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Invited Review

Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors

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This paper is dedicated to the memory of Prof. Donald F. Steiner (1930–2014) – advisor, mentor, and discoverer of proinsulin.

Chemical compounds studied in this article:
Bosutinib (PubMed CID: 5328940)
Dasatinib (PubMed CID: 3062316)
Ponatinib (PubMed CID: 24826799)
Saracatinib (PubMed CID: 10302451)
Vandetanib (PubMed CID: 3062316)

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ABSTRACT

The physiological Src proto-oncogene is a protein-tyrosine kinase that plays key roles in cell growth, division, migration, and survival signaling pathways. From the N- to C-terminus, Src contains a unique domain, an SH3 domain, an SH2 domain, a protein-tyrosine kinase domain, and a regulatory tail. The chief phosphorylation sites of human Src include an activating pTyr419 that results from phosphorylation in the kinase domain by an adjacent Src molecule and an inhibitory pTyr530 in the regulatory tail that results from phosphorylation by C-terminal Src kinase (Csk) or Chk (Csk homologous kinase). The oncogenic Rous sarcoma viral protein lacks the equivalent of Tyr530 and is constitutively activated. Inactive Src is stabilized by SH2 and SH3 domains on the rear of the kinase domain where they form an immobilizing and inhibitory clamp. Protein kinases including Src contain hydrophobic regulatory and catalytic spines and collateral shell residues that are required to assemble the active enzyme. In the inactive enzyme, the regulatory spine contains a kink or a discontinuity with a structure that is incompatible with catalysis. The conversion of inactive to active Src is accompanied by electrostatic exchanges involving the breaking and making of distinct sets of kinase domain salt bridges and hydrogen bonds. Src-catalyzed protein phosphorylation requires the participation of two Mg^{2+} ions. Although nearly all protein kinases possess a common K/E/D/D signature, each enzyme exhibits its unique variations of the protein-kinase reaction template. Bosutinib, dasatinib, and ponatinib are Src/multikinase inhibitors that are approved by the FDA for the treatment of chronic myelogenous leukemia and vandetanib is approved for the treatment of medullary thyroid cancer. The Src and BCR-Abl inhibitors saracatinib and AZD0424, along with the previous four drugs, are in clinical trials for a variety of solid tumors including breast and lung cancers. Both ATP and targeted therapeutic Src protein kinase inhibitors such as dasatinib and ponatinib make hydrophobic contacts with catalytic spine residues and form hydrogen bonds with hinge residues connecting the small and large kinase lobes.

Contents

Abbreviations: AL, activation loop; ALL, acute lymphoblastic leukemia; AS, activation segment; CDK, cyclin-dependent kinase; Chk, Csk homologous kinase; CML, chronic myelogenous leukemia; Csk, C-terminal Src kinase; C-spine, catalytic spine; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated protein kinase; FGFR, fibroblast growth factor receptor; GIST, gastrointestinal stromal tumor; HGFR or c-Met, hepatic growth factor receptor; HΦ, hydrophobic; IGFR, insulin-like growth factor receptor; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; Ph^+, Philadelphia chromosome positive; PKA, protein kinase A; PTP, protein-tyrosine phosphatase; pTyr or pY, phosphotyrosine; R-spine, regulatory spine; Sh, shell; SH1/2/3, Src homology 1/2/3; VEGFR, vascular endothelial growth factor receptor.

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Introduction

Src, a non-receptor protein-tyrosine kinase, has been the subject of intense investigation for three decades owing in part to its association with malignant transformation and oncogenesis. 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. In contrast, Src (the normal cellular homologue) is encoded by a physiological gene, the first proto-oncogene to be described and characterized [2].

Src is expressed ubiquitously in vertebrate cells; however, brain, osteoclasts, and platelets express 5–200 fold higher levels of this protein than most other cells [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 growth factor, integrin, and G-protein-coupled receptors and serve as an essential intermediary in signal transduction [3,4]. The expression of high levels of Src in platelets (anucleate cells) and in neurons (which are postmitotic) indicates that Src participates in processes other than cell division [3].

Protein kinases including Src catalyze the following reaction:

$$MgATP^{1-} + \text{protein–O : H} \rightarrow \text{protein–O : } PO_3{}^{2-} + MgADP + H^+$$

Note that the phosphoryl group ($PO_3{}^{2-}$) and not the phosphate ($OPO_3{}^{2-}$) group is transferred from ATP to the protein substrate. Divalent cations such as Mg^{2+} are required for the reaction. Based upon the nature of the phosphorylated —OH group, these enzymes are classified as protein-tyrosine, protein-serine/threonine, or dual specificity protein-tyrosine/threonine kinases.

Manning et a. identified 478 typical and 40 atypical protein kinase genes in humans (total 518) [5]. The family includes 90 protein-tyrosine kinases, 43 tyrosine-kinase like proteins, and 385 protein-serine/threonine kinases. Of the 90 protein-tyrosine kinases, 58 are receptor and 32 are non-receptor enzymes including Src. A small group of dual-specificity kinases including MEK1 and MEK2 catalyze the phosphorylation of both tyrosine and threonine in target proteins; dual-specificity kinases possess molecular features that place them within the protein-serine/threonine kinase family [6].

Protein phosphorylation is the most widespread class of post-translational modification used in signal transduction [5]. Families of protein phosphatases catalyze the dephosphorylation of proteins thus making phosphorylation-dephosphorylation an overall reversible process [7]. Protein kinases play a predominant regulatory role in nearly every aspect of cell biology [5]. They regulate apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation, development, the immune response, nervous system function, and transcription. Src and the Src family kinases have been implicated in each of these processes [3,4]. Moreover, dysregulation of protein kinases occurs in a variety of diseases including cancer and inflammatory disorders. Considerable effort has been expended to determine the manifold functions of protein kinase signal transduction pathways during the past 50 years.

Manning et al. included 11 members in the human Src kinase family [5]. The four closely related group I enzymes include Src, Fyn, Yes, and Fgr and the four closely related group II enzymes include Blk, Hck, Lck, and Lyn. The three group III enzymes, which are distantly related to these two groups, include Frk, Srm, and Brk. Src, Fyn, and Yes are expressed in all cell types [3]. In contrast, Blk, Fgr, Hck, Lck, and Lyn are found primarily in hematopoietic cells, and Srm is found in keratinocytes. Frk occurs chiefly in bladder, brain, breast, colon, and lymphoid cells. Moreover, Brk occurs chiefly in colon, prostate, and small intestine; however, it was initially isolated from a breast cancer cell line [8].

Organization of Src

SH3, SH2, SH1 domains

From the N- to C-terminus, Src contains an N-terminal 14-carbon myristoyl group, a unique domain, an SH3 domain, an SH2 domain, an SH2-kinase linker, a protein-tyrosine kinase domain (SH1), and a C-terminal regulatory segment (Fig. 1) [3,4]. During biosynthesis, the amino-terminal methionine is removed and the resulting amino-terminal glycine becomes myristoylated following its reaction with myristoyl-CoA.

The human *SRC* gene encodes 536 amino acids and the chicken *Src* gene encodes 533 residues while the avian Rous sarcoma viral *Src* oncogene encodes 526 residues. The human and chicken Src

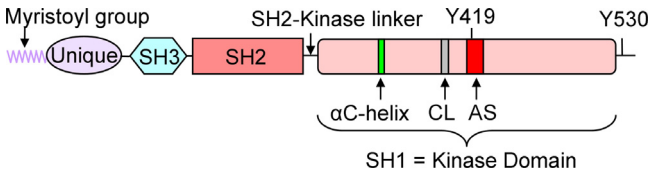

Fig. 1. Organization of human Src. CL, catalytic loop; AS, activation segment.

proteins exhibit 99.6% identity with most of the variation occurring near the N-terminus. The chicken viral Src (v-Src) protein Exhibits 95.8% identity with chicken Src. The protein kinase domains of the chicken/human proto-oncogenes (residues 267/270–520/523) exhibit only two differences: chicken Met354 corresponds to human Thr357 and chicken Asp502 corresponds to human Glu505. Thus, results from studies of the protein kinase domain of chicken Src are expected to accurately reflect those of the human enzyme.

The C-terminal tails (residues 521/524–533/536) of chicken/human Src are identical, but they are completely different from those of Rous v-Src. The Rous viral oncogene protein (v-Src) lacks seven-residues at its carboxyterminus, which include an autoinhibitory tyrosine phosphorylation site, thus accounting for its increased basal activity. Human Src and the Rous v-Src protein kinase domains exhibit 11 residue differences. The chicken numbering system is used in much of the early literature, even when studies were performed with the human enzyme. In this paper, unless specified otherwise, human residue numbers are used for both human and chicken Src reflecting efforts targeting the human enzyme for drug discovery. Three amino acids in the avian protein are deleted after human Src residue 25. To go from the human Src residue to that of chicken, subtract three.

Myristoylation facilitates the attachment of Src to membranes, and myristoylation is required for Src operation in cells [3]. The seven N-terminal amino acids beginning with glycine are required for the myristoylation of Src and v-Src [9,10]. 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 (PKA) and the Abl non-receptor protein-tyrosine kinase are myristoylated, but they are largely cytosolic [11,12]. Myristoylation is thereby not sufficient to ensure protein kinase membrane localization.

SH3 domains (≈60 amino acid residues) bind to sequences that can adopt a left-handed helical conformation [13]. The SH3 domain is a β-barrel consisting of five antiparallel β-strands and two prominent loops called the RT and n-Src loops (Fig. 2). These loops lie at either end of a surface composed of aromatic and hydrophobic residues that make up the recognition site for protein sequences bearing a PxxP motif. These sequences adopt a polyproline type II helical conformation that complexes with the SH3 domain. The prolines interact with aromatic side chains on the SH3 surface. Not all type II left-handed helices contain multiple prolines [13]. For example, the linker between the Src SH2 domain and kinase domain that interacts with the SH3 domain contains Pro249 in a type II helix. This residue interacts with N138 and Y139 of the SH3 domain of human Src.

SH2 domains (≈100 amino acid residues) bind to distinct amino acid sequences C-terminal to phosphotyrosine [14]. 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. X-ray crystallographic studies of the Src SH2 domain indicate that (i) the phosphotyrosine ligand binds to an invariant arginine and (ii) the isoleucine at the P + 3 position binds within a hydrophobic pocket [16]. The acidic residues at the pY + 1 and pY + 2

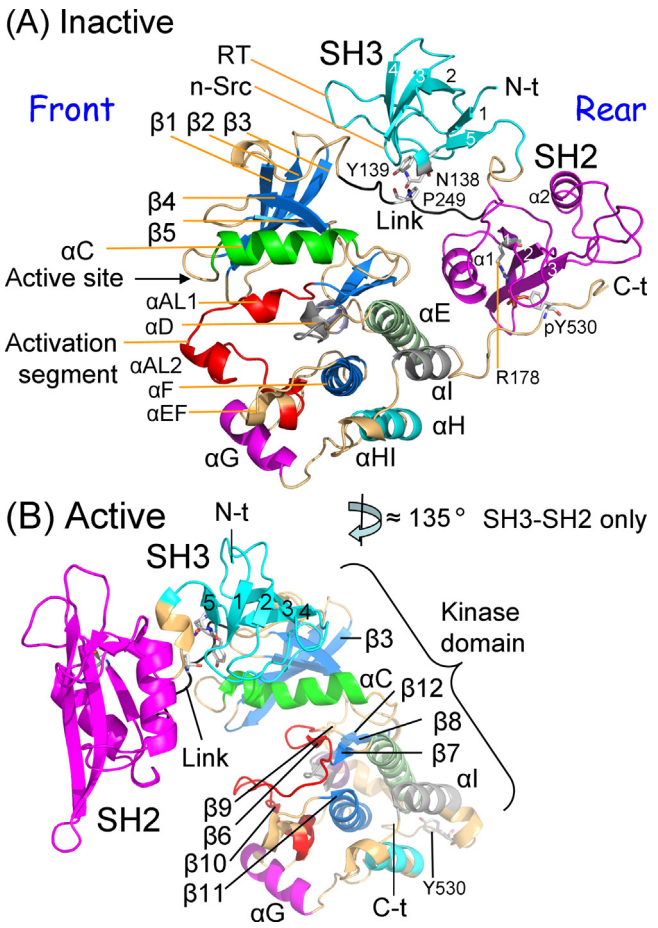

Fig. 2. Secondary structures of (A) inactive and (B) active Src. The SH3 domain is cyan, and the SH2 domain is magenta. C-t, C-terminus; N-t, N-terminus. The figures of inactive human Src (A) and active chicken Src (B) were prepared from PDB ID: 2SRC and 3DWQ, respectively.

