Breakthroughs and Views

Src protein–tyrosine kinase structure and regulation ☆

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Abstract

Src and Src-family protein kinases are proto-oncogenes that play key roles in cell morphology, motility, proliferation, and survival. v-Src (a viral protein) is encoded by the chicken oncogene of Rous sarcoma virus, and Src (the cellular homologue) is encoded by a physiological gene, the first of the proto-oncogenes. From the N- to C-terminus, Src contains an N-terminal 14-carbon myristoyl group, a unique segment, an SH3 domain, an SH2 domain, a protein–tyrosine kinase domain, and a C-terminal regulatory tail. The chief phosphorylation sites of Src include tyrosine 416 that results in activation from autophosphorylation and tyrosine 527 that results in inhibition from phosphorylation by C-terminal Src kinase. In the restrained state, the SH2 domain forms a salt bridge with phosphotyrosine 527, and the SH3 domain binds to the kinase domain via a polyproline type II left-handed helix. The SH2 and SH3 domains occur on the backside of the kinase domain away from the active site where they stabilize a dormant enzyme conformation. Protein–tyrosine phosphatases such as PTPα displace phosphotyrosine 527 from the Src SH2 domain and mediate its dephosphorylation leading to Src kinase activation. C-terminal Src kinase consists of an SH3, SH2, and kinase domain; it lacks an N-terminal myristoyl group and a C-terminal regulatory tail. Its X-ray structure has been determined, and the SH2 lobe occupies a position that is entirely different from that of Src. Unlike Src, the C-terminal Src kinase SH2 and SH3 domains stabilize an active enzyme conformation. Amino acid residues in the αD helix near the catalytic loop in the large lobe of C-terminal Src kinase serve as a docking site for the physiological substrate (Src) but not for an artificial substrate (polyGlu$_4$Tyr).
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Src, a non-receptor protein–tyrosine kinase, has been the subject of intense investigation for decades. These studies stem from work on the Rous sarcoma virus, a chicken tumor virus discovered in 1911 by Peyton Rous [1]. v-Src (a viral protein) is encoded by the avian cancer-causing oncogene of Rous sarcoma virus, and Src (the cellular homologue in humans, chickens, and other animals) is encoded by a physiological gene, the first of the proto-oncogenes. In this review the normal cellular homologue is called Src (not c-Src), and the viral homologue is designated as v-Src.

Src is expressed ubiquitously; however, brain, osteoclasts, and platelets express 5–200-fold higher levels of this protein than most other cells [2]. Alternatively spliced forms of Src, which contain 6- or 11-amino acid insertions in the SH3 domain (18- or 33-nucleotide insertions between exons 3 and 4), are expressed in nerve cells. However, there is little evidence for a functional difference among Src splice variants [3]. In fibroblasts, Src is bound to endosomes, perinuclear membranes, secretory vesicles, and the cytoplasmic face of the plasma membrane where it can interact with a variety of

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growth factor and integrin receptors [2,3]. Src kinase activity is dormant during most of the cell cycle, but is transiently activated by cell-cycle-dependent C-terminal tail dephosphorylation and activation loop phosphorylation.

Protein kinases catalyze the following reaction:

$$MgATP^{1-} + protein–OH \rightarrow Protein–OPO_3^{2-} + MgADP + H^+$$

Based upon the nature of the phosphorylated –OH group, these enzymes are classified as protein–serine/threonine kinases and protein–tyrosine kinases. Manning et al. [4] identified 478 typical and 40 atypical protein kinase genes in humans (total 518) that correspond to about 1.7% of all human genes. The family includes 385 protein–serine/threonine kinases, 90 protein–tyrosine kinases, and 43 tyrosine-kinase like proteins. Of the 90 protein–tyrosine kinases, 58 are receptor and 32 are non-receptor enzymes. The protein kinase family is the second largest enzyme family, and the fifth largest gene family in humans. It follows C_2H_2 zinc finger proteins (3%), G-protein coupled receptors (2.8%), the major histocompatibility complex protein family (2.8%), and the protease enzyme family (1.9%) with 561 members [5,6].

There are 11 members of the Src-family kinases in humans [4]. These include Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes. Moreover, the human genome contains a Yes pseudogene (YESps). Src, Fyn, and Yes are expressed in all cell types [3]. Srm is found in keratinocytes, and Blk, Fgr, Hck, Lck, and Lyn are found primarily in hematopoietic cells. Frk occurs chiefly in bladder, brain, breast, colon, and lymphoid cells. Brk occurs chiefly in colon, prostate, and small intestine; however, it was initially isolated from a breast cell line.

Functions of Src

Src plays important roles in cell differentiation, proliferation, and survival [2,7,8]. For example, stimulation of the epidermal growth factor receptor, which leads to cell proliferation, results in Src activation. Src is activated during the G_2/M transition. The occurrence of Src in platelets (anucleate cells) and in neurons (which are postmitotic) indicates that Src participates in processes other than cell division.

