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High Throughput and Mechanistic Analysis of the HRD Motif of Src Protein Tyrosine Kinase

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HIGH THROUGHPUT AND MECHANISTIC ANALYSIS OF THE HRD MOTIF OF SRC PROTEIN TYROSINE KINASE

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ABSTRACT

Protein tyrosine kinases (PTK) play essential roles in cell signal transduction pathways. PTKs are associated with numerous diseases and promote oncogenic cell signal transduction in unregulated forms. Thus PTKs represent a large group of potential targets for anticancer drug discovery. Src was the first identified oncogene and has been intensively studied for decades. The work described in this dissertation studied the structure-function relationships of Src, to build a knowledge base for the function of conserved residues in the kinase domain. To accomplish this, the first focus of this dissertation was to develop a high throughput screening method to identify kinase functionalities regarding catalytic activity and substrate specificity. The developed method utilizes a bacterial screen system and detects phosphotyrosine in cell lysate by a high throughput manner. Combining the developed functional screening with a rational mutation library, functional mutants can be identified for further analysis. This in vivo strategy was validated by model PTKs and could be universally applied to study kinase catalytic activity and substrate specificity. A successful application of the developed method on the study of the conserved HRD motif in Src led to a more comprehensive understanding of the role of HRD in the kinase function.

The second focus of the dissertation was to study the regulatory function of the arginine (R385) in the HRD motif of Src, since R385 was found unnecessary for catalysis in the first part of this dissertation. The effect of R385 mutation on the key

regulatory mechanisms of Src and binding affinity towards PTK inhibitors was examined. First, autophosphorylation of Tyr416 in Src activates this enzyme, and overrides the negative regulation by Csk that phosphorylates Src on Tyr527. However, mutation of R385 to other residues abolished this override. The presence of the arginine prevents the transition of the active Src to inactivated Src. Second, type II inhibitors bind to the inactive conformation of Abl but not to Src even through Src and Abl are structurally very similar. Mutation of R385 greatly increased the affinity of Src towards type II inhibitors, but had no effect on type I inhibitor binding, which targets the active conformation. All together, when induced by Csk phosphorylation or PTK inhibitors that target the inactive conformation of kinase domain, R385 behaves as an energy barrier in the conformational transition from active to inactive conformation. The replacement of the R385 by any other amino acid made the transition much easier. R385 is the first identified residue among protein tyrosine kinases, that controls conformational transitions. This study sheds light on the fundamental mechanism of regulatory conformational changes and provides more insight on design and development of PTK inhibitors.

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PREFACE

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CHAPTER 1

Introduction

1.1 Introduction to protein kinases

Upon the completion of the Human Genome Project[1], 518 putative protein kinase genes have been identified, which is a strikingly large number and constitutes about 1.7% of all human genes. This makes protein kinases one of the most important enzyme families.

1.1.1 Signal transduction and protein kinases

Protein kinases mediate protein phosphorylation via transferring the γ-phosphate of ATP to a target protein. Protein phosphorylation is a structural modification, which often results in a conformational change of the target protein. Conformational change of the protein further leads to functional and regulatory change. As a post-translational modification, protein phosphorylation mediates signal transduction and intracellular communication that controls diverse cellular processes, including growth, development, division, replication, transcription, differentiation, metabolism, apoptosis, homeostasis, motility and structural rearrangements. It has been estimated that 30% of all cellular proteins are phosphorylated on at least one site [2, 3].

1.1.2 Substrate specificity of protein kinases

All protein kinases phosphorylate target proteins on a select group of amino acid side chains, including serine, threonine, or tyrosine, and thus protein kinases are classified as protein serine/threonine kinases and protein tyrosine kinase. Among identified protein kinases, each has one unique set of physiological substrates and the number of substrates can be very different. For example, the Src family kinases have a wide range of protein substrates while Csk family kinases display extremely narrow range of substrates[4, 5].

Protein kinases phosphorylate their substrates selectively in order to maintain the specificity and fidelity of diverse signaling pathways, which can be achieved by different regulatory mechanisms. The local determinants around the active site can direct the protein kinases to specific consensus phosphorylation sequences. The distal docking parts of protein kinase and other adaptor protein or regulatory proteins can also contribute to the high specificity[6].

1.2 Introduction to protein tyrosine kinases (PTKs)

Among human protein kinases identified, 90 have been identified as protein tyrosine kinases. Despite the relatively large number of tyrosine kinases, phosphorylation of tyrosine only accounts for less than 1% of total protein phosphorylation. Besides, most tyrosine kinases are tightly regulated and only activated under specific conditions. Unlike phosphorylation of serine or threonine kinase, phosphorylated tyrosine rarely plays a structural role in proteins, and its role primarily regulatory[2]. All these imply the importance of the tyrosine kinase.

1.2.1 Receptor tyrosine kinases and non-receptor tyrosine kinases

There are two main classes of protein tyrosine kinases: receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). Humans have 58 known RTKs, falling into 20 subfamilies and 32 nRTKs falling into 10 subfamilies.

All RTKs possess an extracellular domain containing a ligand binding region, a trans-membrane domain, and a cytoplasmic domain containing the kinase domain, thus RTKs can directly receive extracellular signals. Growth factor binding activates RTKs by inducing dimerization of receptor tyrosine kinases, including members of the fibroblast, epidermal, vascular, endothelial, and insulin-like growth factors. Ligand-induced dimerization leads to activation of the intracellular tyrosine kinase domain by trans-phosphorylation, which is the fundamental regulatory mechanism

mediating receptor tyrosine kinase activation. Activated RTKs will then proceed to phosphorylate one or more downstream proteins and enzymes, initiating signaling cascades.

Instead, nRTKs are referred to as cytosolic and do not exclusively associate with the cell membrane. nRTKs receive signals through the association with intracellular domains of other transmembrane receptors, including RTKs[7]. In addition, some nRTKs have specific cytosolic function.

1.2.2 Src family kinases (SFK) and Src

The Src family kinases (SFKs) belong to nRTKs and are known as major components to many growth factor signaling pathways. The SFKs include 8 members: Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, and Yes. Abnormally elevated SFK activity levels are closely associated with oncogenesis in humans, particularly in certain types of colon cancers, melanoma, leukemia, and lymphoma.

Src was the first identified oncogene and has been the subject of intense investigation for decades [8]. Src has been implicated in playing significant roles in both normal and pathological states. Src is found expressed ubiquitously and plays key roles in cell morphology, motility, proliferation, and survival[9]. 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. In breast cancer cells, members of the epidermal growth factor family are active and may lead to activation of Src in the production of neoplasms. Src is also found activated in colon, gastric, lung, pancreatic, neural, and ovarian neoplasms[10-12]. Thus, extensive work on the development of Src inhibitors has been performed.

Unlike BRAF or EGFR mutations or BCR-Abl fusion proteins, Src is not a primary driver of tumorigenesis and Src mutants in tumors are very rare[11]. Most of the time, Src participates in many pathways promoting cell division, invasion, migration and survival and is downstream from the oncogenic drivers.

1.3 Structure of Src tyrosine kinase

1.3.1 Domain organization of Src tyrosine kinase

From amino-terminus to carboxyl-terminus, Src contains six distinct functional domains: Src homologous domain 4 (SH4) with a 14-carbon myristoyl group attached, unique domain, SH3 domain, SH2 domain, SH2-kinase linker, protein tyrosine kinase domain (the SH1 domain), and a C-terminal regulatory segment.

In the SH4 domain, seven amino acids beginning with glycine are required for the myristoylation of Src. Myristoylation facilitates the attachment of Src to membranes and plays essential roles in SFK localization in the cytosol[13, 14]. Myristoylation, subsequent membrane association and the Src protein kinase are involved in transforming cells into a neoplastic state.

The unique domain is unique for each Src family member. The unique domain of Src has been proposed to be important for mediating interaction with receptors or proteins, which are specific for each family member[9].

The SH3 domain, around 60 amino acids, specializes in recognition of proline-rich peptide sequences and adopts a left handed helical conformation, consisting of five antiparallel β sheets and two prominent loops. SH3 contributes greatly to localization and regulation of kinases or other SH3 domains [15].

