domain displacement and autophosphorylation at Y416. Studies with Hck and Src suggest that SH3 and SH2 domains become more accessible after autophosphorylation of the kinase (67,74). These observations imply a bi-directional mode for Src regulation: SH3 and SH2 domains inhibit Src activity, but Src activity can in turn increase the accessibility of these domains to exogenous ligands. Thus, autophosphorylation at Tyr416 renders the kinase more sensitive to further activation by SH3/SH2 domain displacement. Molecular dynamics simulations of the conformational change of the activation loop during kinase activation support this view. These studies show that this transition is communicated to the SH3 and in turn the SH2 domain via the connector between the two (75).

Recently, a new mode of regulation of kinase activity involving the catalytic domain has been observed for beta-arrestin and G-proteins (76,77). While the

stimulation of activity appears to arise from direct binding of these proteins to the catalytic domain, the molecular details of this novel activation are not well understood.

5. ROLE OF DOMAINS IN SUBSTRATE RECOGNITION

Combinatorial peptide libraries have been used to identify primary sequence requirements for protein kinases (40,78,79). Once the optimal sequence for each enzyme has been identified, it can be used to search protein databases for potential protein substrates. This approach has been successful for enzymes relying on primary sequence for substrate specificity. For instance, peptide library studies on cAMP-dependent protein kinase (40,78,79) confirm the presence of the established RRxS consensus (80). Similar studies have been carried out with non-receptor tyrosine kinases. These studies indicate that there is some intrinsic specificity associated with the catalytic domain of tyrosine kinases. For example, a hydrophobic residue N-terminal to the tyrosine substrate is preferred by members of the Src, Csk and Abl families. Members of the receptor tyrosine kinase family prefer an acidic residue in this position (78,81). The residues at P+1 and P+3 (relative to tyrosine) are also important in kinase substrate recognition (82). Although these studies provided information on preferred amino acids at positions surrounding tyrosine, no absolute consensus sequence was established for the catalytic domain of Src kinases, suggesting that the substrate specificity of Src kinases cannot arise from the catalytic domain alone.

It is likely that the *in vivo* substrate specificity of Src-family tyrosine kinases is governed by both the intrinsic specificity of the catalytic domain and the effective local concentrations of protein substrates. In most cases, substrate recognition is dictated primarily by interactions with non-catalytic regions of the enzymes such as SH2 and SH3 domains. These interactions bring the target protein close to the catalytic domain for phosphorylation (83). As a consequence, small deletions and insertions in the SH3/SH2 domains impair the transforming ability of oncogenic forms of Src (84-86). This concept is supported by experiments using chimeric tyrosine kinases containing heterologous SH2 domains with different specificities. The pattern of phosphorylated proteins in cells transfected with these chimeric kinases resembles the specificity of the associated SH2 domain, suggesting that the SH2 domain dictates substrate specificity in this experiment (87). Similarly, many proteins that bind the Src SH3 domain are also substrates (Table 1). Therefore, the SH3 domain can target Src kinases to specific proteins or to subcellular compartments rich in proline-containing proteins such as the cytoskeleton.

6. COUPLING ENZYME ACTIVATION TO SUBSTRATE RECOGNITION

When Src signaling is activated, many Src substrates become phosphorylated on sites that could subsequently bind to the SH2 domain of the kinase. The involvement of the SH2 domain in directing substrate specificity is apparent in a process termed processive phosphorylation. In this process, a tyrosine kinase phosphorylates a site in the substrate that becomes a high affinity binding-site for the SH2 domain. Interaction between this site and the SH2 domain of the kinase facilitates phosphorylation of subsequent tyrosines in the substrate by increasing the local concentration of the substrate. For example, stimulation of the PDGF receptor by its ligand results in receptor dimerization and transphosphorylation of the receptor monomers. This autophosphorylation site in PDGFR becomes a high affinity-binding site for the SH2 domain of Src. The result is both an increase in kinase activity (as a consequence of C-terminal tail displacement) and colocalization of Src with one of its substrates, PDGFR, which in turns facilitates further phosphorylation of the receptor (88,89). Similarly, the lymphocyte-specific Src-family kinase Lck phosphorylates several tyrosine residues in the zeta chain of the T-cell receptor. Each zeta chain contains three immunoreceptor tyrosine-based activation motifs (ITAMs) with the consensus YXXI/LX(6-8)YXXI/L which become good SH2 ligands when phosphorylated. Mutation of amino acids involved in phosphotyrosine recognition in the SH2 domain of Lck reduces receptor hyperphosphorylation and signal transduction, consistent with a processive phosphorylation model (90).