Src plays a role in cell adhesion, cell morphology and motility, and bone resorption. Focal adhesion kinase (FAK) and the Crk and Src associated substrate (Cas) are focal adhesion proteins important for integrin signaling and are substrates of Src. Mice with *Src−/−* null mutations die within the first few weeks of birth. One phenotype of Src-deficient mice is osteopetrosis (an increase in bone density) that results from a defect in osteoclasts (cells that resorb bone) [1]. Src protein kinase inhibitors thus represent a drug target for osteoporosis, which results from excessive bone resorption.

Src-family kinases are controlled by receptor protein–tyrosine kinases, integrin receptors, G-protein coupled receptors, antigen- and Fc-coupled receptors, cytokine receptors, and steroid hormone receptors [3]. Src signals to a variety of downstream effectors including Stat transcription factors. Signal transduction pathways intersect in various ways in v-Src transformed cells. For example, v-Src leads to a decrease in the expression of type I, but not type II, protein kinase A [9].

v-Src was initially described as a cancer-causing gene, and considerable work has been performed relating aberrant Src activity to human cancers. Src protein–tyrosine kinase activity is elevated in several types of human cancer, and this has been attributed to both elevated Src expression and increased specific activity. Members of the epidermal growth factor receptor family are active in breast cancer cells, and these activated receptors may lead to activation of Src in the production of neoplasms. Src is also activated in colon, gastric, lung, pancreatic, neural, and ovarian neoplasms [7,8,10].

Organization of Src protein–tyrosine kinase

From the N- to C-terminus, Src contains a 14-carbon myristoyl group attached to an SH4 domain, a unique domain, an SH3 domain, an SH2 domain, an SH2-kinase linker, a protein–tyrosine kinase domain (the SH1 domain), and a C-terminal regulatory segment (Fig. 1) [2]. During biosynthesis, the amino-terminal methionine is removed, and the resulting amino-terminal glycine becomes myristoylated following a reaction with myristoyl-CoA.

Myristoylation facilitates the attachment of Src to membranes, and myristoylation is required for its operation in cells [2]. The seven terminal amino acids beginning with glycine are required for the myristoylation of Src and v-Src [11]. Mutational studies show that a correlation exists between N-myristoylation, subsequent membrane association, and the ability of v-Src protein kinase to transform cells into a neoplastic state. The catalytic subunit of the serine/threonine protein kinase A and the Abl non-receptor protein–tyrosine kinase are myristoylated, but they are largely cytosolic [12,13]. Myristoylation is thereby not sufficient to ensure membrane localization of Src and v-Src. Many have hypothesized that there is a specific binding protein for the amino terminus of Src, but this hypothetical docking protein has not been identified.
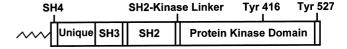

Fig. 1. Organization of Src. Except for the aliphatic myristoyl group attached to the SH4 domain, the relative length of the domains is to scale. The chicken numbering system is displayed.

SH3 domains (≈60 amino acid residues) bind to sequences that can adopt a left-handed helical conformation. These sequences are usually rich in proline. RPLPPLP is an optimal Class 1 Src SH3 ligand [2,14]. ΦPPLPXR is an optimal Class 2 Src SH3 ligand that binds with an inverted orientation on the SH3 surface (Φ represents a hydrophobic residue and X represents any amino acid). The sequence specificity is low, and amino acid substitutions result in small decreases in binding affinity.

SH2 domains (≈100 amino acid residues) bind to distinct amino acid sequences C-terminal to the phosphotyrosine. Songyang and Cantley analyzed the binding of a library of phosphopeptides to SH2 domains to define preferred docking sequences [15]. The SH2 domains of Fgr, Fyn, Lck, and Src select pYEEI in preference to other sequences. In contrast, the Grb2 SH2 domain binds preferentially to pYQNY/Q peptides.

X-ray crystallographic studies of the Src SH2 domain indicate that the phosphotyrosine ligand binds to an invariant arginine, and the isoleucine at the P + 3 position binds to a hydrophobic pocket [16]. The acidic residues at the pY + 1 and pY + 2 positions occur on the surface of the SH2 domain and point toward basic residues on the surface. The Src SH2 domain, however, can bind to a variety of sequences that do not conform to this optimal sequence, and other parts of proteins beyond the vicinity of the phosphotyrosine contribute to binding affinity.

The human Src gene encodes a protein of 536 amino acids, and the chicken Src gene encodes a 533-residue protein. The three additional amino acids in humans are inserted near the amino terminus. For historical reasons, the chicken numbering system is used in most of the literature, even when studies were performed with the human enzyme. The chicken viral oncogene contains 7 fewer amino acids than its physiological proto-oncogene.