The SH2 domain, around 100 amino acids, consists of a central three-stranded β-sheet with a single helix packed against each other. The SH2 domain binds to distinct phosphorylated tyrosine containing peptides and is responsible for

localization to RTKs and other enzymes that have been activated by tyrosine phosphorylation.

Also, SH2 and SH3 have significant importance in Src function regulation. They inactivate catalytic activity via intramolecular contacts and displacing the intracellular SH2 or SH3 domains can activate Src activity. Proteins containing SH2 and SH3 can enhance their ability to function as substrates for Src protein tyrosine kinases[16].

1.3.2 Architecture of Src catalytic domain

The Src catalytic domain consists of the featured bi-lobed protein kinase architecture, a small amino-terminal lobe known as ATP-binding lobe and a large carboxyl-terminal lobe known as peptide binding lobe. The N lobe is composed of a five-stranded β sheet (β1-β5) and a prominent α helix, called helix αC. The C lobe contains six conserved segments (αD-αI) and seven short β sheets (β6-β12). The active site is located in the cleft between these two structural lobes[17]. ATP is bound to the deep cleft and sits beneath a highly conserved loop connecting strands β1 and β2, called the ATP-phosphate binding loop. Peptide substrates bind in an extended conformation of active site with the assistance of a centrally located loop, known as the activation loop. The activation loop typically has around 20~30 amino acids and provides a platform for the peptide substrate. In most protein kinases, it begins with

a conserved DFG motif and ends with a conserved APE motif. Moreover, the initial five residues of the activation loop make up the magnesium-positioning loop. In most kinases, there is an autophosphorylation site, which is responsible for activation of kinase activity.

1.3.3 Catalytic signatures of Src

Protein kinases share highly conserved catalytic domains in the primary sequence and these conserved individual amino acids are implied with significant catalytic importance[17]. The chicken numbering system is used in this dissertation. Among these conserved subdomains, a K/D/D motif is highly featured as the catalytic core in the catalytic domain of Src. Lys295 in the K/D/D motif, forms ion pairs with the α- and β- phosphates of the ATP and with Glu310 in the αC helix. Asp386, as the catalytic base, occurs in the conserved motif HRD in the catalytic loop. It is thought to orient the tyrosyl group of the substrate protein in a catalytically competent state. It functions as a base that abstracts a proton from tyrosine thereby facilitating its nucleophilic attacks of the γ-phosphorus atom of MgATP. Asp404 located in the activation loop, is thought to bind Mg^{2+} or Mn^{2+} to coordinate the β- and γ-phosphate groups of the ATP. Mg^{2+} is more likely the cation catalyst in the physiological state.

Comparison of residues exposed on the surface of active and inactive kinases revealed a highly conserved spatial motif that was found in every active kinase but

missing in inactive kinases. Local Spatial Pattern (LSP) alignment was developed to compare and identify spatially conserved residues[18, 19]. This computational analysis[18, 19] leads to the identification of hydrophobic structural skeletons of catalytic domain, which suggests a catalytic spine with four hydrophobic residues and a regulatory spine with eight hydrophobic residues.

Regulatory spine or R-spine consists of four inconsecutive residues, two from the N-lobe: Leu325 from the β4 strand and Met314 from the αC helix; and two from the C-lobe: Phe405 from DFG in the activation loop and His384 from HRD in the catalytic loop. The backbone of H384 is anchored to the αF helix by a conserved aspartate residue (Asp447), which serves as a base to the R-spine. The correct alignment of the R-spine is essential for substrate positioning.

The catalytic spine or C-spine positions ATP and governs catalysis by facilitating ATP binding. Two residues belongs to C-spine locate in the N lobe, Val281 in β2 and Ala293 in the AXK motif of β3, directly docked onto the adenine ring of ATP. In the C lobe, it is the Leu393, which is directly docked onto the adenine ring. Leu392 and Leu394 flank the Leu393 and rest on a hydrophobic residue from the D-helix, Leu346. Leu346 is in turn bound to the F-helix via Leu451 and Leu455.

The F helix is highly hydrophobic and functions as an organizing element for the entire kinase core: C-spine anchors to the C terminus of the F helix ; R-spine anchors to the N terminus of the F helix.

1.4 Regulation of Src tyrosine kinase

1.4.1 Phosphorylation and dephosphorylation of Src

Protein phosphorylation is a reversible and dynamic process that is mediated by protein kinases and phosphoprotein phosphatases. Phosphorylation by a protein kinase can stimulate or inhibit the activity of the substrate proteins, or provide binding sites for the downstream effectors depending on the substrate and pathway. Phosphoprotein phosphatases, which counteract the action of kinases, can reverse the activity of substrate proteins or eliminate binding sites for downstream effectors. Src and Src family protein kinases are tightly regulated by phosphorylation and dephosphorylation and the dynamic effect of protein phosphatase and protein kinase is crucial for substrate function[20-22].

One of the most important phosphorylation sites of Src is Tyr527, located on the C terminal tail of Src (Figure 1. 1). Under basal conditions in vivo, 90-95% of Src is phosphorylated at Tyr527[23]. Phosphorylation of Tyr527 inhibits Src catalytic activity by engaging the SH2 domain in an intracellular interaction. This interaction positions the SH3 domain and the connecting SH2 linker to the surface of the N lobe of the catalytic domain, stabilizing the helix αC in an inactive conformation. The C-terminal Src kinase (Csk)[24] and the Csk homology kinase (Chk)[25] catalyze the phosphorylation of the inhibitory tyrosine of Src. Csk is expressed in all mammalian cells, whereas Chk is limited to breast, hematopoietic cells, neurons and testes. Whereas Chk inactivates Src by catalytic phosphorylation, Chk forms an inhibitory

non-covalent complex with Src[26].

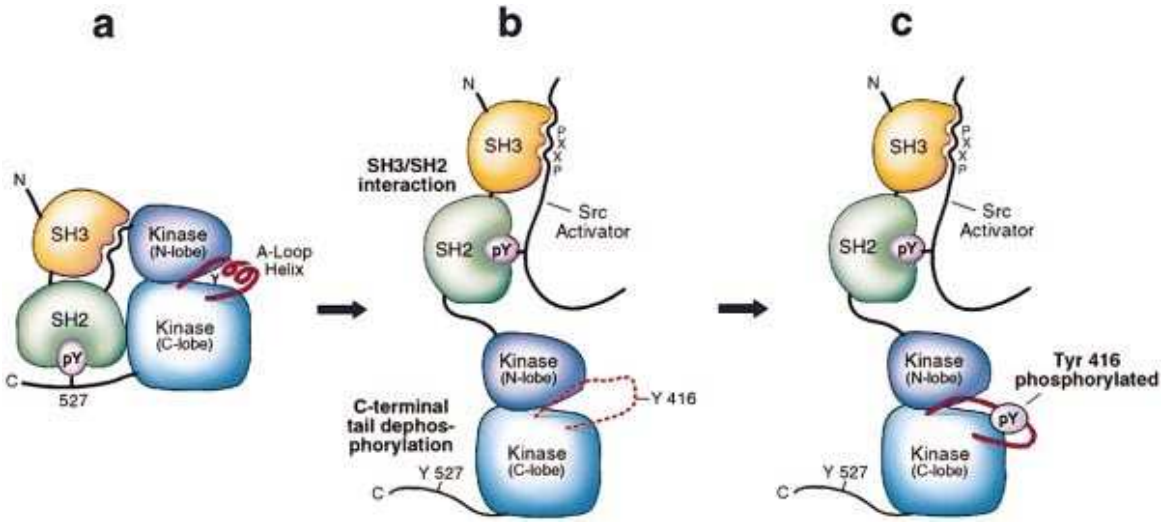
Another major phosphorylation site of Src is Tyr416 (Figure 1. 1). It can undergo autophosphorylation by another Src kinase molecule and then initiate a rearrangement of the activation loop of the Src into its active configuration, which stabilizes Src in its active conformation. When unphosphorylated, the activation loop adopts a conformation blocking the binding of peptide substrate[27-29]. Furthermore, autophosphorylation on the activation loop overrides the inhibitory regulation by the phosphorylation on Tyr527[30]. This regulation provides an additional layer of control beyond the inactivation by Csk. Phosphorylated Tyr416 and rearranged activation loop together play a kinetic proofreading role. The inhibition by Tyr527 phosphorylation is also found to be overridden by Tyr213 phosphorylation[31]. This kind of phenomenon has been well recognized in protein phosphorylation reactions as a way of error correction. Many substrates need to be phosphorylated at multiple sites before their function is altered, or primed by phosphorylation before phosphorylation on the second site of the substrate.