This figure and Figs. 4, 5 and 9 were prepared using the PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.

positions of the SH2 binding partner interact with basic residues on the surface of the SH2 domain.

The Src SH2 domain (Fig. 2) consists of a central three-stranded β-sheet with a single helix packed against each side (α1 and α2). The SH2 domain forms two recognition pockets: one co-ordinates phosphotyrosine and the other binds one or more hydrophobic residues C-terminal to the phosphotyrosine. The phosphotyrosine pocket contains a conserved arginine residue (Arg178 in human Src). The Src SH2 domain, however, can bind to a variety of sequences that do not conform to this optimal pYEEI sequence, and other parts of proteins beyond the vicinity of the phosphotyrosine contribute to the formation of the binding interface. The human Src SH2 domain binds intramolecularly to C-terminal pTyr530 that results in inhibition of protein kinase activity. The sequence of this intramolecular site is pYQPG, which is a nonoptimal Src SH2-binding sequence. As a result, this binding can be readily displaced by more optimal phospholigands that can lead to enzyme activation.

One of the two most important regulatory phosphorylation sites in Src is Tyr530, six residues from the C-terminus. Under basal conditions *in vivo*, 90–95% of Src is phosphorylated at Tyr530 [17], which binds intramolecularly with the Src SH2 domain. SH2 and SH3 binding partners are able to displace the intramolecular association that stabilizes the dormant form of the enzyme [3]. The Tyr530Phe mutant is more active than the wild type enzyme and

can induce anchorage-independent growth *in vitro* and tumors *in vivo* [18,19]. Tyr530 phosphorylation results from the action of other protein-tyrosine kinases including Csk and Chk [20–22]. Src undergoes an intermolecular autophosphorylation catalyzed by another Src molecule at activation loop Tyr419, which promotes kinase activity [3].

The SH2 and SH3 domains have four important functions [3]. First, they constrain the activity of the enzyme *via* intramolecular contacts. Second, proteins that contain SH2 or SH3 binding partners can interact with 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 lead to the activation of Src kinase activity. And fourth, proteins containing SH2 or SH3 binding partners may preferentially serve as substrates for Src protein-tyrosine kinase. The group I and II Src-family kinase members contain an N-terminal myristoyl group and the seven-residue C-terminal regulatory tail [4]. The group III Src family kinases (Frk, Srm, and Brk) lack the myristoyl group and the seven-residue C-terminal regulatory tail, but they possess the SH1, SH2, and SH3 domains.

Secondary structure of the Src protein kinase domain: the protein kinase fold

The small lobe of all protein kinases is dominated by a five-stranded antiparallel β-sheet (β1–β5) and an important regulatory αC-helix (Fig. 2A) [23]. The first X-ray structure of a protein kinase (PKA) [24] contained an αA and an αB-helix proximal to αC (PDB ID: 2CPK), but these first two helices are not conserved in the protein kinase family. The active site of the kinase domain occurs within a cleft that is between the small N-lobe and the large C-lobe.

The large lobe of the Src protein kinase domain is mainly α-helical with six conserved segments (αD–αI) that occur in all protein kinases (Fig. 2A) [23]. The first X-ray structure of a protein kinase (PKA) possessed a short helix between the activation segment and the αF-helix, which was not named [24]. However, this αEF-helix is conserved in all protein kinase structures and represents a seventh-conserved segment in the C-lobe. The αF-helix forms an important hydrophobic core. The large lobe of active Src contains a helix between the αH and αI segment (αHI) (Fig. 2A). The activation segment of inactive Src, which contains the αAL1, αAL2, and αEF-helices, is compact while that of active Src is an extended open loop lacking αAL1 and αAL2, but still containing the αEF-helix.

The large lobe of active Src kinase contains seven short β-strands (β6–β12) (Fig. 2B). The β6-strand, the primary sequence of which occurs before the catalytic loop, interacts with the activation segment β9-strand. The β7-strand interacts with the β8-strand, the primary structures of which occur between the catalytic loop and the activation segment. The kinase domain of PKA and most active protein kinases contain these nine β-strands. However, the active Src kinase domain contains three additional strands (β10–12). The β10-strand from the activation segment interacts with the β11-strand that occurs proximal to the αF-helix. The β12-strand occurs in the initial part of the C-lobe immediately after the hinge residues and it interacts with the β7-strand. Inactive Src contains the β7 and β8-strands (not shown), but it lacks the β6 and β9–12 strands. Note that the β-strand nomenclature follows that of PKA while additional strands in protein kinases are assigned arbitrarily.

There are two general kinds of conformational motions associated with all protein kinases including those of the Src family; one involves conversion of an inactive conformation into a catalytically competent form. Activation typically involves changes in the orientation of the αC-helix in the small lobe and the activation segment in the large lobe. The active kinase then toggles between open and closed conformations as it goes through its catalytic cycle. The open form of the active enzyme binds MgATP and the protein substrate; this is accompanied by the conversion to the closed form as catalysis occurs. After catalysis, phosphorylated protein and then MgADP are released as the enzyme is reconverted to the open form prior to the next catalytic cycle.

Interconversion of the autoinhibited and active conformations of Src

Src regulation by the latch, clamp, and switch

Early biochemical studies led to the suggestion that the SH2 and SH3 domains inhibit Src protein kinase activity by directly blocking the active site. However, its three-dimensional structure showed that the SH2 and SH3 domains occur in the rear of the kinase domain (Fig. 2A). Moreover, the SH2 domain, which binds to the inhibitory pTyr530, is 40 Å from the active site. These structural studies indicated that the inhibitory effects of SH2 and SH3 are indirect.

The apparatus controlling Src activity has three components that Harrison calls the latch, the clamp, and the switch (Fig. 3) [25]. The SH2 domain binds to pTyr530 in the C-terminal tail to form the latch, which stabilizes the attachment of the SH2 domain to the large lobe. The avian oncogenic form of Src, which lacks a tyrosine in its C-terminal tail, and the Tyr530Phe mutant of Src are constitutively activated [3,4,18,19]. The SH3 domain contacts the small lobe. The linker between the SH2 and kinase domains contains proline at position 249 that is part of a motif that binds to the SH3 domain and attaches the SH3 domain to the small kinase lobe. The linker does not possess the classical PxxP signature [13], 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 used various algorithms in attempts to identify a Src sequence that could bind to an SH3 domain, but these were unsuccessful.

The clamp is an assembly of the SH2 and SH3 domains behind the kinase domain that functions in concert. As a result of clamping the SH2 and SH3 domains to the kinase domain, helix αC and its critical Glu313 are displaced that results in an autoinhibited enzyme. A hydrophobic interaction between Trp263 of the SH3 kinase linker and Gln315 of the αC-helix participates in its displacement producing an autoinhibited enzyme (not shown). The switch refers to the kinase-domain activation loop; the activation loop can switch from an inactive to active conformation following its autophosphorylation at Tyr419 as catalyzed by a partner Src molecule.

Unlatching by phosphatases

Dormant Src exists in equilibrium with pTyr530 bound to or free from the SH2 domain with the bound state greatly favored. When pTyr530 is displaced from the SH2-binding pocket, the protein can be unlatched with the clamp no longer locking the catalytic domain in an inactive conformation [25,26]. Furthermore, dissociation of pTyr530 allows dephosphorylation by various protein-tyrosine phosphatases that lead to the unlatched and active enzyme (Fig. 3). Candidate pTyr530 phosphatases include cytoplasmic PTP1B, Shp1 (Src homology 2 domain-containing tyrosine phosphatase-1) and Shp2 and transmembrane enzymes including CD45, PTPα, PTPε, and PTPλ [27].

Switching and unclamping

Following the unlatching of Src as catalyzed by various protein-tyrosine phosphatases, Tyr419 can then undergo autophosphorylation by another Src kinase molecule in a process called switching (Fig. 3). Following autophosphorylation, the enzyme is stabilized

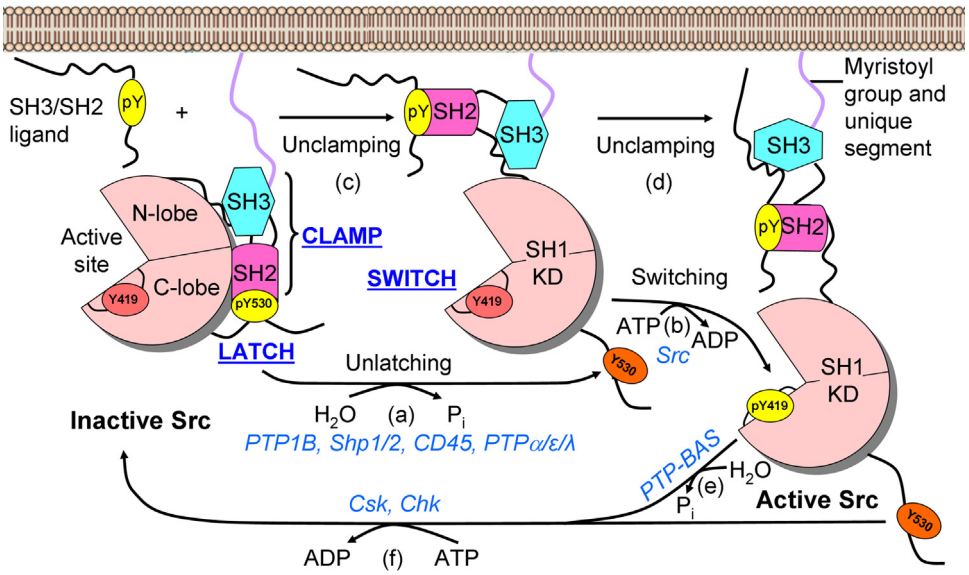

Fig. 3. The Src latch, clamp, and switch. Unlatching, unclamping, and switching lead to the formation of active Src.

in its active state. Exogenous substrates decrease autophosphorylation *in vitro* during activity measurements [28]. This finding is consistent with the notion that activation loop phosphorylation occurs in *trans* and involves two kinase molecules in an intermolecular reaction and not a *cis* intramolecular reaction, which is less likely to be inhibited by competition with exogenous substrates.