One of the two most important regulatory phosphorylation sites in Src is Tyr527, six residues from the C-terminus. Under basal conditions in vivo, 90–95% of Src is phosphorylated at Tyr527 [17], and phosphotyrosine 527 binds intramolecularly with the Src SH2 domain. This intramolecular association stabilizes a restrained form of the enzyme [2]. In the restrained enzyme, neither the SH2 nor the SH3 domain is readily accessible to external ligands. The Tyr527Phe mutant is more active than the wild type enzyme and can induce anchorage-independent growth in vitro and tumors in vivo [18,19]. Tyr527 phosphorylation results from the action of other protein–tyrosine kinases including Csk and Chk [20,21]. Src undergoes an intermolecular autophosphorylation at tyrosine 416; this residue is present in the activation loop, and its phosphorylation promotes kinase activity [2].

The SH2 and SH3 domains have four important functions [2]. First, they constrain the activity of the enzyme via intramolecular contacts. Second, proteins that contain SH2 or SH3 ligands can bind to the SH2 or SH3 domains of Src and attract them to specific cellular locations. Third, as a result of displacing the intramolecular SH2 or SH3 domains, proteins can activate Src kinase activity. And fourth, proteins containing SH2 or SH3 ligands can enhance their ability to function as substrates for Src protein–tyrosine kinase. Other Src-family kinase members also contain an N-terminal myristoyl group, a unique segment, an SH3 domain, an SH2 domain, a kinase domain, and a C-terminal regulatory tail [3].

The intramolecular interactions that maintain Src-family kinases in the inactive state are of relatively low affinity. For example, the SH2 domain ligand in the C-terminal regulatory tail of Hck (pYQQQ) does not conform to the sequence of the high-affinity SH2 ligand pYEEI. However, the intramolecular ligands for the SH2 and SH3 domains possess an entropic advantage in binding because they are on the same polypeptide. The intramolecular interactions in Src-family kinases must be strong enough to maintain the catalytic domain in an inactive conformation, but weak enough to allow for activation by exogenous ligands. Porter and co-workers generated a mutant form of Hck with the high-affinity pYEEI in the C-terminal tail [22]. They found that this mutant cannot be activated by exogenous SH2 ligands.

Structure of Src protein kinase

The Src kinase domain consists of the characteristic bilobed protein kinase architecture [23–26]. Residues 267–337 make up the small amino-terminal lobe of the kinase; residues 341–520 make up the large carboxyl-terminal lobe (Fig. 2). The smaller amino-terminal lobe of protein kinases is primarily involved in anchoring and orienting ATP; the G-rich loop forms part of the nucleotide-phosphate binding site. This smaller lobe has a predominantly antiparallel β-sheet structure. The larger carboxyl-terminal lobe is responsible for binding the protein substrate. However, part of the ATP-binding site occurs in the large lobe. The large lobe is predominantly α-helical in nature. As described for protein kinase A [27], the catalytic site of Src lies in a cleft between the two lobes. The two lobes move relative to each other and can open or close the cleft. The open form is necessary to allow access of ATP to the catalytic site and release of ADP; the closed form is necessary to bring residues into the catalytically active state. Any process that can block the interconversion of the open and closed forms of the cleft will be inhibitory. Moreover, active site residues are derived from both the small and large lobes of the kinase, and changes in the orientation of the two lobes can promote or restrain activity.

Hanks et al. [28] identified 12 subdomains with conserved amino acid residue signatures that constitute the catalytic core of protein kinases. Of these, the three following residues, which constitute a K/D/D motif,
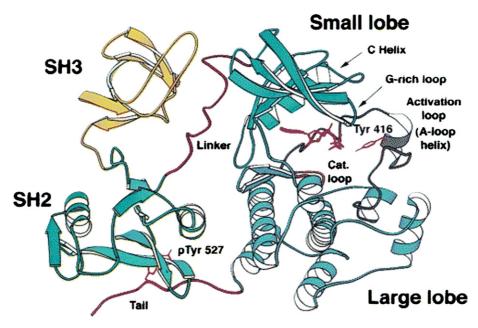
Fig. 2. Ribbon diagram illustrating the structure of human Src. AMP-PNP (red) is bound in the active site. The A loop helix occurs between the small and large lobes of the kinase and sequesters Tyr416. This figure is reproduced from [26] with copyright permission from Elsevier.
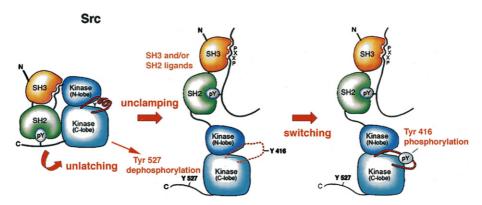

Fig. 3. Src activation by unlatching, unclamping, and switching. This figure is reproduced from [30] with copyright permission from Elsevier.