In addition to the two major phosphorylation sites of Src discussed above, the platelet-derived growth factor receptor (PDGF receptor) mediates the phosphorylation of Tyr 213 and actives Src activity. Tyr138 of Src can also be phosphorylated by PDFG receptor in vitro, but the role of it is not clear yet. Src is also phosphorylated by protein serine/threonine kinases.

Phosphorylated Tyr527 dephosphorylation by phosphatases is another

important mechanism for Src activation, including cytoplasmic PTP1B, Shp1 (Src homology 2 domain-containing tyrosine phosphatase 1) and Sph2, and transmembrane enzymes including CD45, PTPα, PTPε, and PTPλ[11]. PTP-BL also has been shown to dephosphorylate Src phosphotyrosine 416 in a regulatory manner. It is suggested that the cellular expression of phosphotyrosine phosphatase has a role on signal fidelity.

Figure 1. 1



Adapted from: Xu, W., et al., *Crystal structures of c-Src reveal features of its autoinhibitory mechanism.* Mol Cell, 1999. **3**(5): p. 629-38

Figure 1.1 A Model for Src Activation

(a) The inactive conformation of Src is stabilized by intramolecular interactions among the kinase domain, the SH2/SH3 domains, and the phosphorylated C-terminal tail. In this state, an inhibitory configuration of the activation loop helps disrupt the kinase active site by stabilizing a displacement of the helix αC, which interferes with substrate binding and protects Tyr416 from phosphorylation.

(b) Displacement of SH2 and/or SH3 domains, either by C-terminal tail dephosphorylation or by competitive binding of optimal SH2/SH3 domain ligands, allows the kinase domain to open, disrupting the inhibitory configuration of the activation loop and exposing Tyr416 to phosphorylation.

(c) Phosphorylation of Tyr416 initiates a conformational reorganization of the whole activation loop, relieving the steric barrier for substrate binding, allowing the helix αC to move back into the active site, and reconstituting a fully active tyrosine kinase.

1.4.2 Key elements regulating different conformations

Protein kinases can adopt different conformations through complicated regulations. Two extreme conformations can be a fully active conformation and a full inactive conformation[17, 32-35]. Structurally, the N lobe and C lobe can be oriented differently, which results in an open or closed active site cleft. The two lobes can move flexibly towards each other to open and close the cleft. The open form of the cleft is necessary to allow the access of the ATP to the catalytic site and the release of the ADP, and the closed form of the cleft is necessary to bring residues into the catalytic domain.

Within each lobe, there is an essential segment that contributes the conformation status of active site. In the N lobe, this segment is the major α-helix, known as the αC helix. The αC helix rotates and orients with respect to the rest of the kinase domain, stabilizing or destabilizing the active catalytic site. Glu310 in helix αC, as an absolute conserved residue located, plays an important role in regulating the conformational changes. In active conformation, the side chain of Glu310 forms a salt bridge with the side chain of Lys295 that coordinates the α- and β- phosphates of ATP. In addition, the helix αC makes direct contact with the N-terminal region of the activation loop, linking the conserved DFG motif. The interaction between the DFG motif and the Glu310-Lys295 salt bridge directly contribute to the conformation of helix αC to ATP binding. In the inactive conformation of kinases, the Glu310-Lys295 salt bridge is broken and Glu310 forms an alternative salt bridge with Arg385 or Arg409 in Src[29].

Different regulatory mechanisms often modulate kinase activity by altering the integrity of these interactions, thereby altering the conformation of helix αC.

In the C lobe, the activation loop adjusts to make or break part of the catalytic site. The activation loop has the capacity to undergo large conformational changes when the kinases switch between inactive and active state[36]. Phosphorylation of the activation loop stabilizes kinase in an open and extended conformation that is permissive for substrate binding, which is featured in two aspects. First the Asp404 in the DFG motif at the amino-terminal base of the activation loop binds to the magnesium ion. 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 the inactive conformation of Src[29], residues 413-418 of the activation loop form a short αhelix between the small and large loop of the kinase domain, which buries the side chain of Tyr416. 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.

1.5 Oncogene and therapeutic targets

Imatinib is the first developed small molecule compound used to treat Leukemia caused by the Philadelphia chromosome, which is a specific genetic defect resulting in an unregulated form of Abl. This unregulated Abl, known as BCR-ABL, alters the signal transduction and leads to leukemia ultimately. BCR-ABL represents a typical example of therapeutic targets and the success of imatinib guides continued investigation and development of small molecule inhibitors[37].

Currently, 27 orally effective direct kinase inhibitors have been FDA-approved targeting a limited number of enzymes[38]. Nearly all of the approved protein kinase inhibitors are steady-competitive enzyme inhibitors with respect to ATP and they interact with the ATP-binding pocket. Drugs can be tailored to bind specifically to targets that exhibit differences. Structural studies have exploited differences within the ATP-binding site and contiguous regions that can be used to provide specificity for protein kinase inhibitors.

1.5.1 Classification of protein kinase inhibitors

Inhibitor classification can be complicated given diverse interactions between inhibitor and protein tyrosine targets and the interaction of each inhibitor with a protein kinase target is unique. Nevertheless, it is useful to classify these interactions in order to apply them to the drug discovery process.

Small molecular protein kinase inhibitors have been characterized and classified

based on the binding property towards the target protein kinase [38-42]. Here we use the classification Robert Roskoski Jr proposed based on the structures of drug-enzyme complex determined by X-ray crystallography. These interactions are classified by the activation state of target protein kinases that specifically include the status of DFG-Asp and activation loop, the αC helix, and the regulatory spine. Generally, most inhibitors are ATP competitive and fall into type I or type II inhibitors, which are classified based on the states of the target enzyme. Type I protein kinase inhibitors are characterized to bind with the active form of their protein kinase target and type II inhibitors bind to the inactive conformation of the target protein kinase. The specific states of the essential elements of the active site further subdivide the type I inhibitors or type II inhibitors. Type III inhibitors are non-ATP competitive, occupying a site next to the ATP binding pocket so that both ATP and the allosteric inhibitor can bind to the target protein simultaneously. Type IV inhibitors bind covalently to their protein kinase target.

1.5.2 Development of protein kinase inhibitors in the future

Regarding the significant importance of the protein kinase in both physiological and pathological processes, we can foresee the demand on more targeted inhibitors for numerous illnesses. The continued study of protein kinase structure and its binding specificity towards current protein kinase inhibitors will serve as a basis for the fundamental and applied biomedical breakthroughs.

1.6 Objectives of this research

With the aim to study the structure-function relationship of PTKs regarding catalytic activity and substrate specificity, the first project was to develop a rapid high throughput screening method to select functional mutants of PTK. The second project was focused on the functional study of Arg385 regarding the regulation of Src, which was a functional mutant selected from the first project.

1.6.1 Development of a high throughput screening method to select functional mutants

Site-directed mutagenesis is a method widely used as a common biochemical approach to study structure-function relationship of PTKs. However, this strategy is time consuming and less informative. Thus, the aim of the first project was to screen catalytic activity and substrate specificity of PTKs in a high throughput manner with no need to purify each mutant. The rapid screening method enabled the detection of catalytic activity and the differentiation of substrate specificity among closely related PTKs. A successful application of this strategy on the functional study of the HRD motif of Src further validated the developed screening method. The strategy described can be broadly applicable to other protein kinases of interest and freely tailored based on distinct functional studies.