The catalytic advantage conferred by the presence of an SH2 ligand in a Src substrate has been investigated using synthetic peptides (91). An SH2 binding sequence in a substrate can increase the rate of phosphorylation of the substrate by 10-fold. This enhanced phosphorylation is imparted by a reduction in the Km of the peptide substrate and requires the integrity of the SH2 domain of the kinase. An additional boost in phosphorylation efficiency would be expected when the same SH2-binding sequence is also used to activate the kinase by SH2 domain displacement. SH2-substrate interactions therefore impart an additional level of specificity in Src-dependent signaling pathways.

Processive phosphorylation can arise from polyproline-SH3 interactions as well. The SH3 domains of Src-family kinases recognize proline-rich sequences which can be found in a large number of Src substrates. An example of enhanced phosphorylation involving SH3-polyproline interactions comes from studies of the focal adhesion protein p130Cas. Cas (Crk Associated Substrate) was first identified as a highly phosphorylated protein in v-Src and v-Crk transformed cells (92). A C-terminal polyproline region in Cas is necessary for interaction with Src kinases and for efficient phosphorylation of the multiple tyrosine residues in Cas (72,73,93). Disruption in the C-terminal polyproline of Cas completely abolishes interaction between the two proteins and results in impaired phosphorylation of Cas. Kinetic analysis and *in vitro* phosphorylation studies indicate that the polyproline region of Cas serves to anchor the kinase allowing multiple phosphorylation events to occur before the two dissociate (73). Similarly, the integrity of the polyproline motif in the actin filament-associated protein AFAP is necessary for stable complex formation with Src. Impaired

Table 1. Proteins that associate with Src kinases via the SH3-domain.

Protein	Substrate	Activation	Sequence	References
Class I consensus			XpΦPpXP	33
Src library consensus			RXLPPLP	38
P130Cas	Y	Y	RPLPSPP	93
SHP2	Y	Y	PLPPCTPTPP	103
Cbl	Y	Y	RDLPPPP	104
AFAP	Y	Y	MPLPEIP	94
Synapsin	Y	Y	RALPSIPKL	105
CD 28	N.D.	Y	PYQPYAPAR	106
CD 2	N.D.	Y	QKGPPLPR	106
Connexin 43	Y	N.D.	PTAPLSP*	107
K-Channel v1.5	Y	N.D.	RPLPPLP*	108
K-Channel v1.3	Y	N.D	RYEPLPP*	108
RasGAP	Y	N.D.	PPLPPPPPQLP	109
Arrestin	N.D.	Y	PPAPEDKK…PPNLP	96
Sin	Y	Y	RPLPPPP	68
HIV-Nef	No	Y	RPQVPLPP	61
Sam68	Y	Y	PPLPHRSR	110
Herpesvirus TIP	Y?	Y	TPPLPPRP	111

SH3-mediated interactions have been demonstrated by mutation of the SH3 domain or by disruption of polyproline regions in the binding partner. Sequences in the protein necessary for binding are shown. An asterisk in the sequence indicates that there are predicted SH3 binding motifs in the protein, but they have not been shown experimentally to be the site of interaction. Proteins that are known substrates are shown, as well as proteins known to activate Src kinases by SH3 domain displacement. N.D.: not determined

phosphorylation *in vivo* is observed in a mutant lacking this polyproline (94). Peptide phosphorylation studies support this concept: introduction of a polyproline sequence into a peptide substrate of v-Src reduces the Km of the substrate by 3-fold (95). Thus, there is a sizable gain in phosphorylation efficiency when substrates possess SH3 domain binding motifs. As described above, polyproline sequences in substrates have dual functions: they serve as an anchors between the kinase and the substrate, and also activate the kinase by SH3 domain displacement (both of which promote efficient phosphorylation) (73). When both processes are combined, the overall increase in phosphorylation efficiency of a substrate such as Cas can be up to 10-fold (73).

Table 1 presents a list of proteins known to bind Src kinases though SH3-polyproline interactions. Some of the SH3-binding proteins in Table 1 possess polyproline regions that do not conform well to the consensus. For example, in the case of arrestin, two distant prolines had to be mutated in order to eliminate binding to c-Src (96). This raises the possibility that, for some proteins, SH3-interacting regions may be separated in the polypeptide chain of the protein. The crystal structure of p53 complexed with p53BP2 shows that prolines from different regions of p53 interact with the peptide-binding groove of the SH3 domain of p53BP2 (97).