illustrate the inferred catalytic properties of Src. Lys295 represents an invariant residue of protein kinases that forms ion pairs with the α- and β-phosphates of ATP (Table 1). Asp386 orients the tyrosyl group of the substrate protein in a catalytically competent state. Asp386 may function as a base that abstracts a proton from tyrosine thereby facilitating its nucleophilic attack of the γ-phosphorus atom of MgATP; Asp386 is called the catalytic base. This base occurs in the catalytic loop (Fig. 2) that generally has the sequence HRDLRAAN in non-receptor protein–tyrosine kinases including Src. Asp404 is the first residue of the activation loop found in the large lobe. Asp404 binds Mg^{2+} or Mn^{2+}, which in turn coordinates the β- and γ-phosphate groups of ATP. Mg^{2+} or Mn^{2+} supports catalysis by the Src-family protein kinases, but Mg^{2+} is the likely physiological cation.

The small and large lobes can adopt a range of relative orientations, opening or closing the active site cleft

[26,29]. Within each lobe is a polypeptide segment that has an active and a restrained conformation. In the small lobe, this segment is the major α-helix designated as the αC-helix (it is preceded by minor A and B helices in protein kinase A) [27]. The αC-helix in some kinases rotates and translates with respect to the rest of the lobe, making or breaking part of the active catalytic site. In the large lobe, the activation loop adjusts to make or break part of the catalytic site. In most kinases, phosphorylation of the activation loop stabilizes the active conformation; this corresponds to Tyr416 in Src. Src kinase and members of the Src-kinase family thus have two important phosphorylation sites: phosphorylation of Tyr416 is stimulatory and phosphorylation of Tyr527 is inhibitory [2]. The amino acids at the C-terminal tail of v-Src are missing, and the absence of the inhibitory phosphotyrosine from this segment results in a constitutively active form of the enzyme.

Table 1
Important amino acid residues in Src and Csk protein kinases

Residue or motif	Chicken Src	Chicken v-Src[a]	Human Src	Human Csk
N-Myristoyl glycine	2	2	2	None
SH3 domain	81–142	81–142	84–145	9–70
SH2 domain	148–245	148–245	151–248	82–171
SH2-kinase linker	246–266	246–266	249–269	172–194
Protein–tyrosine kinase domain	267–520	267–517	270–523	195–449
Glycine-rich nucleotide-binding site	273–281	273–281	276–284	201–209
Phosphate-binding lysine	295	295	298	222
αC helix glutamate	310	310	313	236
Catalytic aspartate	386	386	389	314
Activation loop DFG	404–406	404–406	407–409	332–334
Activation loop APE	430–432	430–432	433–435	354–356
Stimulatory activation loop phosphotyrosine	416	416	419	None
Inhibitory tail phosphotyrosine	527	None	530	None
Total number of encoded amino acids	533	526	536	450
Swiss-Prot Accession No.	P00523	P00524	P12931	P41240

[a] Schmidt-Rupin strain.

The structures of dormant, or restrained, human and chicken Src kinases and human Hyk, a Src-kinase family member, have been solved by X-ray crystallography [23–26]. The conformation of the activation loop differs between active and inactive kinases [29]. The activation loop of nearly all protein kinases begins with DFG and ends with APE. In protein kinases that are dormant, the activation loop has various compact conformations. In structures of enzymes that are in an active state, the activation loop is in an extended conformation. There are two crucial aspects to this active conformation. First, the aspartate residue (Asp404 in Src) within the conserved DFG motif at the amino-terminal base of the activation loop binds to the magnesium ion as noted above. Second, the rest of the loop is positioned away from the catalytic center in an extended conformation so that the C-terminal portion of the activation loop provides a platform for protein substrate binding.

In dormant Src kinase, residues 413–418 of the activation loop form a short α-helix (the A loop helix) between the small and large lobes of the kinase domain (Fig. 2). As a result, the A loop helix buries the side chain of Tyr416 (the site of activating phosphorylation) between the lobes of the kinase domain [26]. Residues 404–411 of the activation loop push the αC-helix out of its active state so that Glu310 in the helix cannot form a critical salt bridge with Lys295 (Table 1), and the enzyme is inactive. This A loop helix is an important autoinhibitory component. The helix precludes protein/peptide substrate recognition, it sequesters Tyr416, and it stabilizes the inactive conformation of the kinase domain [26].

Intramolecular regulation of Src kinase activity

Mutations in the SH3 and SH2 domains usually activate Src kinase; the Tyr527Phe mutation also promotes Src enzyme activity [2]. It was surmised that the intramolecular contacts of the SH2 and SH3 domains directly block the active site of the enzyme. However, its three dimensional structure showed that the inhibitory effects of the two domains are indirect. The SH3 and SH2 domains occur on the backside of the kinase domain (Fig. 2). Moreover, the SH2 domain binds to phosphotyrosine 527, which is 40 Å from the active site.