1.6.2 Functional study of R385 in the HRD motif regarding Src regulation

The first project led to a further study on the regulatory function of R385 and thus the importance of R385 on conformational transition of the active site was proposed and examined. The role of R385 on major Src regulations was explored and then the affinity of several PTK inhibitors was determined for both the R385 mutation and wild type. These experiments revealed the importance of R385 in controlling conformational changes and shed light on the selectivity of type II inhibitors.

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MANUSCRIPT 1

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Development of a High Throughput Method to Screen Functional Mutants of PTKs and its Application on the Study of the Functionality of the HRD Motif in Src

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2.1 Abstract

Protein Tyrosine Kinases (PTK) play important roles in both physiological and pathological signaling pathways and have been studied extensively as potential drug targets. Site-directed mutagenesis is one of the most traditional approaches for structure-function analysis of PTKs. However, this approach requires the generation and analysis of individual mutants and is time-consuming. In this study, a new strategy was proposed to study kinase functionality using a high throughput screening method. This new strategy utilizes a bacterial expression system to co-express kinase mutants and substrates, and screens for catalytic activity and substrate specificity by detecting phosphotyrosine in cell lysate in a high throughput manner. Combining a rapid functional screening with a rational mutation library, functional mutants can be identified for further analysis. This in vivo strategy has been validated by model PTKs and can be universally applied to study kinase catalytic activity and substrate specificity. A successful application of this strategy to study the functionality of the HRD motif in Src demonstrated the power of this strategy and led to further detailed understanding of the mechanism underlying the HRD motif.

2.2 Introduction

As a post-translational modification, protein phosphorylation plays essential roles in signal transduction and intracellular communication that control diverse cellular processes [2, 3]. Protein kinases mediate protein phosphorylation via transferring the γ-phosphate of ATP to the target proteins. Around 518 putative protein kinase genes were identified with completion of the Human Genome Project, and 90 of them have been identified as protein tyrosine kinases (PTK)[1]. Aberrant tyrosine kinase activities play crucial roles in numerous diseases and malignant transformation. Indeed, 51 out of the 90 tyrosine kinases are implicated in cancer either through mutation or altered expression[43]. These associations greatly encourage the development of inhibitors that would block the action of such protein tyrosine kinases. The stunning success of imatinib in treating chronic myelogenous leukemia (CML) further promotes that[44].

Src, as the first identified oncogene, has been the subject of intense investigation for decades[45], and serves as a model in the study of other PTKs. A full understanding of the oncogenic roles of protein tyrosine kinases on signal transduction and rational drug design require the knowledge of the structural basis of PTKs underlying the catalysis and substrate specificity. The catalytic domain of Src is highly conserved among protein tyrosine kinases and the conservation of certain amino acids suggests that they play functional or structural roles[46]. The Src catalytic domain consists of the featured bi-lobed protein kinase architecture, a small

amino-terminal lobe known as the ATP-binding lobe and a large carboxyl-terminal lobe known as the peptide-binding lobe. The cleft of these two essential lopes is the active site of Src[45]. The well-known catalytic loop is a highly conserved stretch of amino acid residues located right in the catalytic cleft of the kinase domain. The HRD motif located in the catalytic loop is considered as one of the most highly conserved motifs and predicted to have significant functional importance[47]. Further investigations are necessary for a full understanding of HRD motif function.

Researchers working on protein kinases widely use site-directed mutagenesis as a conventional biochemical approach to study the structure-function relationships of protein. However, the current site-directed mutagenesis studies experience great difficulty. Routinely, site-directed mutants are generated, expressed, purified and then characterized for functional analysis. This whole process is tedious and challenging, while the result is not informative some of the time. Another part of the reason for the complexity is that it requires a large number of mutants to fully evaluate the structural feature required for the function of just a few residues. Thus we proposed a new strategy to investigate the structural mechanisms of kinase activity and substrate specificity.

In this study, we developed a rapid high throughput screening method to probe the functionality of important conserved protein residues. To apply this developed screening method to study the functionality of the HRD motif, each residue was probed by catalytic activity screening of a saturated mutagenesis library. Functional

mutants from the screening were selected for further study. Screening analyses of H384 and D386 are consistent with previous findings and support the model of functionality proposed previously. Interestingly, we found that a wide range of structurally diverse amino acids were able to replace the HRD arginine and maintain approaching full catalytic activity. This discovery may indicate significant importance of R385 on regulation of kinase activity, which requires further analysis.

2.3 Results

In this study, a rapid screening method to probe kinase activity and substrate specificity in a high throughput manner was developed to overcome the drawbacks of traditional site-directed mutagenesis method. The proposed screening strategy using the developed method to identify functional mutants is shown in Figure 2.1. A library harboring random mutations in a kinase of interest will be introduced into a bacterial screening system. The colonies expressing an active kinase were be selected through colony blot using an antibody against the specific phosphotyrosine. Functional mutants were investigated for further functional analysis. Unlike site-directed mutagenesis, this strategy provides functional mutants for further analysis.

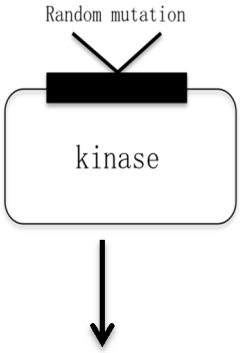
2.3.1 Bacterial proteins are phosphorylated on tyrosine residues when PTKs are heterogeneously expressed in E *coli*.

To demonstrate *E. coli* as a good screening system for catalytic activity, four representative protein tyrosine kinases were chosen, Abl, Csk, FGFR, and Lck. Each kinase was cloned into pGEX-4T-1 in DH5α and expressed at 37^0C for 24 hours. The level of tyrosine phosphorylated bacterial proteins was detected by a monoclonal antibody specific for general tyrosine phosphorylation, using an empty vector without a kinase as a control. As shown in Figure 2.2, a large range of bacterial proteins with different molecular weights got phosphorylated on tyrosine. This demonstrates that PTKs, when expressed in *E. coli,* are able to phosphorylate bacterial proteins on tyrosine to a higher level than the bacteria's own

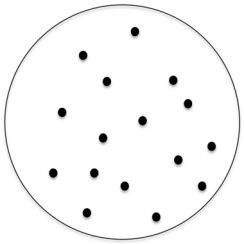
phosphorylation level.

Figure 2.1

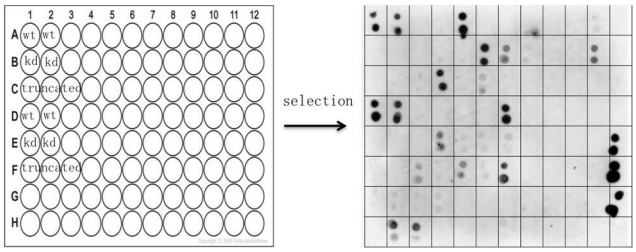
A) A library harboring random mutants will be created with rational design



B) The library will be introduced into a bacterial screening system developed for specific functional selection



C) Functional mutants will be selected through the developed screening method



D) Further functional analysis of selected mutants

Figure 2.1 Scheme of the screening strategy

The screening strategy couples a bacterial based screening with a random mutation library of interest. A) A library harboring random mutants is created with rational design. B) The library is introduced into a screening system developed for specific functional selection. C) Functional mutants are picked through the developed screening method. D) Further functional analysis of selected mutants