The majority of the proteins listed in Table 1 are also phosphorylated by the kinase *in vivo* or *in vitro*. Many of the proteins have been proven to elevate kinase activity through the process of SH3 domain displacement. Overall, the existence of a Src SH3 domain-binding site (in many cases together with an SH2 binding sequence) may be a good predictor of whether the protein will be a substrate for Src kinases *in vivo*. It is likely that all Src family kinase substrates are phosphorylated by either an SH3 domain-dependent or an SH2 domain-dependent process. Substrates can possess the SH3/SH2 ligand within the same polypeptide, or, alternatively, an adapter molecule can act as a bridge between the kinase and the substrate. Substrates possessing multiple phosphorylation sites are often processively phosphorylated. Clearly, Src kinases and their substrates have co-evolved a mechanism in which the processes of kinase activation and substrate recognition and anchoring (via SH3/SH2 domains) are strongly coupled with each other. The coupling of Src activation to substrate recognition ensures that the kinases are released from their dormant, inactive state only at the right time and place, and in the presence of the appropriate cellular substrates.

If SH3/SH2 binding couples Src activation to substrate phosphorylation, how is the intial binding event regulated? In the case of SH2 domains, regulation can be achieved by phosphorylation or dephosphorylation of the SH2 binding sequence. Since unphosphorylated SH2 binding sequences do not bind SH2 domains, dephosphorylation of this sequence can serve to turn the switch off. Phosphorylation of the PDGF receptor by Src is a well-characterized example of SH2-mediated substrate control of phosphorylation. Binding of Src to autophosphorylation sites on PDGFR elevates kinase activity by SH2 domain displacement. Subsequently, Src phosphorylates tyrosine residues in the PDGFR proximal to the catalytic domain.

The basis for control of polyproline-SH3 interactions, however, is less apparent. Because SH3 displacement is a potent activation mechanism, constitutive binding of the Src SH3 domain to polyproline-containing

partners would presumably result in constitutively active Src kinases. Indeed, co-transfection of Nef and Hck leads to cellular transformation (98). Thus, the availability of the polyproline sequence to Src kinases must be a tightly regulated process. The accessibility of polyproline sequences may be regulated by upstream signal transduction processes. In this scenario, upon receiving a signal, a substrate could undergo a conformational change that exposes its polyproline sequence, recruiting and activating Src kinases, and therefore inducing its own phosphorylation. Phosphorylation of the substrate itself could result in a change in conformation of the substrate that makes its polyproline no longer accessible, turning the process off. Phosphorylation-dependent presentation of a polyproline sequence has been described for the SH3 domain containing protein p47phox and its binding partner p22phox, both part of the NADPH oxidase system. P47phox contains a polyproline intramolecularly bound to its own SH3 domain. Phosphorylation of three serine residues within the polyproline region disrupts this intramolecular interaction, freeing the SH3 domain to interact with p22phox and thereby activating the NADPH oxidase (99). A related example has been observed for Src kinases in the case of arrestin, a protein that is involved in terminating G-protein coupled receptor signaling (96). Several prolines in arrestin are involved in recruiting c-Src to the complex through an SH3 domain-dependent interaction. This SH3-polyproline interaction results in activation of c-Src. However, phosphorylation of arrestin on serine renders it unable to interact with c-Src. In general, SH3-binding sites have consensus sites for phosphorylation by proline-directed kinases, such as MAP kinase (100). In the case of arrestin, the phosphorylated serine is located at a site distant from its proline rich region, suggesting that phosphorylation indirectly affects presentation of the polyproline region. Other examples of regulation of SH3-polyproline binding by phosphorylation have been described for the Sos and Grb2 complex (101), and between the Wiskott-Aldrich syndrome protein WASP and the cytoskeletal protein PST-PIP (102). Thus, SH3 ligand formation or disassembly can also be a signal-dependent regulated process.

The potent combination of anchor-activator motif in substrates of Src-family kinases has likely evolved to maximize signaling efficiency *in vivo*. An understanding of the complex interactions between Src and its substrates may be essential in the design and implementation of novel strategies aimed at the inhibition of Src kinases *in vivo*.

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Abbreviations: SH: Src homology**,** PKA: protein kinase A, PDGFR: platelet derived growth factor receptor, CSK: C-terminal Src kinase, Cas: Crk associated substrate

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