The apparatus controlling Src has three components that Harrison calls the latch, the clamp, and the switch [30]. The SH2 domain binds to phosphotyrosine 527 in the C-terminal tail to form the latch, and the latch stabilizes the attachment of the SH2 domain to the large lobe. The SH3 domain contacts the small lobe (Fig. 3). The linker between the SH2 and kinase domains contains proline at positions 246, 250, and 263 that function as a motif that binds the SH3 domain and attaches ("glues") the SH3 domain to the small kinase lobe. The linker does not resemble the classical PXXP motif [14], but this stretch of residues readily forms a left-handed (polyproline type II) helix. Prior to the determination of the three-dimensional structure of Src, investigators looked unsuccessfully for a Src sequence that could bind to an SH3 domain. The clamp is an assembly of the SH2 and SH3 domains behind the kinase domain. As a result of clamping the SH2 and SH3 domains to the kinase domain, helix αC is displaced along with the critical Glu310. Moreover, the clamp prevents the opening and closing of the cleft between the small and large lobes. The switch is the kinase-domain activation loop; the activation loop can switch between active and inactive conformations as described above.

The crystal structure represents a static form of Src. An equilibrium exists between the restrained and active forms of the enzyme. Since Src activity is strictly regulated, the equilibrium favors the inactive bound conformation. The dormant form of the enzyme is destabilized by dephosphorylation of Tyr527 and by phosphorylation of the activation loop Tyr416.

In the inactive state, Tyr 416, which occurs in the activation loop, is sequestered and is not a substrate for phosphorylation by another kinase. When phosphotyrosine 527 dissociates or is displaced from the SH2-binding pocket, the protein is unlatched and the clamp no longer locks the catalytic domain in an inactive conformation [26,30]. Furthermore, dissociation of phosphotyrosine 527 may allow dephosphorylation by enzymes such as protein tyrosine phosphatase-α (PTPα) [2], and the αC-helix and the activation loop can assume their active conformations. Tyr416 can then undergo autophosphorylation by another Src kinase molecule. Following autophosphorylation, the enzyme is stabilized in its active state. Exogenous substrates decrease autophosphorylation in vitro during activity measurements [31]. This finding is consistent with the notion that activation loop phosphorylation occurs in *trans*, and this process is inhibited by competition with exogenous substrates. Whether inhibition of activation loop phosphorylation by exogenous substrates occurs in vivo remains to be established.

This structural design allows for Src regulation at multiple levels including competition between intramolecular and external ligands [2]. The intramolecular interactions maintain an inactive state, and external ligands promote an active state. Proteins that bind to the SH2 domain of Src disrupt the clamp, activating the kinase. Similarly, proteins that bind to the SH3 domain of Src domain also disrupt the clamp, activating the enzyme (Fig. 3).

There are four possible Src enzyme forms: (i) nonphosphorylated, (ii) tyrosine 527 phosphorylated, (iii) tyrosine 416 phosphorylated, and (iv) both tyrosine 527 and 416 phosphorylated enzymes. Only the structure of the tyrosine 527 phosphorylated enzyme has been determined, and this inhibited species is the predominant form in cells. Src with phosphorylated tyrosine 527 cannot undergo autophosphorylation; the residue must first be dephosphorylated. However, Src with tyrosine 416 autophosphorylation is a substrate for C-terminal Src kinase, but the doubly phosphorylated enzyme is active, so that tyrosine 416 phosphorylation overrides inhibition produced by tyrosine 527 phosphorylation [32].

Regulation of Src activity by Csk (C-terminal Src kinase or c-Src kinase)

Src and members of the Src-family of protein kinases are rigidly regulated and occur in vivo in a restrained state in which the phosphorylated C-terminal regulatory tyrosine binds intramolecularly to the SH2 domain. Csk, a cytoplasmic protein–tyrosine kinase, controls the phosphorylation of the regulatory tyrosine. Okada and Nakagawa [20] isolated this enzyme from neonatal rat brain and demonstrated that it catalyzes the phosphorylation of Src at Tyr527. Following phosphorylation, they showed that the K_m of Src for ATP and for acid-denatured enolase is unchanged, but the V_{max} is decreased 50%. Using purified Src, the activity of the Tyr527 phosphorylated enzyme in vitro ranges from 2% to 20% of the unphosphorylated enzyme depending upon the experimental conditions.

Wang et al. [33] performed site-specific mutagenesis to address the selectivity and specificity of Csk for Src. As parent enzyme, they used Src kinase that had a Lys295Met mutation in the catalytic domain that abolishes its kinase activity. This strategy avoids the complication of non-Csk catalyzed phosphorylation. Mutation of the Src SH3 and SH2 domains does not alter the activity of Csk for Src. The sequence surrounding Tyr 527 is EPQYQPGEN (residues 524–532). A Glu524Ala mutation markedly reduces the ability of Src to serve as substrate. Csk requires glutamate at residue 524; even Glu524Asp and Glu524Gln mutants are unable to serve as good substrates. Furthermore, the Gln526Ala mutant is a poor substrate for Csk, whereas the Gln526Ile mutant is a good substrate. Mutation of residue 525 and each of the residues from 528 to 532 to alanine yields proteins that are good substrates for Csk.