The structural design of Src allows for its regulation at multiple levels including competition between intramolecular and external binding partners [25]. The intramolecular interactions maintain an inactive state and external interactions promote an active state. Proteins that bind to the Src SH2 domain, the SH3 domain, or both disrupt the clamp, activating the kinase (Fig. 3).

Conversion of active to inactive Src

To return to the immobilized inactive state, the activation segment phosphate (pY419) is liberated by PTB-BAS [27] and the intramolecular SH2/SH3 binding partners replace the intermolecular binding partners. Human PTP-BAS is a ubiquitously expressed cytosolic phosphatase that contains a FERM domain, five PDZ domains, and a PTP domain. FERM is the acronym of four point one/ezrin/radixin/moesin. FERM proteins associate with F-actin and with the plasma membrane. PDZ domains are modular protein interaction domains of 80–90 residues in signaling proteins that bind to the C-terminus of other specific proteins. PTP-BAS was derived initially from human white blood cell basophils (BAS refers to basophil) and BL (mouse) refers to basophil like. PTP-BAS co-localizes in membrane fractions with Src family kinases.

PTP-BL is the mouse homologue of human PTP-BAS. Palmer et al. found that the bacterially expressed mouse PTP-BL phosphatase domain, but not a catalytically inactive mutant, catalyzes the dephosphorylation of mouse Src specifically at pTyr419 [29]. In contrast, this enzyme does not act upon pTyr530. These investigators found that ephrinB2, an important regulator of morphogenesis, leads to the activation of Src kinase in mouse NIH3T3 cells. Activation is apparent at 10 min and is absent at 30 min. The deactivation is associated with the recruitment of PTP-BL and dephosphorylation of Src pTyr419. As noted above, pTyr419 occurs in the activation segment and is associated with increased Src activity. It is possible that other phosphatases are involved in regulatory pTyr419 dephosphorylation.

Csk or Chk catalyze the phosphorylation of Tyr530 so that the latch can reform. Csk, a cytoplasmic protein-tyrosine kinase, was the first enzyme discovered that catalyzes the phosphorylation of the regulatory C-terminal tail tyrosine of Src (Fig. 3). Okada and Nakagawa isolated this enzyme from neonatal rat brain and demonstrated that it catalyzes the phosphorylation of Src at Tyr530 [20]. Following phosphorylation, the K_m of Src for ATP and for acid-denatured enolase is unchanged, but the k_{cat} is decreased 50%. Using purified Src, the activity of the Tyr530 phosphorylated enzyme *in vitro* ranges from 0.2–20% that of the unphosphorylated enzyme depending upon the experimental conditions.

Chk is a second enzyme that catalyzes the phosphorylation of the inhibitory tyrosine of Src-family kinases [22]. Csk is expressed in all mammalian cells, whereas Chk is limited to breast, hematopoietic cells, neurons, and testes [3]. Csk and Chk consist of an SH3, SH2, and kinase domain; these enzymes lack the N-terminal myristoyl group and the C-terminal regulatory tail phosphorylation site found in Src [30]. Besides inactivating Src by catalytic phosphorylation, Chk forms a noncovalent inhibitory complex with Src. The association of Chk with the activated and autophosphorylated form of Src inhibits Src kinase activity [31]. The action of Chk thereby overrides that of Src. Chk can also bind to unphosphorylated Src and prevent its activation segment phosphorylation.

There are four possible Src enzyme forms: (i) nonphosphorylated, (ii) Tyr530 phosphorylated, (iii) Tyr419 phosphorylated, and (iv) both Tyr530 and 419 phosphorylated enzymes. Src with phosphorylated Tyr530 cannot undergo autophosphorylation; the residue must first be dephosphorylated. However, Src with Tyr419 autophosphorylation is a substrate for C-terminal Src kinase, but the doubly phosphorylated enzyme is active, so that Tyr419 phosphorylation overrides inhibition produced by Tyr530 phosphorylation [32].

Structure of the Src kinase domain skeleton

The regulatory spine

Taylor and Kornev [33] and Kornev et al. [34] analyzed the structures of active and inactive conformations of about two dozen protein kinases and determined functionally important residues by a local spatial pattern (LSP) alignment algorithm. In contrast to the protein kinase amino acid signatures noted later such as DFG

or HRD, the residues that constitute the spines were not identified by sequence analyses *per se*. Rather, they were identified by their three-dimensional location based upon a comparison of the X-ray crystallographic structures [33,34].

The local spatial pattern alignment analysis revealed a skeleton of four nonconsecutive hydrophobic residues that constitute a regulatory or R-spine and eight hydrophobic residues that constitute a catalytic or C-spine (Fig. 4C and D). The R-spine interacts with a conserved aspartate (D447) in the αF-helix. As noted later in this section, there are three conserved "shell" residues that interact with the R-spine. Altogether each protein kinase contains 16 amino acids that make up this protein kinase skeletal assembly. Each spine consists of residues derived from both the small and large lobes. The regulatory spine contains residues from the activation segment and the αC-helix, whose conformations are important in defining active and inactive states. The catalytic spine governs catalysis by facilitating ATP binding. The two spines dictate the positioning of the protein substrate (R-spine) and ATP (C-spine) so that catalysis results. The proper alignment of the spines is necessary for the assembly of an active kinase.

The Src regulatory spine consists of a residue from the beginning of the β4-strand (Leu328, human Src residue number), from the C-terminal end of the αC-helix (Met317), the phenylalanine of the activation segment DFG (Phe408), along with the HRD-histidine (His387) of the catalytic loop. Met317 and comparable residues from other protein kinases are four residues C-terminal to the conserved αC-glutamate. The backbone of His387 is anchored to the αF-helix by a hydrogen bond to a conserved aspartate residue (Asp447). The protein-substrate positioning segment, the activation segment, and the αHI-loop of protein kinase domains, including the Src family, form hydrophobic contacts with the αF-helix [34].

Src has been observed in both inactive (PDB ID: 2SRC) and active (PDB ID: 3DQW) conformations by X-ray crystallography. The active form of the chicken enzyme, which contains a Thr to Ile gatekeeper mutation, was chosen instead of an active wild type human enzyme (*e.g.*, 1Y57) because the chicken enzyme contains (i) an activation segment phosphotyrosine and (ii) a bound ATP-γ-S that were needed for analyses as described later. Although the kinase domains of the active and autoinhibited enzyme are nearly superimposable, the αC-helices and the activation segments of the active and inactive enzyme forms differ from one another (root mean square deviation > 6 Å). Note the subluxation, or kink, at the RS3 residue of the Src regulatory spine in inactive Src (Fig. 4D and F). This abnormality is associated with the αC out and catalytically inactive structure of the Src kinase domain.

The catalytic spine

The catalytic spine of protein kinases consists of residues from the small and large lobes and is completed by the adenine base of ATP [33,34]. The two residues of the small lobe of the Src protein kinase domain that bind to the adenine group of ATP include Val284 from the beginning of the β2-strand and Ala296 from the conserved Ala-Xxx-Lys of the β3-strand. Furthermore, Leu396 from the middle of the large lobe β7-strand binds to the adenine base in the active enzyme. Val284, Ala296, and Leu396 characteristically make hydrophobic contacts with the scaffolds of ATP-competitive small molecule inhibitors. Ile395 and Val397, hydrophobic residues that flank Leu396, bind to Leu349 at the beginning of the αD-helix.

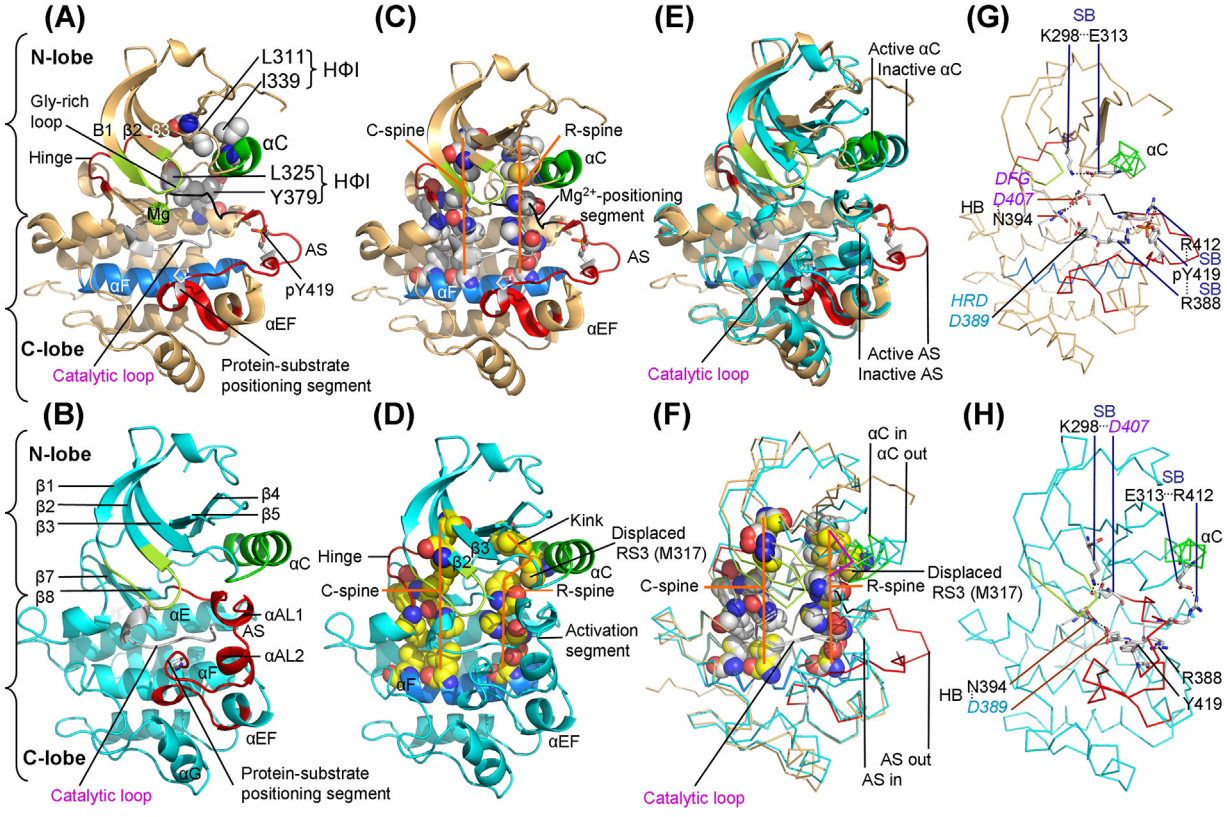

Fig. 4. Overview of the structure of the (A) active and (B) inactive Src kinase domain. Location of the C- and R-spines of (C) active and (D) inactive Src. (E) Superposition of active and inactive Src and (F) their C- and R-spines. Important salt bridges (SB) and hydrogen bonds (HB) in (F) active and (G) inactive Src. AL, activation loop; AS, activation segment; HΦI, hydrophobic interaction.

The figures of active chicken Src were prepared from PDB ID: 3DQW and inactive human Src from PDB ID: 2SRC.

Table 1
Src and PKA R-spine (RS), R-shell (Sh), and C-spine residues.