Figure 2.2

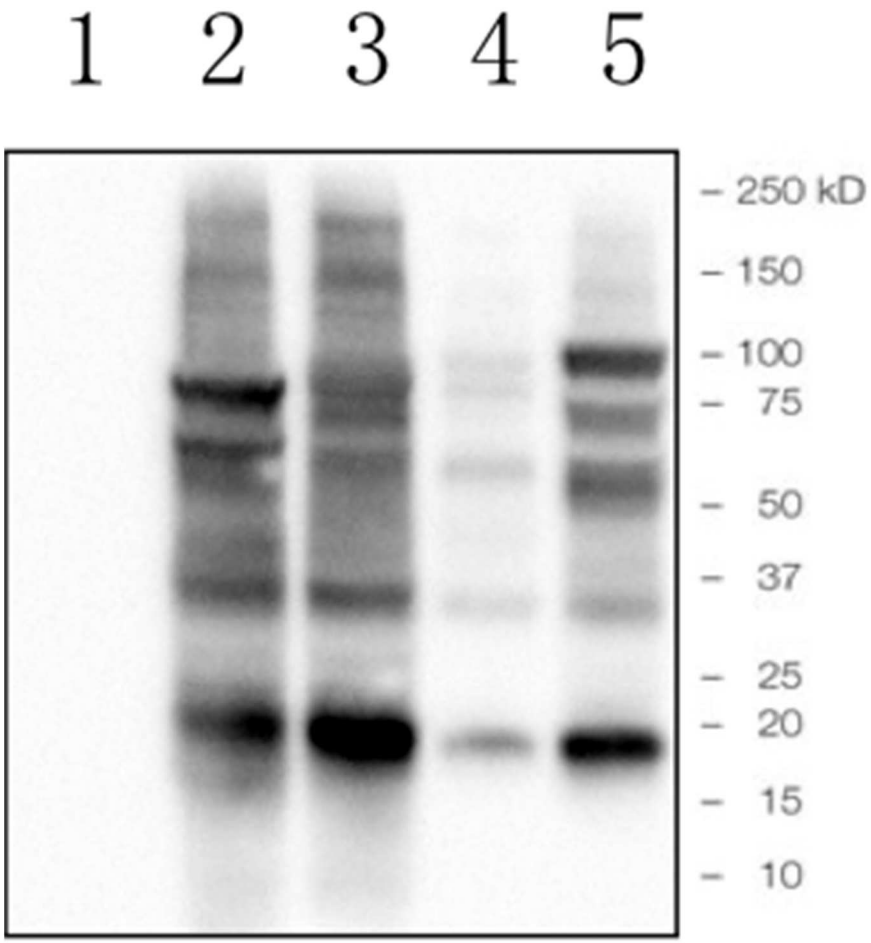


Figure 2.2 General tyrosine phosphorylation on bacterial proteins by representative PTKs when expressed in *E. coli*

As described in the Materials and Method section, samples were collected from overnight cultures and analyzed with a monoclonal antibody specific for general tyrosine phosphorylation. The same amount of soluble cell lysate for each PTK sample was compared with that when just empty vector expressed in DH5α: Lane 1, empty pGEX-4T-1 vector; line 2, Abl; line 3, Csk; line 4, FGFR; line 5, Lck.

2.3.2 Substrate specificity can be screened when a protein tyrosine kinase and a specific substrate are co-expressed in the *E. coli* expression system

Substrate specificity of protein kinases is essential for the fidelity of signal transduction. Thus we further developed and optimized this bacterial screen system to analyze substrate specificity of PTKs. To achieve this, a specific substrate should be co-expressed with kinases. After rounds of development and optimization, it was determined that specific substrate could be expressed using a high copy number plasmid while kinase could be expressed using a low copy number. This setup was chosen with the idea that in a typical kinase reaction, there should be a much higher concentration of the substrate than that of the kinase.

To validate the proposed expression strategy for substrate specificity screening, Abl, Csk and Src were used as models of PTKs with very different substrate specificity. Abl recognizes Crk-L as a physiological substrate and phosphorylates it on Tyr207 specifically[48]. KdSrc served as substrate for both Csk and Src: Csk phosphorylates KdSrc on Tyr527; Src phosphorylates KdSrc on both Tyr416 and Tyr527[49].

To screen substrate specificity towards Tyr207 on Crk-L, the PTKs, Abl, Csk and Src each encoded in pCDF-1B, a low copy number plasmid, were co-expressed respectively with Crk-L encoded in pRSET-A, a high copy number plasmid. The phosphorylation levels on Tyr207 of Abl, Csk, and Src were compared with empty pCDF-1B vector expressed using a monoclonal antibody specific for pY207 in Crk-L. To screen substrate specificity towards Tyr416 on Src, Abl, Csk and Src encoded in

pCDF-1B, using empty pCDF-1B as a control, were co-expressed respectively with KdSrc encoded in pRSET-A. The phosphorylation levels of Tyr416 were determined by western blot using a monoclonal antibody specific for pY416 on Src. To screen substrate specificity towards Tyr527 of Src, Abl, Csk and Src were co-expressed respectively with KdSrc as above and the phosphorylation levels of Tyr527 were determined by western blot using a monoclonal antibody specific for pY527 of Src. As shown in Figure 2.3, Abl phosphorylated Crk-L significantly more than Csk and Src; Src phosphorylated KdSrc on Tyr416 significantly more than Abl and Src; Src and Csk phosphorylated KdSrc on Tyr527 significantly more than Abl. These results demonstrate that when specific substrate is co-expressed with specific PTKs, substrate specificity can be differentiated among PTKs.

Figure 2.3

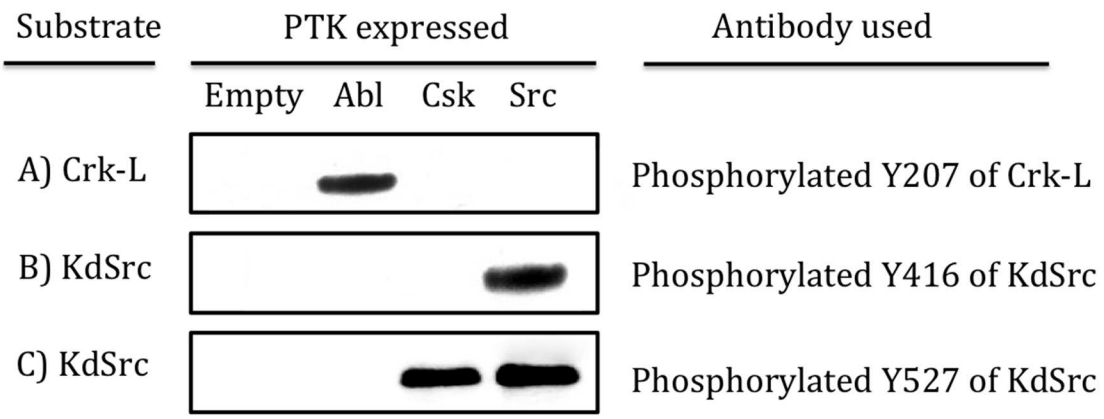

Substrate
PTK expressed
Antibody used
Empty
Abl
Csk
Src
A) Crk-L
Phosphorylated Y207 of Crk-L
B) KdSrc
Phosphorylated Y416 of KdSrc
C) KdSrc
Phosphorylated Y527 of KdSrc

Figure 2.3 Substrate specificity screening of model PTKs in developed method

Model PTKs, Abl, Csk, and Src were cloned into pCDF-1B plasmid respectively and substrates Crk-L and KdSrc were cloned into pRSET-A plasmid respectively. (A) Substrate Crk-L was co-expressed with PTKs and the phosphorylation levels of Y207 on Crk-L were compared between PTKs and empty vector pCDF-1B. (B) Substrate KdSrc was co-expressed with PTKs and the phosphorylation levels of Y416 on KdSrc were compared between PTKs and empty vector pCDF-1B. (C) Substrate KdSrc was co-expressed with PTKs and the phosphorylation levels of Y527 on KdSrc were compared between PTKs and empty vector pCDF-1B.

2.3.3 Adaption of the proposed strategy to study the HRD motif in Src

Protein kinases share conserved features with functional importance[17]. HRD is a highly conserved motif in the active site and predicted with important function. In this study, the proposed screening strategy was applied to study the catalytic function of HRD motif in Src. Each residue of the HRD motif was random mutated to create a mutation library as described in the method section. Each mutation library was screened thoroughly for catalytic activity and functional mutants were selected for structure-function analysis.