Takeuchi et al. [34] described a transmembrane Cbp (Csk binding protein) that can recruit Csk to the membrane where Src and other Src-kinase family members are localized. Phosphorylated (but not unphosphorylated) Cbp not only attracts Csk, but it activates the kinase by reducing the K_m of Csk for Src to 1/6th of the normal value. The Csk-Cbp complex has greater affinity for Src than free Csk. Cbp is involved in the regulation of Csk by both recruiting the enzyme to the membrane and promoting its catalytic activity.

Structure of Csk

The Csk crystals produced by Ogawa et al. [35] contain three pairs of dimers per asymmetric unit. Two pairs have an active conformation, and one pair has an inactive conformation. The three-dimensional structure of Csk shows that the protein is organized differently from Src and other Src-family members. The SH2 and SH3 domains are diametrically opposite on the top of the small lobe of the kinase domain. The position of the SH3 domain resembles that of the SH3 domain of inactive Src, but the position of the SH2 domain is entirely different (Fig. 4). Moreover, there is no direct contact between the SH2 and SH3 domains in Csk as there is in Src [35].

The peptide-binding pockets of the Csk SH2 and SH3 domains are oriented outward permitting intermolecular interactions. There is no interaction between the SH3 domain and the SH2-kinase linker as is found in the
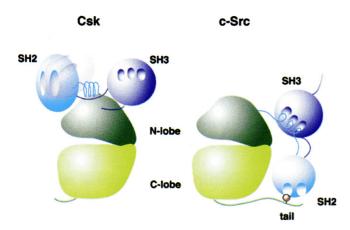
Fig. 4. Comparison of the domain structures of Csk and Src. The indentations represent peptide binding pockets of the SH2 and SH3 domains. The phosphate in the C-terminal tail of Src is pink. The SH2-kinase linkers are light blue, and the SH3–SH2 connectors are gray. The figure is reproduced from [35] with copyright permission from the Journal of Biological Chemistry.

Src-family kinases. In the unrestrained state, the SH2-kinase linker and the SH3–SH2 connector interact with the small Csk kinase lobe and stabilize the active conformation.

The positions of the important catalytic residues of four molecules in the asymmetric unit assume the active conformation. The K/D/D motif in Csk corresponds to Lys222, Asp314, and Asp332. Lys222 forms a critical salt bridge with Glu236 of the αC helix in the active

state. Asp314 is the catalytic base that occurs in the catalytic loop of the kinase (Table 1). Most non-receptor protein–tyrosine kinases such as Src contain HRDLRAAN in the catalytic loop, and most receptor protein–tyrosine kinases contain HRDLAARN. Curiously, Csk (a non-receptor kinase) contains the latter sequence. Asp332, which occurs at the beginning of the activation loop, binds Mg^{2+}.

The similar positioning of the important catalytic residues is remarkable owing to the differences in the activation loop of Csk and other protein kinases such as Src. The activation loop of Csk (332–356) is four residues shorter than the activation loop of Src (406–434), and that of Csk lacks a tyrosine. In Src and members of the Src-kinase family, autophosphorylation of the activation loop is essential for full catalytic activity. v-Src also requires an activation loop tyrosine for its transforming, or tumorigenic, activity. Instead of an activating phosphotyrosine in Csk, the interactions of the kinase domain, the SH3–SH2 connector, and the SH2-kinase linker stabilize the active conformation. In the two molecules with the restrained conformation in the asymmetric unit cells, the critical salt bridge between Glu236 and Lys222 is absent.

One can hypothesize that there is an equilibrium between the restrained and unrestrained conformations of Csk. It is possible that ligands for the Csk SH2 and SH3 domains can stabilize the unrestrained conformation. The regulation of Csk activity differs from those
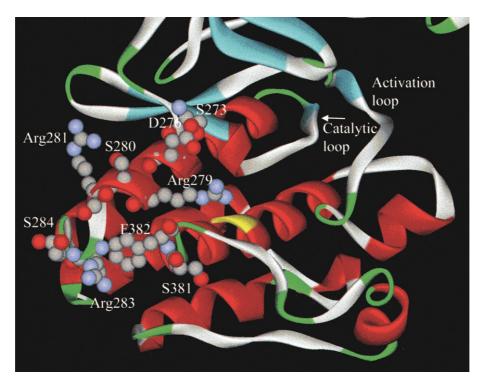

Fig. 5. Location of important substrate docking residues in the large protein kinase lobe of Csk. Ser273, Arg279, Ser280, Arg281, and Arg283 are located in the αD helix, and Phe382 is next to the helix in the tertiary structure. The figure is reproduced from [39] with copyright permission from the National Academy of Sciences, USA.

of Src and the Src-family kinases owing to the absence of C-terminal tail inhibitory phosphorylation (corresponding to Tyr527 of Src) and activating autophosphorylation (corresponding to Tyr416 of Src).