	Symbol	Chicken Src	Human Src	Murine PKA[a]
Regulatory spine				
β4-strand (N-lobe)	RS4	Leu325	Leu328	Leu106
C-helix (N-lobe)	RS3	Met314	Met317	Leu95
Activation loop F of DFG (C-lobe)	RS2	Phe405	Phe408	Phe185
Catalytic loop His or Tyr (C-lobe)[b]	RS1	His384	His387	Tyr164
F-helix (C-lobe)	RS0	Asp444	Asp447	Asp220
R-shell				
Two residues upstream from the gatekeeper	Sh3	Ile336	Ile339	Met118
Gatekeeper, end of β4-strand	Sh2	Thr338	Thr341	Met120
αC-β4 loop	Sh1	Val323	Val326	Val104
Catalytic spine				
β2-strand (N-lobe)		Val281	Val284	Val57
β3-AXK motif (N-lobe)		Ala293	Ala296	Ala70
β7-strand (C-lobe)		Leu393	Leu396	Leu173
β7-strand (C-lobe)		Ile392	Ile395	Leu172
β7-strand (C-lobe)		Val394	Val397	Ile174
D-helix (C-lobe)		Leu346	Leu349	Met128
F-helix (C-lobe)		Leu451	Leu454	Leu227
F-helix (C-lobe)		Leu455	Leu458	Met231

[a] From Ref. [34,35].
[b] Part of the HRD (His-Arg-Asp) or YRD (Tyr-Arg-Asp) sequence.

The αD-helix Leu349 binds to Leu454 and Leu458 in the αF-helix. Note that both the R-spine and C-spine are anchored to the αF-helix, which is a very hydrophobic component of the enzyme that is entirely within the protein and not exposed to the solvent. The αF-helix serves as a sacrum that supports the spines, which in turn anchor the protein kinase catalytic muscle. Table 1 lists the residues of the spines of human and chicken Src and the catalytic subunit of murine PKA.

Spinal collateral ligaments or shell residues

Using site-directed mutagenesis and sensitive radioisotopic enzyme assays, Meharena et al. identified three residues in murine PKA that stabilize the R-spine, and they referred to them as shell residues [35]. Going from the connecting aspartate at the bottom in the αF-helix up to the spine residue in the β4-strand at the top, these investigators labeled the regulatory spine residues RS0, RS1, RS2, RS3, and RS4 (Fig. 5 and Table 1). The three shell residues are labeled Sh1, Sh2, and Sh3. Sh3 interacts with RS4, and Sh1 interacts with RS3 and Sh2. Sh2, which is the classical gatekeeper residue, interacts with Sh1 below it and with RS4 next to it. The term gatekeeper refers to the role of such residues in allowing or disallowing access to a hydrophobic pocket adjacent to the adenine binding site [36,37] that is occupied by portions of many small molecule inhibitors as described later. Using the previous local spatial pattern alignment data [34], only three of 14 amino acid residues in PKA surrounding RS3 and RS4 are conserved, and these are the shell residues that serve as collateral spinal ligaments that stabilize the protein kinase vertebral column or spine [35]. The V104G mutation (Sh1) decreased the catalytic activity of PKA by 95%. The M120G (Sh2) and M118G (Sh3) double mutant was devoid of catalytic activity. These results provide evidence for the importance of the shell residues in stabilizing the spine and maintaining protein kinase activity.

A comparison of the active and inactive Src R-spines shows that RS3 of the dormant enzyme is displaced when compared with active Src, a result that is consistent with the displaced αC-helix of the inactive enzyme (Fig. 5). The RS3 and RS4 α-carbon atoms of the active and inactive kinase domains differ in location by 2.6 Å and 1.2 Å, respectively, and the terminal methyl carbon atoms of Met317 (RS3) differ in location by 6.2 Å. The Sh1 and Sh2 residues of active and autoinhibited Src are nearly superimposable while the α-carbon atoms of Sh3 are modestly displaced (1.9 Å). Moreover, the C-spines of active and inactive Src are nearly superimposable.

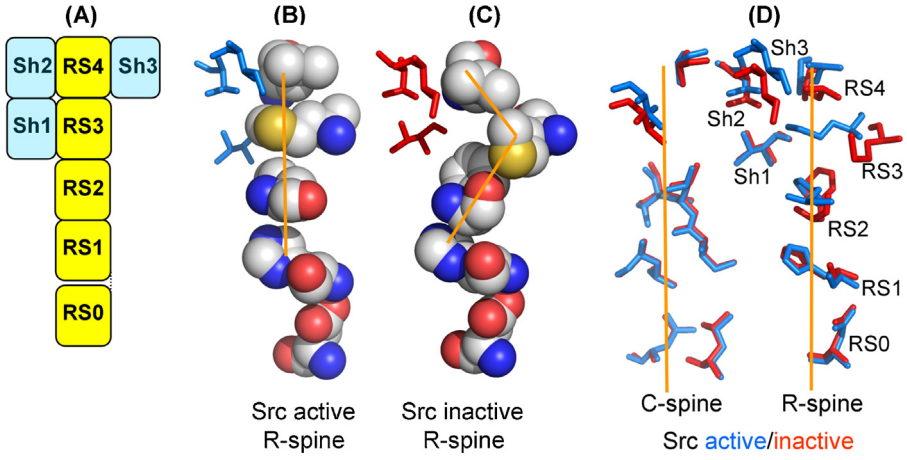

Fig. 5. The Src regulatory and catalytic spines and shell residues. (A) Interaction of the shell (Sh) residues with those of the regulatory spine (RS). The R-spine is depicted as spherical CPK residues and the shell residues are shown as sticks in (B) active and (C) inactive Src. (D) Superposition of the (i) spine residues and (ii) shell residues from active (PDB ID: 3DQW in blue) and inactive (PDB ID: 2SRC in red) Src.

Since the RS3 residues of the R-spine of active and inactive enzyme forms differ in location, it is natural to expect that the surrounding shell residues will also vary in their three-dimensional location.

The 18 residues of the Src family skeletal assembly are conserved. Of the group I (Src, Yes, Fyn, Fgr) and group II (Blk, Lck, Lyn, Hck) members of the Src family, nearly 97% of the skeletal assembly residues are identical. This compares with only 70% identity of the kinase domain residues (Src 270–523) in the eight Src kinase family members. The four nonidentical C-spine residues correspond to Src Leu458 within the hydrophobic αF-helix. These residues are all leucine in the group I members of the Src family, but consist of valine (Blk) or isoleucine (Lck, Lyn, Hck) in the group II Src family. The evolutionary conservation of the Src family kinase domain skeletal assembly underscores its importance.

Src catalytic residues

Properties of the small and large lobes

Like all protein kinases, the Src protein kinase domain has a small amino-terminal lobe and large carboxyterminal lobe (Fig. 4A and B) first described by Knighton et al. for PKA [24]. The two lobes form a cleft that serves as a docking site for ATP. The small lobe of protein kinases contains a conserved glycine-rich (GxGxΦG) ATP-phosphate–binding loop, which is the most flexible part of the lobe. The glycine-rich loop is near the phosphates of the ATP substrate as described later. The β1 and β2-strands of the N-lobe harbor the adenine component of ATP and they interact with ATP-competitive small molecule inhibitors. The β3-strand typically contains an Ala-Xxx-Lys sequence, the lysine of which in Src (K298) forms a salt bridge with a conserved glutamate near the center of the αC-helix (E313) of protein kinases. The presence of a salt bridge between the β3-lysine and the αC-glutamate is a prerequisite for the formation of the activate state and corresponds to the "αC-in" conformation (Fig. 4G). By contrast, Lys298 and Glu313 of the dormant form of Src fail to make contact and this structure corresponds to the displaced "αC-out" conformation (Fig. 4H). The αC-in conformation is necessary, but not sufficient, for the expression of full kinase activity.

The large lobe contains a mobile activation segment with an extended conformation in active enzymes and closed conformation in dormant enzymes. The first residues of the activation segment of protein kinases consist of DFG (Asp-Phe-Gly). The DFG exists in two different conformations in the protein kinase family. In the dormant activation segment conformation of many protein kinases, the aspartate side chain of the conserved DFG sequence faces away from the active site. This is called the "DFG-Asp out" conformation. In the active state, the aspartate side chain faces into the ATP-binding pocket and coordinates Mg^{2+}. This is called the "DFG-Asp in" conformation. This terminology is better than "DFG-in" and "DFG-out" because, in the inactive state, the DFG-phenylalanine may move into the active site (while the DFG-aspartate moves out) [38]; it is the ability of aspartate to bind (Asp-in) or not bind (Asp-out) to Mg^{2+} in the active site that is crucial. However, the inactive conformation of the Src kinase activation segment exists in a closed conformation but with the DGF-Asp directed inward. The distinctive αC out and DGF-Asp in combination is labeled as the Src family kinase-like inactive conformation. The Mg^{2+}-positioning segment (Fig. 4C) of Src consists of the first five residues of the activation segment (DFGLA).

The activation segments of protein kinases including Src typically ends with APE (Ala-Pro-Glu). The last eight residues of the activation segment of Src are PIKWTAPE, which make up the protein-substrate positioning segment (Fig. 4A and B). The R-group of the first proline in this sequence serves as a platform that interacts with the tyrosyl residue of the peptide/protein substrate that is phosphorylated [39]. In protein-serine/threonine kinases, the serine or threonine interacts with backbone residues near the end of the activation segment and not with an R-group. The activation segment of Src contains a phosphorylatable tyrosine and its phosphorylation, like that of most other protein-tyrosine kinases [34], is required for enzyme activation [27]. As noted previously, Harrison referred to this phosphorylation as switching [25].

Table 2
Important structural and functional residues in Src.

	Chicken Src	Human Src
SH3 domain	81–142	84–145
SH2 domain	148–245	151–248
SH1 catalytic domain	267–520	270–523
N-lobe		
Glycine-rich loop: GQGCFG	274–279	277–282
β-3 lysine (K of K/E/D/D)	K295	K298
αC-Glu (E of K/E/D/D)	E310	E313
αC-helix β5-strand HΦ interaction	L308–I336	L311–I339
αC-β4 loop and αE helix HΦ interaction	L322–Y376	L325–Y379
Hinge residues: EYMSKG	339–344	342–347
C-lobe		
αE-activation segment loop and activation segment HΦ interaction	Y382–L410	Y385–L413
Catalytic loop HRD (first D of K/E/D/D)	386	389
Intracatalytic loop hydrogen bond	R388–N391	R391–N394
Catalytic loop–activation segment hydrogen bonds	R385–pY416; N391–D404	R388–pY419; N394–D407
Catalytic loop N	391	394
Activation segment DFG (second D of K/E/D/D)	404	407
Activation segment	404–432	407–435
Mg^{2+}-positioning loop:DFGLA	404–408	407–411
Activation segment tyrosine phosphorylation site	416	419
Substrate protein-positioning loop	424–432	427–435
End of the activation segment:ALE	430–432	433–435
ALE-E and αH-αI loop salt bridge	E432–R506	E435–R509
UniProt KB entry	P00523	P12931

Two conserved hydrophobic interactions in Src and other protein kinases contribute to kinase domain stability. A hydrophobic contact between Leu311, which is two residues N-terminal to the Glu313 in the αC-helix, with Ile339 near the N-terminus of the β4-strand helps to stabilize the N-terminal lobe. Moreover, another hydrophobic contact from Leu325 in the αC-β4 loop of the small lobe and Tyr379 near the carboxyterminal end of the αE-helix in the large lobe (eight residues upstream from HRD of the catalytic loop) further stabilizes the interaction between the two lobes (Fig. 4A).