It has been demonstrated that fully active Src is toxic to the bacterial host and can't be expressed and purified in bacteria. Co-expression with a protein tyrosine phosphatase (PTP) can reduce the toxicity to allow the fully active Src to be expressed. However, the co-expressed phosphatase may remove the phosphorylation of bacterial proteins to an undetected level for low activity kinase. Low activity Src mutants can be expressed without the phosphatase[50]. Thus we decided to use a PTP system and a non-PTP system for screening of Src kinase mutants: the PTP system expressed Src mutants with PTP1B and GroESL in BL21(DE3)RIL as described [51]; the non-PTP system expressed Src mutants with GroESL in BL21(DE3)RIL. The combination of the two expression systems would be suitable for screening both high activity and low activity mutants. There wasn't a positive control for the non-PTP system since wild type Src was toxic for bacterial growth without co-expression of a phosphatase. Thus, in the non-PTP system, any mutant that showed a higher signal than the negative

control was selected as a functional mutant for further analysis. Additionally, two Src mutants were generated to validate the screening system for catalytic activity of Src, KdSrc and SDSrc. KdSrc was a kinase-defective mutant of Src (kdSrc), which was different from active Src with a point mutation (Lys295Met) and SDSrc was a truncated mutant at R385 position, which had only half of the catalytic domain of Src. Neither KdSrc nor SDSrc should have any phosphorylation of bacterial proteins other than bacteria's own phosphorylation.

WtSrc, KdSrc and SDSrc were expressed in PTP system for 24h at 37^0C. Soluble fractions from cell culture were analyzed by western blot using a monoclonal antibody specific for general tyrosine phosphorylation. Src is known to autophosphorylate on Tyr416 in the activation loop and thus the phosphorylation levels of Tyr416 was also explored in the soluble fractions[45]. WtSrc was shown to phosphorylate the bacterial proteins and the Tyr416, whereas neither KdSrc nor SDSrc were detected with any phosphorylation (Figure 2.4). In the Tyr416 phosphorylation blot, comparison between WtSrc and KdSrc indicated that substrate specificity towards Tyr416 of Src was also retained in the screening, in which Src served as a specific substrate for itself (Tyr416 was lost in the truncated form of Src, SDSrc). In the general phosphorylation blot, WTSrc phosphorylated a wide range of bacterial proteins compared with KdSrc and SDSrc, in addition to autophosphorylation. All together, the results demonstrate that in the PTP system, general tyrosine phosphorylation blot screens general catalytic activity of Src and

phosphorylated Tyr416 blot screens substrate specificity activity of Src.

Figure 2.4

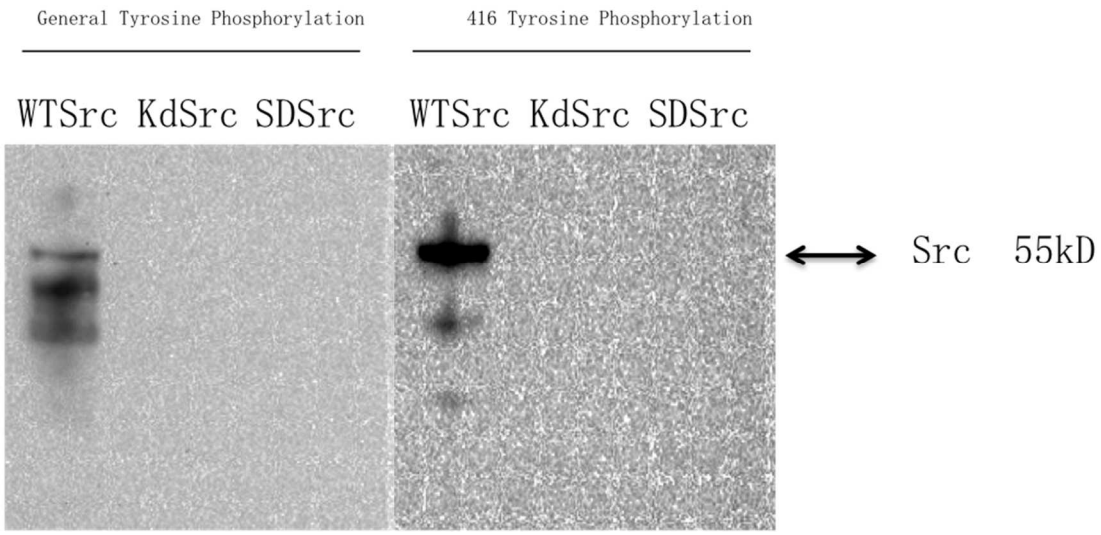

General Tyrosine Phosphorylation
416 Tyrosine Phosphorylation
WTSrc KdSrc SDSrc
WTSrc KdSrc SDSrc
Src 55kD

Figure 2.4 Validation of developed catalytic screening method for HRD motif in PTP system

WtSrc and Src mutants were co-expressed with GroESL and PTP1B in PTP system. WtSrc (wild type), KdSrc (a kinase defective form of Src (Lys295Met)) and SDSrc (a truncated form of Src (truncated at Arg385 position)) were expressed at 37^0C for 24 hours. The same amounts of soluble fractions were analyzed by western blot with monoclonal antibodies specific for general tyrosine phosphorylation and Tyr416 phosphorylation, respectively.

2.3.4 Screening study of H384 residue on catalysis

An H384 mutation library was created and screened for general catalytic activity as described in the method section. Representative screening results from PTP system and non-PTP system, respectively, are shown in Figure 2.5. From the screening in PTP system, there was only one or none functional mutant selected on average for each 96-well screening (Figure 2.5A). With ~650 random mutants screened, 5 mutants were selected as functional mutants all together. All five mutants had histidine on H384 position and had different genetic codons, which indicated that only wild type was selected for this position from the created mutation library. In non-PTP system, ~20-40 mutants on average had significant phosphorylation signal compared with KdSrc and SDSrc for each 96-well screening (Figure 2.5B). Fifty random functional mutants were selected and sequenced, and led to the identification of nine different mutations, including H384Q, H384Y, H384T, H384S, H384F, H384L, H384V, H385I and H384G. Wild type Src was not selected from the screening in non-PTP system because the lack of phosphatase.

The phosphorylation signal produced in screening may not be quantitative due to many factors including culture density, growth phase, expression level of kinase, sensitivity of antibody and other unknown factors. Based the screening results from the two screening systems described, the catalytic activity of H385 mutants would have relatively low activity because the mutants were selected only within non-PTP system. In the PTP system, the co-expressed phosphatase might remove the

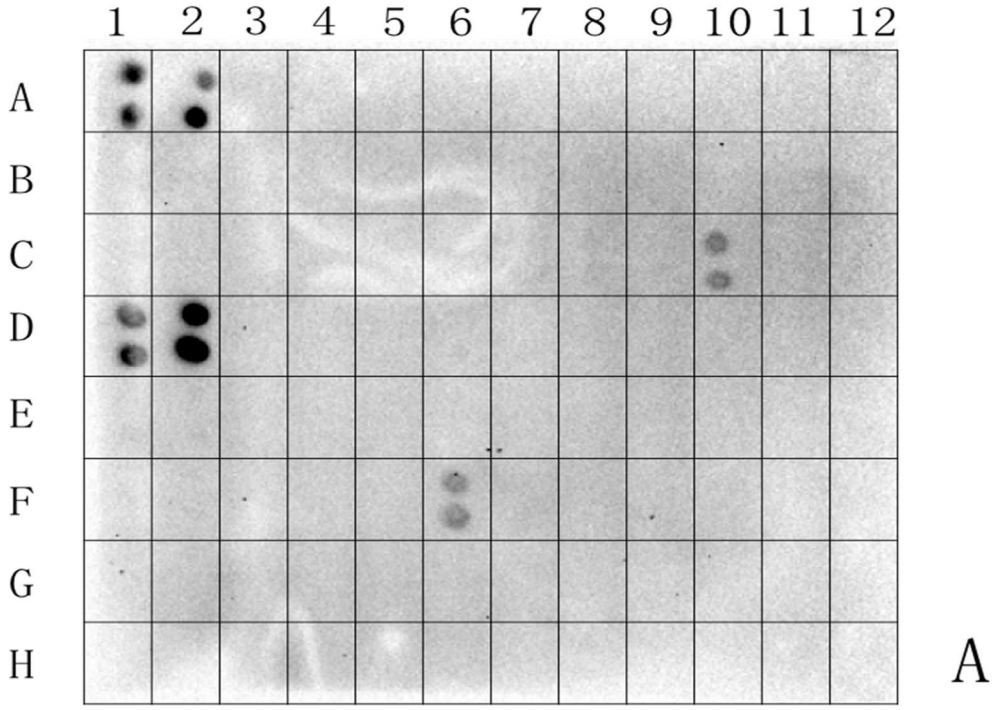
phosphorylation of the bacterial proteins to an undetected level because of the low kinase activity.