The Csk activation loop

In protein kinases that are regulated by autophosphorylation, the phosphotyrosine in the activation loop is stabilized by interaction with two arginine residues—one in the activation loop and the other in the catalytic loop. In active Src, this phosphotyrosine (Tyr416) is predicted to coordinate with Arg385 and Arg409, and thus stabilize the active enzyme. In Csk, however, the side chain of Lys337, which corresponds to Arg409 of Src, has no apparent coordination to a specific partner, yet the segment assumes an active enzyme conformation. The Lys337Ala mutation results in an enzyme that retains about 80% of its activity toward Src and polyGlu$_4$Tyr [36], where the latter is a random copolymer with a ratio of glutamate to tyrosine of 4:1. The catalytic loop arginine in Csk is Arg313. The Arg313Ala mutation retains full kinase activity. This contrasts with Src where each of these mutations decreases activity to less than 0.1% of the wild type enzyme.

Replacement of the Csk loop with the Src activation loop yields an enzyme with 80% of the activity with polyGlu$_4$Tyr and 130% with Src [36]. This mutant enzyme does not undergo autophosphorylation. However, incubation of the Csk mutant containing the Src activation loop yields a protein that undergoes phosphorylation by wild type Src. This reaction is consistent with the *trans* phosphorylation mechanism for Src autophosphorylation. Activation loop phosphorylation in the mutant Csk has no discernable effect on enzyme activity. It seems likely that replacement of the activation loop of Src with that of Csk would decrease Src kinase activity owing to the elimination of the tyrosine phosphorylation site.

The replacement of all 11 activation loop residues of Csk with glycine results in an enzyme that exhibits 1% of wild type activity toward polyGlu$_4$Tyr and 14% toward Src [36]. Deletion of the activation loop alanine (ΔA339) gives similar results. Although activation loop mutations give different results with physiological and artificial substrates, the K_m values are altered much less than the k_{cat} values. When a thrombin cleavage site (LVPRGS) was substituted for ASSTQD in the activation loop, the enzyme retains 2% of its activity toward polyGlu$_4$Tyr and 5% of its activity toward Src. When the mutant activation loop is cleaved by thrombin, enzyme activity is completely restored. Proteolysis of the mutant activation loop thus enables an inactive enzyme conformation to assume an active state. Based upon the three-dimensional structure of Csk, Lin et al. [36] suggest that hydrophobic interactions involving helices αE

(from the 36 kDa chain) and αF and αI (from the 12 kDa chain) hold the two enzyme fragments together to enable catalysis.

C-terminal Src kinase (Csk)-homologous kinase

Chk catalyzes the phosphorylation of the inhibitory tyrosine in the Src-family kinases (Tyr527 of Src) [21,37]. Csk is expressed in all mammalian cells, whereas Chk expression is limited to breast, hematopoietic cells, neurons, and testes [2]. Like Csk, Chk consists of an SH3, SH2, and kinase domain. Besides inactivating Src by catalytic phosphorylation, Chk forms an inhibitory non-covalent complex with Src. The association of Chk with the activated and autophosphorylated form of Src inhibits Src kinase activity [37]. The action of Chk thereby overrides that of Src. Chk can also bind to unphosphorylated Src and prevent its autophosphorylation.

The substrate-docking site of C-terminal Src kinase

The C-terminal tails of Src-family kinases have a consensus sequence of TATEXQYQXQ/G where the X represents a variable residue. Early efforts to understand Csk substrate specificity relied on peptides based upon this consensus sequence. However, these peptides exhibit 1/1000th the substrate activity of Src. Using a random peptide library, Sondhi et al. [38] identified EEEIIYFFF as an optimal substrate for C-terminal Src kinase (Csk). This substrate is nearly as efficient as Src, but this sequence bears little resemblance to the physiological phosphorylation site. These studies suggest that contacts between Csk and Src in addition to the C-terminal tail are important for substrate recognition.

Lee et al. [39] performed site-specific mutagenesis to determine the substrate-docking site of Csk. Initial work indicated that Arg279, Arg281, and Arg283 are important. These residues were chosen for this study because they are conserved in Csk and Chk. Mutation of these residues to alanine decreases the ability of kinase defective Src (Lys295Met) to serve as substrate without significantly decreasing Csk kinase activity with polyGlu$_4$Tyr. These residues form a triangle and are located in the αD helix in the C-terminal lobe (Fig. 5). Based upon the three-dimensional structure of Csk, they modified residues near the arginine triangle, and identified Ser273, Ser280, and Phe382 as additional residues that constitute the docking site. Most of these residues are conserved in Csk and Chk, the only two enzymes thought to catalyze the phosphorylation of the C-terminal tail tyrosine of Src-family kinases.