The K/E/D/D protein kinase signature

The Src kinase domain consists of the characteristic bilobed protein kinase architecture [26,40–42]. Residues 270–341 make up the small amino-terminal lobe of the kinase; residues 348–523 make up the large carboxyterminal lobe (Fig. 4A). As described for PKA [33], ATP binds in the cleft between the small and large lobes of Src and the protein substrate binds to the larger carboxyterminal lobe. Furthermore, 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. identified 11 subdomains with conserved amino acid residue signatures that constitute the catalytic core of protein kinases [43]. Of these, the four following residues, which constitute a K/E/D/D motif, illustrate the inferred catalytic properties of Src. Lys298 (the K of K/E/D/D) represents an invariant residue of protein kinases that generally forms ion pairs with the α- and β-phosphates of ATP and with Glu313 in the αC-helix (the E of K/E/D/D) (Table 2). Asp389 orients the tyrosyl group of the substrate protein in a

catalytically competent state. Asp389 functions as a base that abstracts a proton from tyrosine thereby facilitating its nucleophilic attack of the γ-phosphorus atom of MgATP; Asp389 (the first D of K/E/D/D) is called the catalytic base. This base occurs within the catalytic loop (Fig. 4A and B) that generally has the sequence HRDLRAAN in non-receptor protein-tyrosine kinases including Src. Asp407 is the first residue of the activation segment found in the large lobe (the second D of K/E/D/D). Asp407 binds Mg^{2+}, which in turn coordinates the β- and γ-phosphate groups of ATP.

The small and large lobes can adopt a range of relative orientations, opening or closing the active site cleft [26,44]. Within each lobe is a polypeptide segment that has an active and an inactive conformation. In the small lobe, this segment is the αC-helix [44]. 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 segment adjusts to make or break part of the catalytic site. In most protein kinases, phosphorylation of a residue within the activation segment stabilizes the active conformation; in human Src, this residue corresponds to Tyr419.

The structures of active and dormant Src kinases including the SH3, SH2, and SH1 (kinase) domains have been solved by X-ray crystallography [26,40–42]. The conformation of the activation loop differs between active and inactive kinases [44]. In protein kinases that are inactive, 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 (Asp407 in Src) within the conserved DFG motif at the amino-terminal base of the activation segment 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 segment provides a platform for protein substrate binding.

In dormant Src kinase, residues 410–413 and 417–421 of the activation segment form short α-helices (αAL1 and αAL2) (Fig. 4B). As a result, αAL1 displaces the αC-helix into its inactive out conformation so that Glu313 in the helix cannot form a critical salt bridge with Lys298. The αAL2-helix helps to bury the side chain of Tyr419 (the site of activating phosphorylation). The αAL1 and αAL2-helices are thus important autoinhibitory components. They (i) preclude protein/peptide substrate recognition, (ii) sequester Tyr419, and (iii) stabilize the inactive conformation of the kinase domain [26].

The interconversion of the inactive and active forms of Src kinase also involves an intricate electrostatic switch. In the dormant enzyme, the β4-lysine (K298) forms a salt bridge with the DFG-Asp (D407) residue, and the αC-Glu313 forms a salt bridge with Arg412 (the sixth residue of the activation segment). Moreover, the –NH of Asn394 hydrogen bonds with a carboxylate of Asp389 (the D of HRD). The conversion to the active enzyme form entails an electrostatic switch: the β4-lysine (K298) now forms a salt bridge with the αC-Glu (E313) with the concomitant formation of the αC-in conformation and the –NH of Asn394 now hydrogen bonds with a carboxylate of Asp407 (the D of DFG). Following the phosphorylation of Tyr419 in the activation segment, the phosphate forms salt bridges with Arg388 within the catalytic loop and with Arg412 within the activation segment (Fig. 4G and H).

Stabilizing the Src activation segment

The phosphorylation of one or more residues in the activation segment of the majority of protein kinases is required to generate their active conformation. In the case of the catalytic subunit of murine PKA, this corresponds to the phosphorylation of activation loop Thr197 as catalyzed by PKA [45]. This activation loop phosphate interacts with four different sections of PKA including (i) His87 of the αC-helix, (ii) Arg165 of the catalytic loop H/YRD, (iii) Tyr215 in the αEF/αF-loop, and (iv) Lys189 and Thr195 within the activation segment [33]. The glutamate at the end of the activation segment forms a conserved salt bridge with Arg280 in the αHI-loop. Within the R-spine, Phe185 of DFG within the PKA activation segment makes hydrophobic contacts with Leu95 of the αC-helix and with Tyr164 of the catalytic loop (equivalent to the histidine HRD of most protein kinases).

The activation segment Tyr419-phosphate of Src differs from the Thr197-phosphate of PKA in its interactions within the kinase domain. The Src Tyr419-phosphate does not interact with any residues within the αC-helix, but it does form a salt bridge with Arg388 of the catalytic group HRD (Fig. 4 G). The Src Tyr419-phosphate forms a salt bridge with Arg412 within the activation segment, but it fails to make contact with residues within the αEF/αF-loop. Like PKA, Src Glu435 at the end of its activation segment forms a salt bridge with Arg509 that lies within its αHI-loop. The DFG-Phe407 makes a hydrophobic contact with Met317 of the N-lobe αC-helix and the HRD-His387 of the C-lobe catalytic loop as part of the R-spine. Also, like PKA, the activation segment β9-strand interacts with the β6-strand near the catalytic loop. The activation segment β10-strand interacts with the β11-strand just proximal to the αF-helix; however, this interaction is lacking in PKA. Thus, the stabilization of the Src activation segment differs in detail from that observed in PKA. Moreover, the β6 and β9-strand and the β10 and β11-strand interactions are lacking in the inactive conformations of Src.

Hydrophobic interactions occur within the Src activation segment involving (i) Phe407 (the Phe of DFG) and Leu410 and (ii) Phe427, Ala425, and Phe442. However, these hydrophobic interactions occur in both (i) active (PDB ID: 3DQW) and (ii) inactive Src (PDB ID: 2SRC); accordingly, they fail to explain any additional stabilization of the activation segment in its active conformation. Using molecular dynamics simulations, Meng and Roux reported that phosphorylation of the activation loop tyrosine of Src helps to stabilize the R-spine and the HRD motif [46]. They conclude that this phosphorylation helps to lock the enzyme into its catalytically active conformation.

Knowledge of the active and inactive conformations of protein kinases can serve as an aid in drug discovery [38]. Although the tertiary structures of catalytically active protein kinase domains are strikingly similar, Huse and Kuriyan reported that the crystal structures of inactive enzymes reveal a multitude of distinct protein kinase conformations [44]. The practical consequence of this is that drugs targeting specific inactive conformations may be more selective than those targeting the active conformation [47]. Huse and Kuriyan noted that protein kinases usually assume their less active conformation in the basal or non-stimulated state and the acquisition of their activity may involve several layers of regulatory control [44].

Role of magnesium ions in the protein kinase catalytic process

Participation of two magnesium ions in catalysis

Nearly all protein kinases require a divalent cation such as Mg^{2+} or Mn^{2+} for expression of their activity. Because the cellular content of Mg^{2+} is much greater than that of Mn^{2+}, Mg^{2+} is considered to be the physiologically important cation. The magnesium ion plays a dual role in protein kinase reactions. First, the physiological nucleotide substrate is MgATP. Second, another magnesium ion interacts with the enzyme/metal-nucleotide complex to increase the catalytic efficiency (k_{cat}/K_{MgATP}), where K_{MgATP} is the K_m for ATP.

The k_{cat}/K_{MgATP} is an apparent second-order rate constant (M^{-1} s^{-1}) that relates the reaction rate to the concentration of free, rather than total, enzyme (the present discussion ignores the protein substrate of protein kinases). At low substrate concentrations most of the enzyme is free and the reaction velocity is given by v = [Enzyme][MgATP] × k_{cat}/K_{MgATP} [48]. This constant (k_{cat}/K_{MgATP}) is called the specificity constant when it is used to compare the effectiveness of multiple substrates for a given enzyme. For enzyme reactions that are limited only by the rates of diffusion of the enzyme and substrate, the upper limit of the value for kinetic efficiency is ≈10^8 M^{-1} s^{-1}. In contrast, the k_{cat}/K_{MgATP} for avian Src [49] is 2310 M^{-1} s^{-1}, that for human Csk [49] is 500 M^{-1} s^{-1}, and that for the catalytic subunit of bovine PKA [50] is 2.3 × 10^5 M^{-1} s^{-1} where the citations denote the sources of data upon which the calculations are based. Unlike general metabolic enzymes, protein kinases function as dynamic molecular switches that are turned on or off. Thus, protein kinases are not continuously active as, for example, metabolic enzymes such as hexokinase [23]. Protein kinases are able to perform their physiological functions despite having low catalytic efficiencies.

The function of Mg^{2+} and other divalent cations in protein kinase-mediated reactions is intricate. The role of Mg^{2+} has been examined in numerous receptor and non-receptor protein-serine/threonine and protein-tyrosine kinases (Table 3). Variable effects on the k_{cat} and K_{MgATP} have been observed in response to increasing the concentration of magnesium ion. For Src, Csk and leucine-rich repeat kinase-2 (LRRK2), the k_{cat} is increased whereas the K_{MgATP} is unchanged. For CDK5, ERK2, interleukin-1 receptor associated kinase-4 (IRAK-4) and FGFR1, the k_{cat} is increased and the K_{MgATP} is decreased. For the insulin and Fps receptor protein-tyrosine kinases, the k_{cat} is unchanged and the K_{MgATP} is decreased. And for the catalytic subunit of PKA and for CDK2, we have the unusual situation where both the k_{cat} and the K_{MgATP} are decreased. In all of these cases, however, the kinetic efficiency (k_{cat}/K_{MgATP}) increases at higher [Mg^{2+}]. Studies with Bruton's protein-tyrosine kinase, EGFR, ErbB2, ErbB3, Yes, and VEGFR2 also indicate that the k_{cat}/K_{MgATP} is increased, but studies on the k_{cat} and K_{MgATP} as a function of [Mg^{2+}] were not reported.

The initial studies on the role of divalent cations on the protein kinase reaction were performed with PKA and the results are now seen to be somewhat atypical. Using nuclear magnetic resonance and steady-state kinetic studies, Armstrong et al. observed that the catalytic subunit of PKA in the presence of a nucleotide such as ADP contains two binding sites for Mn^{2+} (K_d = 6–10 μM and 50–60 μM) or for Mg^{2+} (both about 1.6 mM) [62]. They observed that increasing the Mn^{2+} concentration first increases the reaction rate, but further increases (Mn^{2+} > 75 μM) lead to a decline. They suggested that the more tightly bound Mn^{2+} is an essential metal ion activator while the more weakly bound Mn^{2+} is an inhibitor of catalytic activity.

Our steady-state kinetic analysis of bovine PKA indicated that a high (10 mM) Mg^{2+} concentration resulted in a k_{cat} that is about one-fifth that at low (0.5 mM) Mg^{2+} concentration. Even though these studies led to the terminology of an inhibitory Mg^{2+} site, the catalytic efficiency (k_{cat}/K_{MgATP}) at a high Mg^{2+} concentration increased by 13-fold as a result of the decreased K_{MgATP}. It is important to note that in the absence of a nucleotide, divalent cation binding affinity to PKA is very weak [62]. This indicates that both metal binding sites are greatly augmented by ADP/ATP. We found that MgATP and peptide substrate bound randomly to PKA, but the release of product was ordered (phosphopeptide before MgADP) [50].