To confirm the catalytic activity of H384 mutants, all of the mutants selected from the screening were expressed and purified. The relative Src activity of H384 mutants was determined using polyE4Y as substrate (Figure 2.6). All mutants maintained active kinase activity and H385Q had the highest catalytic activity among H385 mutants, ~14% of wild type activity. Among the selected mutants, H384F (10.2%), H384L (1.1%), H384V (0.6%) H385I (0.6%) and H384G (0.2%), are hydrophobic amino acids that can maintain marginal catalytic activity, supporting the proposed hydrophobic interaction of His384 in stabilizing the active conformation[18]. H384Q (14.2%), H384Y (6.2%), H384T (6.0%) and H384S (2.6%) are polar amino acid replacements, which may still maintain the hydrophobic interaction and hydrogen bond formation as histidine. The contribution of the polarity of the side chain is not clear and needs further study of it[47].

Some of the H384 mutants have been characterized previously. The histidine mutation to leucine in Src64 reduces the kinase activity to 27% of the wild type and produces significant defects in its biological function on development[52]. The histidine mutations to glutamine, tyrosine and alanine in Src also reduce the kinase activity[50]. The replacements of the corresponding histidine to tyrosine, phenyalanine and arginine in Aurora A impair the kinase activity and the ability of autophosphorylation is only maintained in the histidine to tyrosine mutant[53]. The

crystal structure of the histidine to arginine mutant of Aurora A displays a disruption in the hydrophobic structural skeletons of the catalytic core. In stead, the histidine to tyrosine mutant displays a changed geometry of it. The results of H384 screening are consistent with previous research.

Figure 2.5



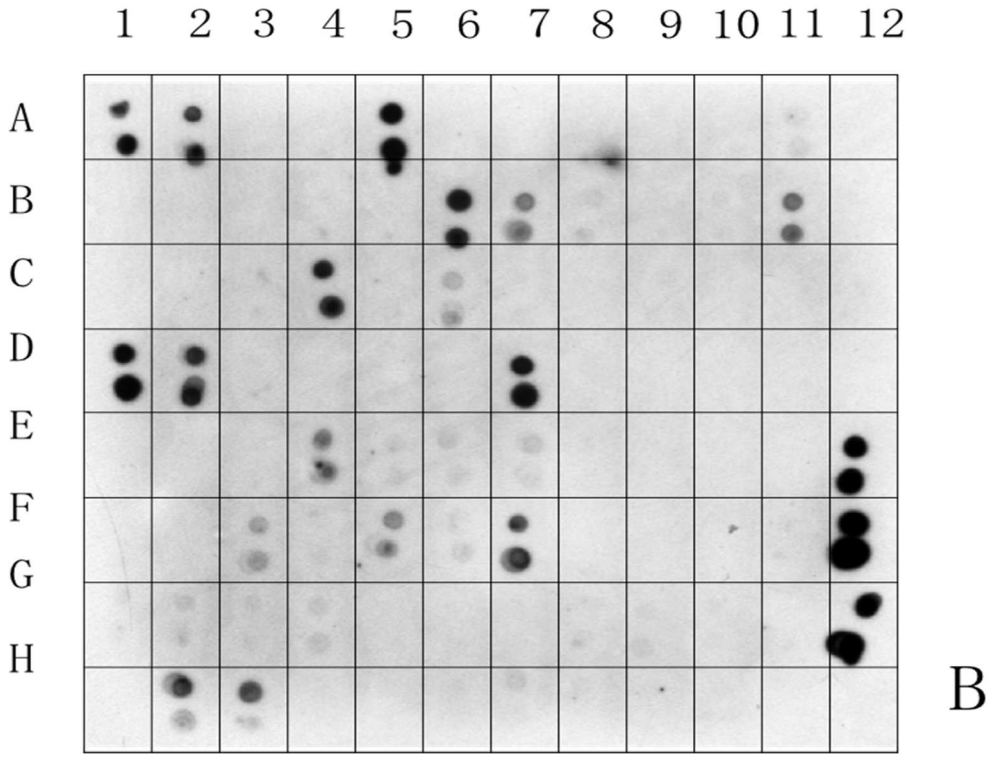


Figure 2.5 Representative catalytic activity screening results of an H384 mutation library

An H384 mutation library was constructed as described in Method section. Controls and H384 library mutants were inoculated into a 96-well plate and blotted by a monoclonal antibody specific for general tyrosine phosphorylation. Each control was inoculated repeatedly in two wells and each mutant was inoculated once into one well. The cell lysate from each well was blotted twice in the nitrocellulose membrane. The blot results were labeled in a 96-well manner. (A) A representative screening blot of H384 library in PTP system: A1, A2, D1 and D2 had WtSrc expressed as positive controls; B1, B2, E1 and E2 had KdSrc expressed as negative controls; C1, C2, F1 and F2 had SDSrc expressed as negative controls. The rest of the wells had random H384 mutants expressed for screening selection. (B) A representative screening blot of H384 library in non-PTP system: A1 and A2, had WtSrc expressed in PTP system as positive controls; B1 and B2 had KdSrc expressed as negative controls; C1 and C2 had SDSrc expressed as negative controls. The rest of the wells had random H384 mutants expressed for screening selection.

Figure 2.6

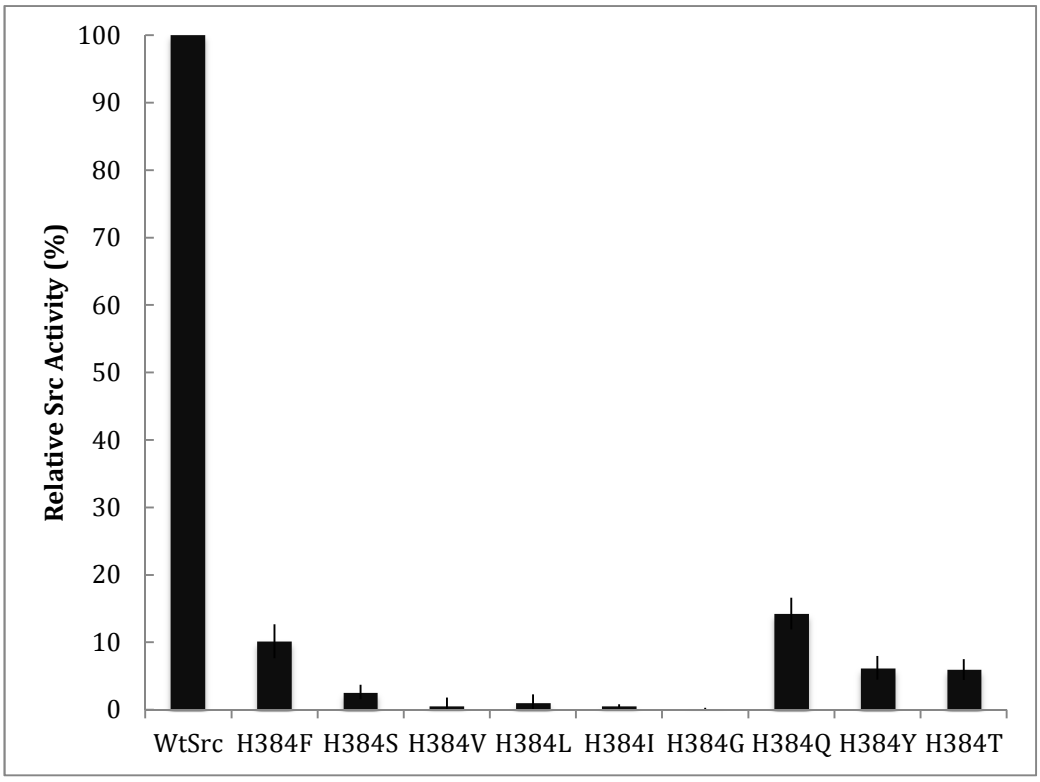

Relative Src Activity (%)
100
90
80
70
60
50
40
30
20
10
0
WtSrc
H384F
H384S
H384V
H384L
H384I
H384G
H384Q
H384Y
H384T

Figure 2.6 Relative catalytic activity of Src H384 mutants

The catalytic activity of selected H384 mutants toward an artificial substrate, polyE_4Y was determined. WtSrc at 3nM concentration were incubated with 200uM ATP, 12mM $MgCl_2$ and 1mg/ml polyE_4Y in the kinase assay buffer for 30min. Catalytic activity of H384 mutants were determined at 30nM or higher dependent on the activity of the mutant. The relative Src catalytic activity was determined by ADP production as described in the method section.