Double and quadruple mutants of Csk catalyze the phosphorylation of kinase defective and wild type Src

at only 5% of the efficiency as wild type Csk, whereas mutants are able to phosphorylate polyGlu$_4$Tyr with only a modest decrease in activity [39]. While wild type Csk binds Src tightly in the absence of catalysis, the loss of Csk activity correlates with decreased binding to Src. The docking site is located near the active site of Csk, but it is not clear which parts of Src interact with the docking residues. Site-specific mutations of Src that decrease binding to wild type Csk may aid in identifying the residues that participate in docking. Furthermore, a peptide mimicking the docking site of Csk inhibits the phosphorylation of Src much more efficiently ($IC_{50} = 21\,\mu M$) than it inhibits phosphorylation of the artificial substrate ($IC_{50} = 422\,\mu M$).

The structure of the αD helix is highly variable among protein–tyrosine kinase members, and it is possible that this portion of the enzyme is important for substrate selection by other kinases. This hypothesis warrants testing with other protein–tyrosine kinases. One puzzling result of this work is that docking mutations affect the k_{cat} more than the K_m. Lee and co-workers suggest that the docking interaction is critical for the Csk transition state complementarity [39].

Epilogue

Protein kinases participate in cell signaling and the regulation of many biochemical activities. Second messengers such as cAMP, Ca^{2+}, and diacylglycerol activate their cognate protein kinases. Epidermal growth factor, transforming growth factor-α, and other factors activate the epidermal growth factor receptor protein kinase [40]. The epidermal growth factor receptor was the first receptor protein–tyrosine kinase and Src was the first non-receptor protein–tyrosine kinase to be described. The receptor for transforming growth factor-β is a protein–serine/threonine kinase. Specific regulatory molecules activate these kinases, and a comparison of enzymes with and without the activating compound aids in enzyme characterization. In contrast, non-receptor kinases and kinases without an activating compound or protein are more difficult to delineate.

There are major differences in substrate recognition between protein–serine/threonine kinases and protein–tyrosine kinases. Protein kinase A, for example, shows high specificity for short linear peptide sequences that correlate well with sequences of residues that are naturally phosphorylated in proteins. Studies with short peptides that correspond to the phosphorylation site of liver pyruvate kinase (LRRASLG) indicate that basic side chains at P-2 and P-3 (relative to serine) are important substrate determinants [41]. In contrast, there is significant disparity between peptide site phosphorylation selectivity and protein site phosphorylation specificity for Src and other protein–tyrosine kinases. Using pep-

tide libraries, Src prefers EEIYGEF as substrate [15]. However, these preferences are not very stringent, and protein–tyrosine kinases can tolerate amino acid substitutions without drastic consequences. While the catalytic domains target certain sequences, the substrates for a given kinase may differ in their primary structure while substrates for different kinases can be similar. Other determinants beside the local primary structure are required to explain the specificity of protein–tyrosine kinase signaling pathways in vivo.

Protein–tyrosine kinases are found in animals. They are lacking in bacteria, yeast, and plants. Tyrosine phosphorylation is regulated by the balanced action of kinases and protein–tyrosine phosphatases. The specific activity of protein–tyrosine phosphatases is 10–1000 that of protein kinases in vitro [42]. Although protein–tyrosine phosphatases play an active role in setting the levels of tyrosine phosphorylation, we know considerably less about the mechanisms for regulating phosphatase activity.

Joan Brugge and Ray Erikson [43] demonstrated that v-Src was a phosphoprotein with a molecular mass of 60 kDa. Early work indicated that v-Src was a protein kinase [44,45]. Initially it was thought to be a protein–threonine kinase, but Tony Hunter and Bartholomew Sefton showed that v-Src was a protein–tyrosine kinase, the first member of this class of enzymes [46]. Moreover, the characterization of SH2 and SH3 domains of Src has provided insights into modular protein–protein interactions that have wide application and have greatly advanced the field of signal transduction. Michael Bishop, Harold Varmus, and co-workers demonstrated that uninfected cells possess a protein homologous to v-Src called c-Src (pp60$^{c\text{-src}}$, or Src), and Src represents the first proto-oncogene [47]. Subsequent work indicated that there are a bevy of oncogenes that are derived from their normal cellular homologue or proto-oncogene [1,3]. The work that Peyton Rous initiated nearly a century ago on a breast muscle tumor, or sarcoma, in a Plymouth Rock chicken has paved the way to fundamental discoveries on the cell cycle, cell growth and death, cell–cell signaling, cell morphology and motility, and cancer biology.

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