Zheng et al. determined the X-ray crystal structure of the catalytic subunit of murine PKA bound to Mg^{2+}, ATP, and a heat-stabile protein kinase inhibitor that mimics a protein substrate [63]. Crystals were prepared under conditions of low [Mg^{2+}] and high [Mg^{2+}]. They observed that MgATP is found between the small and large lobes. Under low [Mg^{2+}] conditions, a single Mg^{2+} is bound to the β and γ-phosphates and to the aspartate of the DFG sequence; this magnesium ion is labeled 1: Mg^{2+} (1). Under high [Mg^{2+}] conditions, a second Mg^{2+} is bound to the α and γ-phosphates and to the asparagine amide nitrogen within the catalytic loop downstream from the Y/HRD conserved sequence of the catalytic loop. This magnesium ion is labeled 2: Mg^{2+}(2).

The role of each Mg^{2+} has been the subject of numerous studies during the past two decades. Initially, many investigators thought that Mg^{2+}(1) was the key divalent cation required for the protein kinase reaction [64]. More recently, Jacobsen et al. used steady-state kinetics, X-ray crystallography, and molecular dynamics simulations to investigate the role of two cations in the CDK2-mediated reaction [57]. They demonstrated that two Mg^{2+} ions are essential for efficient phosphoryl transfer. Their studies showed that the ADP phosphate mobility is more restricted when ADP is bound to two Mg^{2+} ions when compared to one. The cost that is paid to accelerate the chemical process is the limitation in the velocity of ADP release, which is the rate-limiting step in the overall process [65,66]. Jacobsen et al. provide evidence that Mg^{2+}(1) is released prior to ADP-Mg^{2+}(2) [57].

Table 3
Effect of high Mg^{2+} concentrations on steady-state kinetic parameters of various protein kinases.

Enzyme[a]	Class	Specificity	Substrate[b]	k_{cat}	K_{MgATP}	K_{cat}/K_{MgATP}	References
Chicken Src	Non-receptor	Tyr	Poly-E_4Y	↑	No Δ	↑	[49]
Human Csk	Non-receptor	Tyr	Poly-E_4Y	↑	No Δ	↑	[49]
Human LRRK2	Non-receptor	Ser/Thr	Peptide I	↑	No Δ	↑	[51]
Human CDK5	Non-receptor	Ser/Thr	Peptide II	↑	↓	↑	[52]
Rat ERK2	Non-receptor	Ser/Thr	Ets138	↑	↓	↑	[53]
Human IRAK-4	Receptor	Ser/Thr	Peptide III	↑	↓	↑	[54]
Xenopus FGFR-1	Receptor	Tyr	Poly-E_4Y	↑	↓	↑	[49]
Rat insulin receptor	Receptor	Tyr	Poly-E_4Y	No Δ	↓	↑	[55]
Avian v-Fps	Non-receptor	Tyr	EAEIYEAIE	No Δ	↓	↑	[56]
Bovine PKA	Non-receptor	Ser/Thr	LRRASLG	↓	↓	↑	[50]
Human CDK2	Non-receptor	Ser/Thr	Histone H1	↓	↓	↑	[57]
Human Bruton's tyrosine kinase	Non-receptor	Tyr	Poly-E_4Y	↑	?	↑	[58]
Human EGFR (ErbB1)	Receptor	Tyr	Peptide A	?	?	↑	[59]
Human ErbB2	Receptor	Tyr	Peptide B	?	?	↑	[59]
Human ErbB3	Receptor	Tyr	Peptide C	?	?	↑	[59]
Human Yes	Non-receptor	Tyr	Poly-E_4Y	?	?	↑	[60]
Human VEGFR2	Receptor	Tyr	Poly-E_4Y	?	?	↑	[61]

[a] LRRK2, leucine-rich repeat kinase-2; IRAK-4, interleukin-1 receptor associated kinase-4.
[b] Peptide I, RLGRDKYKTLRQIRQ; Peptide II, PKTPKKAKKL; Peptide III, KKARFSRFAGSSPSQSSMVAR; Peptide A (biotin-(aminohexanoate)-EEEEYFELVAKKK-$CONH_2$); Peptide B (biotin-(aminohexanoate)-GGMEDIYFEFMGGKKK-$CONH_2$); Peptide C (biotin-(aminohexanoate)-RAHEEIYHFFFAKKK-$CONH_2$).

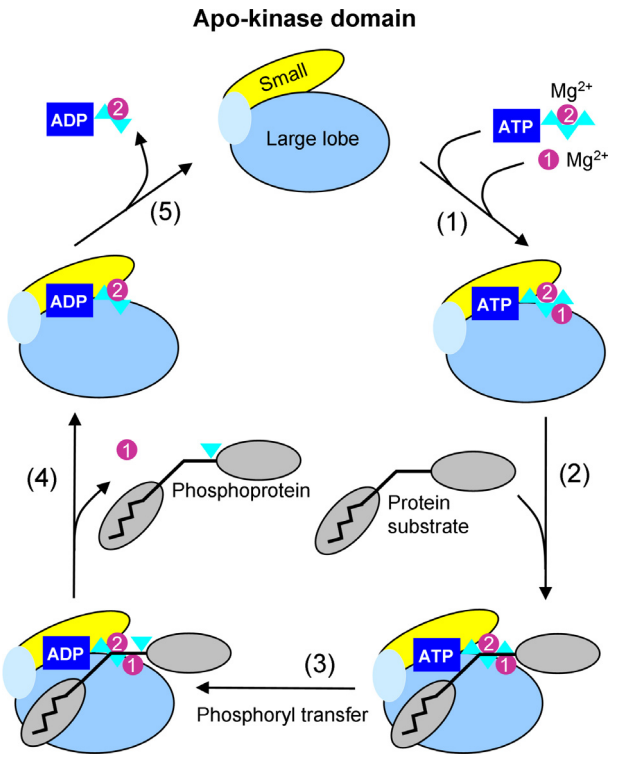

Fig. 6. Proposed protein kinase catalytic cycle including $Mg^{2+}(1)$, $Mg^{2+}(2)$-ATP, protein substrate, and $Mg^{2+}(2)$-ADP.
Source: Adapted from Bastidas et al. [67].

Bastidas et al. performed X-ray crystallographic studies with the catalytic subunit of murine PKA, Mg^{2+}, and an ATP analog (AMP-PNP, 5'-adenylyl-β, γ-imidodiphosphate) [67]. This is a stable ATP congener that ordinarily fails to react; surprisingly these investigators found that the analog transfers its terminal phosphoryl group to a peptide substrate. They examined crystals that contained 55% intact AMP-PNP and an unphosphorylated peptide substrate and 45% displaying transfer of the γ-phosphoryl group onto the substrate peptide yielding a phosphorylated peptide and AMP-PN. These structures implicated $Mg^{2+}(2)$ as the more stably-bound ion. Following the transfer reaction, $Mg^{2+}(2)$ recruits a water molecule to retain its octahedral coordination geometry and it remains in the active site while $Mg^{2+}(1)$ is released. Before the studies of Jacobsen et al. [57] and Bastidas et al. [67], the evidence seemed to indicate that $Mg^{2+}(2)$ was released first and $Mg^{2+}(1)$ was released afterward [63,64,66].

The catalytic cycle for VEGFR2 [61], which is similar to that proposed for PKA [57,67] is summarized in Fig. 6. $Mg^{2+}(1)$ and $Mg^{2+}(2)$-ATP bind to the protein kinase, but the order of their binding was unclear [61]. The results of Armstrong et al. indicate that binding of any divalent metal is very weak in the absence of ADP or ATP [62] and this suggests that $Mg^{2+}(2)$-ATP binds before $Mg^{2+}(1)$, which is then followed by the binding of the protein substrate under turnover conditions. The enzyme then catalyzes the transfer of the phosphoryl group to the protein substrate. $Mg^{2+}(1)$ and the phosphorylated protein substrate are released, but the order of their release is unclear. $Mg^{2+}(2)$-ADP is released during the last and rate-limiting step of the catalytic cycle. Bastidas et al. suggest that the mechanism of all protein kinases will require two magnesium ions for catalysis [67]. The studies summarized in Table 3 are consistent with this notion for protein kinases in general and for Src in particular. However, additional X-ray crystallographic work with bound nucleotide and peptide/protein substrate or inhibitor under conditions of high $[Mg^{2+}]$ would be helpful in confirming a role for two magnesium ions in Src biochemistry.

In contrast to the above studies, Gerlits et al. reported that PKA can mediate the phosphorylation of a high affinity peptide (SP20) in the absence of a divalent cation [68]. This study demonstrated that divalent metals greatly enhance catalytic turnover. Moreover, in the absence of Mg^{2+} or other metal, these investigators showed that PKA mediates but a single turnover from ATP to the high-affinity peptide. In another study, Mukherjee et al. reported that Ca^{2+}/calmodulin-activated Ser-Thr kinase (CASK) functions without a divalent cation [69]. This enzyme, which lacks the critical D of DFG that binds to Mg^{2+}, was thought to be an inactive pseudokinase. However, CASK exhibits catalytic activity and, surprisingly, catalysis is actually inhibited by Mg^{2+}, Mn^{2+}, or Ca^{2+}.

Targeting the Mg^{2+} binding sites

The elucidation of the role of two Mg^{2+} ions suggests other strategies for the development of Src inhibitory drugs. The design of ligands that bind to the ATP-binding pocket with an extension that interacts with either the metal-binding asparagine within the catalytic loop or the metal-binding aspartate at the beginning of the activation segment promises to yield new types of protein kinase inhibitor. Along these lines, Peng et al. developed EGFR inhibitors that form a salt bridge with Asp831 of its DFG-motif [70], which may interfere with the binding of $Mg^{2+}(1)$ to the enzyme. It remains to be established whether this strategy will have general applicability.

Src signaling and cancer

Src is a non-receptor protein tyrosine kinase that participates in numerous signaling pathways [4]. Src interacts with several protein-tyrosine kinase receptors at the plasma membrane. The flow of information is bi-directional with the receptors affecting Src activity and *vice versa.* Src interacts with EGFR (ErbB1) and ErbB2, two key protein-tyrosine kinase receptors. EGFR mutations occur in NSCLC and ErbB2 overexpression is associated with breast cancer. The ErbB family is also implicated in colorectal, stomach, head and neck, and pancreatic carcinomas as well as glioblastoma [71,72]. c-MET, or hepatocyte growth factor receptor (HGFR), is a protein-tyrosine kinase receptor that participates in embryonic development, wound healing, cell migration, and angiogenesis [73,74]. c-Met is produced by cells of epithelial origin while HGF is produced by mesenchymal cells. c-Met is dysregulated in many types of human malignancies, including cancers of bladder, brain, breast, kidney, liver, pancreas, prostate, stomach, and NSCLC. Aberrant activation of the pathway has frequently been found in human cancers *via* protein overexpression, mutation, gene amplification, and paracrine or autocrine up-regulation of the receptor ligands. Somatic c-MET mutations in the tyrosine kinase domain (21 mutations), juxtamembrane domain (5 mutations), and extracellular domain (16 mutations) have been described in many solid tumors, including hereditary and sporadic human papillary renal carcinomas, lung cancer, ovarian cancer, childhood hepatocellular carcinomas, squamous cell carcinoma of the head and neck, and gastric cancer [73]. c-Met overexpression is associated with invasion, migration, and metastatic disease.

Platelet-derived growth factor (PDGF) signaling participates in cell division, growth, migration, survival, and in angiogenesis [75]. PDGF signals *via* its receptor protein-tyrosine kinases (PDGFRα/β). PDGFR activity is implicated in breast, colorectal, and prostate cancers, GIST, glioblastoma, osteosarcoma, NSCLC, and neuroblastoma. Point mutations in the PDGFRα gene occur in about 5% of human GISTs. Moreover, increased expression of this gene occurs in 5–10% of glioblastomas. Amplification of PDGFRα has also been observed in oligodendrogliomas, esophageal squamous cell carcinoma, and artery intimal sarcomas. PDGFRα/β activation participates in cellular invasion and metastasis.

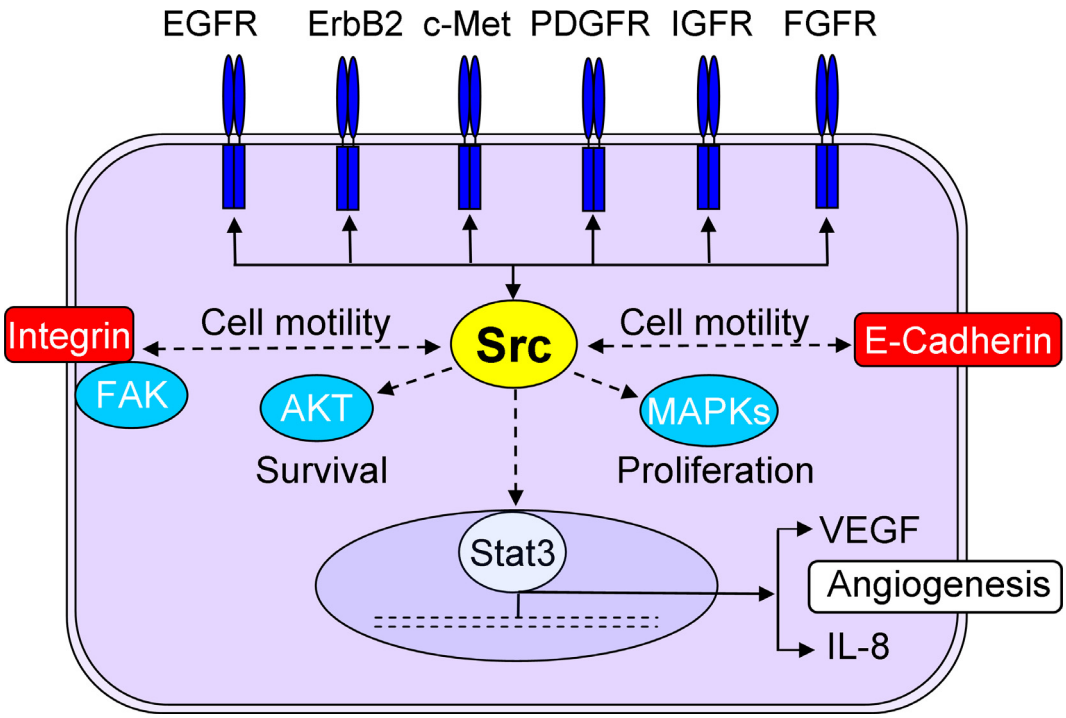

Fig. 7. Src signaling pathways. FAK, focal adhesion kinase; MAPKs, mitogen-activated protein kinases.

Insulin-like growth factors (IGF-1 and -2) participate in several processes including cell division, growth, survival, angiogenesis, wound healing, and embryonic development [76,77]. These growth factors bind to the insulin-like growth factor receptor (IGFR) and to the insulin receptor; they bind to IGFR with higher affinity than they bind to the insulin receptor. IGFR dysregulation is implicated in a variety of human cancers including breast, colorectal, and prostate cancers, NSCLC, and sarcomas. IGFR dysregulation is related in part to increased production of IGF-1 and -2.

In humans, 22 members of the fibroblast growth factor (FGF) family and four protein-tyrosine kinase receptors (FGFR1–4) have been identified. FGFR signaling regulates cell growth and division in many cell types in addition to fibroblasts. FGFR signaling is involved in angiogenesis, wound healing, and embryonic development. FGF family signaling is implicated in hepatocellular carcinoma, melanoma, lung, breast, bladder, endometrial, head and neck, and prostate cancers [78]. Point mutations and gene amplification/overexpression of members of the FGFR family are responsible for dysregulation and oncogenesis. Point mutations have been described in 50–60% of urothelial carcinomas and gene amplification has been described in 10% of breast carcinomas.

Besides protein-tyrosine kinases, Src-family kinases are controlled by integrin receptors, G-protein coupled receptors, antigen- and Fc-coupled receptors, cytokine receptors, and steroid hormone receptors [4]. Src participates in cell migration and motility by interacting with integrins, E-cadherin, and focal adhesion kinase (Fig. 7) [30]. Src participates in pathways regulating cell survival, proliferation, and regulation of gene expression [4]. The enzyme also plays an essential role in bone formation and remodeling and may play a role in breast, prostate, and lung cancer metastasis to the skeleton.

Therapeutic small molecule Src inhibitors

Src as a drug target

The role of v-Src in oncogenesis eventually led to the discovery of the Src proto-oncogene and then to the discovery of all of the other members of the Src family of protein kinases. Src drug discovery has been aimed at the role of Src in oncogenesis. Indeed, most of the FDA-approved small molecule inhibitors of protein kinases are directed toward neoplastic diseases (www.brimr.org/PKI/PKIs.htm). Unlike BRAF or EGFR mutants or BCR-Abl fusion proteins, Src is not a primary driver of tumorigenesis, but rather it is a participant in many pathways promoting cell division and survival. Moreover, Src mutants in tumors are very rare. Thus, it is unlikely that anti-Src monotherapy will be efficacious in the treatment of cancers. Since Src is a participant in many aspects of cell division, invasion, migration and survival, Src inhibition may play an important auxiliary role in various cancer treatments as described in the next section.

Src inhibitors that are FDA-approved or in clinical trials

As noted above, Src and Src family kinases have been implicated in the neoplastic process for three decades and extensive work on the development of Src inhibitors has been performed [79]. Src is downstream from such oncogenic drivers as EGFR, ErbB2, and BCR-Abl. Signals downstream from these oncogenic drivers include the Ras/Raf/ERK cell division pathway and the phosphatidylinositol 3-kinase and protein kinase B (Akt) cell survival pathway [80], which are pathways that involve Src. Thus far there appears to be no prognostic biomarkers related to Src activity that can be used for patient selection in clinical trials. Moreover, Src-specific kinase inhibitors have not made their way into the clinic.

Four orally effective Src/multikinase inhibitors are FDA-approved for the treatment of various malignancies (Table 4). Bosutinib is a BCR-Abl, Src, Lyn, Hck, Kit, and PDGFR inhibitor that is approved for the treatment of Ph^+ (i) CML and (ii) ALL (Fig. 8). This drug is currently in clinical trials for the treatment of breast cancer and glioblastoma. Dasatinib is an inhibitor of BCR-Abl, Src, Lck, Fyn, Yes, PDGFR, and other kinases that is approved for the treatment of CML. This drug is undergoing numerous clinical trials for various solid tumors and for ALL. Ponatinib is an inhibitor of BCR-Abl, PDGFR, VEGFR, Src family and other kinases that is approved for

Table 4
Selected orally effective Src/multikinase small molecule inhibitors.

Drug	Known targets	PubChem CID[a]	Clinical indications[b]	References
Bosutinib	BCR-Abl, Src, Lyn, Hck, Kit, PDGFR	5328940	Ph^+ CML[b], Ph^+ ALL[b], breast cancer, glioblastoma	[81–83]
Dasatinib	BCR-Abl, Src, Fyn, Yes, Lck, Arg, Kit, EphA2, EGFR, PDGFRβ	3062316	Ph^+ CML[b], Ph^+ ALL, breast, colorectal, endometrial, head and neck, ovarian, and small cell lung cancers, glioblastoma, melanoma, and NSCLC	[81,84]
Ponatinib	BCR-Abl, Src family kinases, VEGFR, PDGFR, FGFR, Eph, Kit, RET, Tie2, Flt3	24826799	Ph^+ CML[b], Ph^+ ALL[b], endometrial, GIST, hepatic biliary, small cell lung, and thyroid cancers	[81,85]
Vandetanib	RET, Src family kinases, EGFR, VEGFRs, Brk, Tie2, EphRs	3081361	Medullary thyroid cancer[b], breast, head and neck, kidney cancers, NSCLC, and several other solid tumors	[86–88]
Saracatinib (AZD0530)	Src, BCR-Abl	10302451	Colorectal, gastric, ovarian, small cell lung cancers, NSCLC, and metastatic osteosarcoma in lung	[81,89]
AZD0424	Src, BCR-Abl	None	Early clinical trials for numerous solid tumors	http://www.clinicaltrials.gov/

[a] The PubChem CID (chemical identification no.) from the National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed) provides the chemical structure, molecular weight, number of hydrogen-bond donors/acceptors, and bibliographic references.
[b] Indication approved by FDA, otherwise in clinical trials.

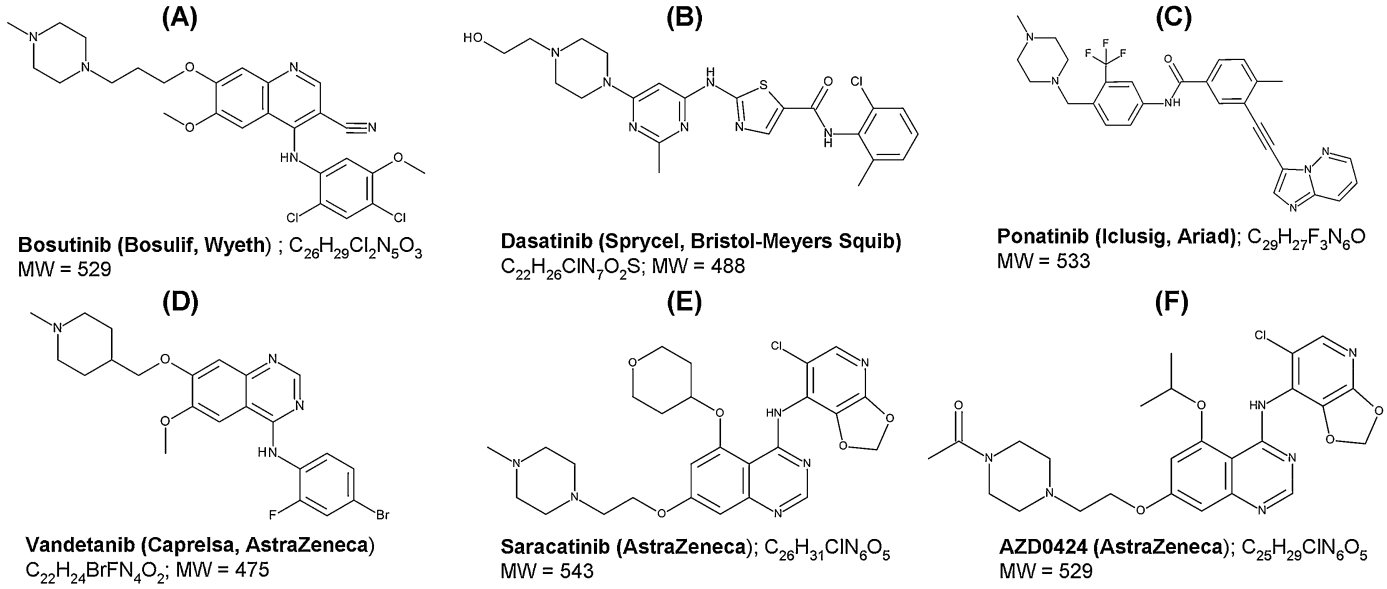

Fig. 8. Structures of selected Src/multikinase inhibitors approved by the FDA or in clinical trials.

the treatment of CML and ALL. It too is undergoing clinical trials for several solid tumors.

Vandetanib is an inhibitor or EGFR, VEGFR, RET, Src family and other kinases that is approved for the treatment of medullary thyroid carcinoma, and it is in clinical trials for numerous solid tumors (Table 4). With the exception of vandetanib, the currently FDA-approved disease targets of these drugs are hematologic in nature and not directed against solid tumors. Saracatinib (AZD0530) is a Src and BCR-Abl inhibitor that is undergoing clinical trials for colorectal, gastric, ovarian, small cell lung cancers, NSCLC, and metastatic osteosarcoma in lung (www.clinicaltrials.gov). A related drug (AZD0424) is in stage I clinical trials for numerous solid tumors. KX01, KX2-391, XL228, XL99, and XLI-999 are Src inhibitors that were in clinical trials against various disorders, but they have a low likelihood of advancing in the clinic.

Bosutinib [90], dasatinib [91], ponatinib [92], saracatinib [93], and AZD0424 [94] were initially developed as Src/Abl inhibitors and vandetanib [95] was initially developed as a VEGFR2 inhibitor. Inhibition of Abl by bosutinib, dasatinib, and ponatinib accounts for their approval for the treatment of Ph^+ CML. The data in Table 4 indicate that these drugs inhibit more than their initial targets, and this property is shared by most of the FDA-approved kinase inhibitors (www.brimr.org/PKI/PKIs.htm). Whether these drugs are clinically effective for the treatment of various solid tumors and whether such effectiveness is related primarily to Src inhibition or to the inhibition of other protein kinases remains to be determined.

The ATP-binding pocket of Src

The glycine-rich loop occurs universally in protein kinases and consists of a canonical GxGxΦG sequence where Φ refers to a hydrophobic residue. In Src this sequence consists of GQGCFG (Table 2). The glycine-rich loop, which forms a lid above the ATP phosphates, is characteristically one of the most mobile portions of the protein kinase domain. This mobility may be due to the requirement that the enzyme binds ATP and then releases ADP following catalysis. The penultimate phenylalanine and third glycine of the glycine-rich loop of PKA anchor the β-phosphate of ATP (PDB ID:

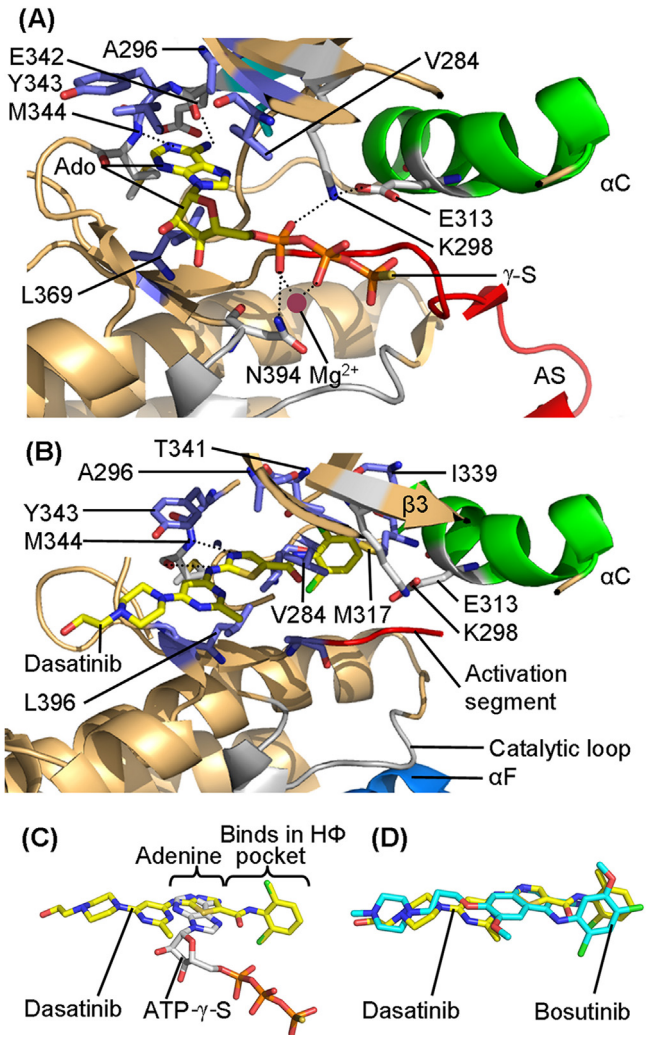

Fig. 9. (A) Binding of ATP-γ-S to active chicken Src (PDB ID: 3DQW). (B) Binding of dasatinib to active human Src (PDB ID: 3QLG). (C) Superposition of ATP-γ-S and dasatinib bound to Src. (D) Superposition of dasatinib (PDB ID: 3QLG) and bosutinib (PDB ID: 4MXO) bound to Src. The dotted lines indicate polar contacts. Ado, adenosine; AS, activation segment; HΦ, hydrophobic.

4DH3) [96]. These glycine residues in Src are close to the phosphates of ATP, but they do not form polar contacts with it (PDB ID: 3DQW). Additional structural studies of Src with (i) ATP or an ATP analog along with (ii) high [Mg^{2+}] and (iii) a peptide substrate or inhibitor would provide a better understanding of ATP-phosphate interaction with the glycine-rich loop.

The exocyclic amino group of ATP characteristically interacts with the peptide backbone of one or more hinge residues, which connect the small and large lobes. The 6-amino group of the adenine ring of an ATP analog (ATP-γ-S) forms a hydrogen bond with the carbonyl oxygen of Glu342, the first hinge residue of Src (PDB ID: 3DQW) [97]. The N-1 of the adenine ring forms a hydrogen bond with the main chain —NH group of the Met344 hinge residue (Fig. 9A). The α-phosphate binds to Lys298 of the β3-strand, which in turn forms a salt bridge with Glu313 of the αC-helix. The α- and β-phosphates of ATP bind to Mg^{2+}, which coordinates with Asn394 of the HRD catalytic loop. The adenine base extends only to the β3-strand (A296), but not to the distal β4-strand or the adjacent αC-helix, which is in contrast to most small molecule ATP-competitive inhibitors.

ATP-competitive Src inhibitors

The two main classes of reversible ATP-competitive protein kinase inhibitors are named type I and type II [98]. Type I inhibitors bind to the DFG-Asp in enzyme conformation and the type II inhibitors bind to the DGF-Asp out conformation. The X-ray crystallographic structures of dasatinib [99] and bosutinib bound to Src demonstrate that these are type I inhibitors. The structures of ponatinib, vandetanib or saracatinib bound to Src have not been reported (www.pdb.org). The interactions of dasatinib and bosutinib with Src are similar so that only dasatinib is considered in detail here.

Most, if not all, ATP-competitive protein kinase inhibitors interact with the peptide backbone of hinge residues, and dasatinib and bosutinib are not exceptions. The thiazole nitrogen of dasatinib forms a hydrogen bond with Met344 of the hinge (Fig. 9B). ATP-competitive protein kinase inhibitors generally interact with the nearby C-spine residues. In the case of Src, dasatinib interacts with Ala296 and Leu396. It also forms hydrophobic contacts with Tyr343, Thr341, Ile339, and Val284. Thr341 is the gatekeeper residue and Ile339 is the Sh3 shell residue. Dasatinib also extends to the αC-helix and makes hydrophobic contacts with Met317, which is RS3 of the regulatory spine. Bosutinib has hydrophobic interactions with all of these residues with the exception of Ile339 (PDB ID: 3QLG) (not shown).

Adenine interacts with residues within the β1, β2, and β3-strand in the N-lobe while most ATP-competitive inhibitors including dasatinib and bosutinib extend into a region called hydrophobic pocket II or the back pocket [98] that continues past the β5 and β4-strands to the αC-helix. Both dasatinib and bosutinib bind to the active form of Src with the αC-helix in conformation (Lys298 and Glu313 are kissing) and with the activation segment in its open conformation. The superposition of bound ATP-γ-S and dasatinib depicts the extension of the drug that extends into the hydrophobic pocket (Fig. 9C). Moreover, the superposition of dasatinib and bosutinib bound to Src illustrates their binding similarities (Fig. 9D).

Epilogue

Protein kinases participate in cell signaling and the regulation of many biochemical activities [5]. Protein-tyrosine kinases occur in animals, but are lacking in bacteria, yeast, and plants. In 1979, Eckhart et al. identified phosphotyrosine in polyoma T antigen precipitates [100]. In 1980, Hunter and Sefton reported that the transforming gene product of Rous sarcoma virus (v-Src) catalyzes the phosphorylation of tyrosine, which represents the first study explicitly demonstrating protein-tyrosine kinase activity [101].

Czernilofsky et al. reported the amino acid sequence of the Schmidt-Rupin strain of v-Src in 1980 based upon its nucleotide sequence [102,103], and Shoji et al. reported the sequence of the catalytic subunit of bovine PKA in 1981 using Edman degradation of cyanogen bromide and tryptic peptides [104]. Owing to the incomplete nature of the protein sequence databases at the time, the identification of v-Src as a protein kinase was not made until 1982 [105]. The signatures that enabled this association were the glycine-rich loop and sequence similarity near Src Lys298 and PKA Lys71, the latter of which Zoller and Taylor identified as the residue that reacts with *p*-fluorosulfonylbenzoyl-5′-adenosine, an ATP analog [106]. We can now identify DFG, APE, and H/YRD as common components in each of these published sequences [102–105]. Thus, the first known primary structure of any protein kinase was that of v-Src, another noteworthy distinction for Src kinase.

There are currently 250 or more protein kinase inhibitors in various stages of clinical development worldwide. Such drug discovery

programs have led to the approval of more than two dozen orally effective protein-kinase inhibitors by the FDA during the past dozen years (www.brimr.org/PKI/PKIs.htm) Nearly all of these approved drugs are used for the treatment of neoplastic disorders. However, we can expect advances in clinical efficacy and subsequent approval of new drugs targeting additional protein kinases and illnesses such as hypertension, Parkinson's disease, and autoimmune diseases [107].

Peyton Rous initiated work on a sarcoma, or skeletal muscle tumor, in a Plymouth Rock chicken more than a century ago [1]. He demonstrated that injection of a cell-free tumor filtrate into the muscle of susceptible fowls produced sarcomas. Rous considered the notion that the neoplastic cells might elaborate a chemical stimulant that could produce the tumor in another host. He also suggested that this transmission may be caused by an "ultramicroscopic organism", which we now equate with the Rous sarcoma virus. Rous was nominated for a Nobel Prize as early as 1926, but he did not receive it until 1966. His early work led to fundamental discoveries on the cell cycle, cell growth and death, cell-cell signaling, cell morphology and motility, cancer biology, and cancer therapy.

Conflict of interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.01.003.

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