2.3.5 Screening study of R385 on catalysis

An R385 library was created and screened for general catalytic activity, and representative screening results for PTP system and non-PTP system respectively are shown in Figure 2.7. In both expression systems, a large number of mutants had significant phosphorylation signals compared with negative controls (Figure 2.7A and 2.7B). From each screening system, ~50 functional mutants were selected and sequenced. There was an overlap on the selection of replacement amino acids between the PTP system and non-PTP system, while other mutations only showed up in either one. R385Q and R385Y were mutants selected only from non-PTP system, while R385K, R385V, R385N, R385L, R385C and WtSrc were only found in the PTP system. R385A, R385G, R385S, and R385T were selected from both systems. These results indicated that a variety of R385 mutants maintained significant catalytic activity.

Selected mutants from the PTP system were purified and the catalytic activity was determined by the kinase assay using polyE4Y as substrate (Figure 2.8). As shown, mutants selected from the PTP system including R385N (88.3% of wild type Src activity), R385V (85.4%), R385C (70.0%) and R385K (43.6%), R385L (35.9%), R385T (30.8%), R385A (27.9%), R385G (19.3%) and R385S (17.2%), had catalytic activity ranging from ~17.2 to 100% of wild type Src kinase activity. This result indicated that selected R385 mutants maintained significant catalytic activity in the standard kinase assay and the selective range of the PTP system was at least ~17% of

the wild type Src activity or above. While R385 mutants selected from both systems included R385A (27.9%), R385G (19.3%), R385S (17.2%), and R385T (30.8%), which indicated that the selective range for non-PTP system was at most ~31% of the wild type activity or below. This was also consistent with the finding that no His384 mutants but WtSrc was selected from the PTP system and the most active H385Q screened from non-PTP system had 14.2% of the wild type Src activity. Based on the observation that the H384 mutants selected only from non-PTP system had catalytic activity ranging from ~0.5 to 14.2%, the selection range for non-PTP system should be ~0.5 to 30.8% of the wild type Src kinase activity.

The results of R385 screening are consistent with the previous report that mutation of the HRD arginine to cysteine on Src64 has very little effect on kinase activity[52]. Besides, the R385 is not as universally conserved as D386 in all kinases[46]. All together, based on the results from the R385 random mutation screening and the standard kinase assay on selected R385 mutants, it is clearly shown that R385 is not essential for catalysis.

Figure 2.7

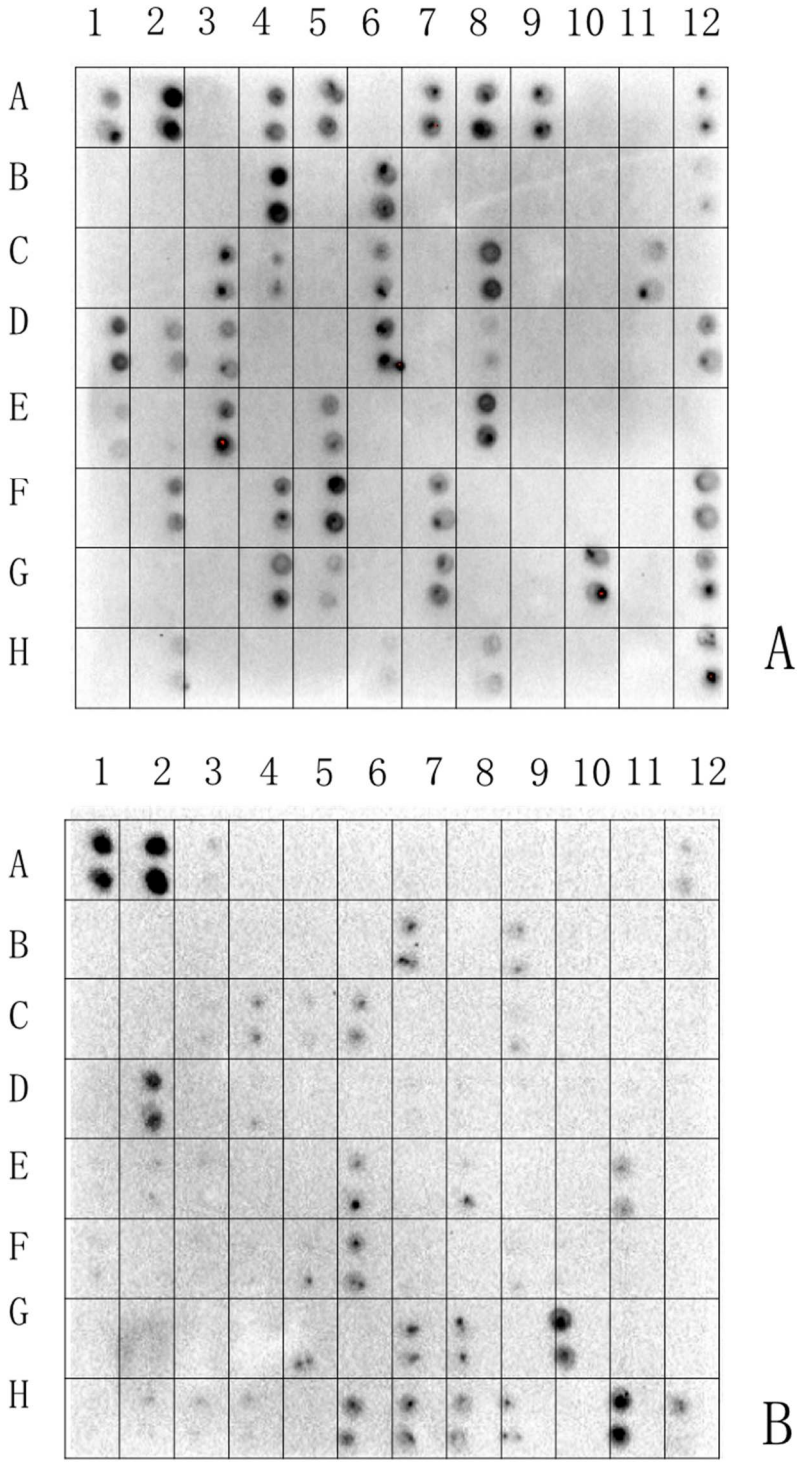


Figure 2.7 Representative catalytic activity screening results of an R385 mutation library

An R385 mutation library was constructed as described in the Methods section. Controls and R385 library mutants were inoculated into a 96-well plate and blotted by a monoclonal antibody specific for general tyrosine phosphorylation. Each control was inoculated repeatedly in two wells and each mutant was inoculated once into one well. The cell lysate from each well was blotted twice in the nitrocellulose membrane. The blot results were labeled in a 96-well manner. (A) A representative screening blot of the R385 library in the PTP system: A1 and A2 had WtSrc expressed as positive controls; B1 and B2 had KdSrc expressed as negative controls; C1 and C2 had SDSrc expressed as negative controls. The rest of the wells had random R385 mutants expressed for screening selection. (B) A representative screening blot of R385 library in the non-PTP system: A1 and A2 had WtSrc expressed in the PTP system as positive controls; B1 and B2 had KdSrc expressed as negative controls; C1 and C2 had SDSrc expressed as negative controls. The rest of the wells had random R385 mutants expressed for screening selection.

Figure 2.8

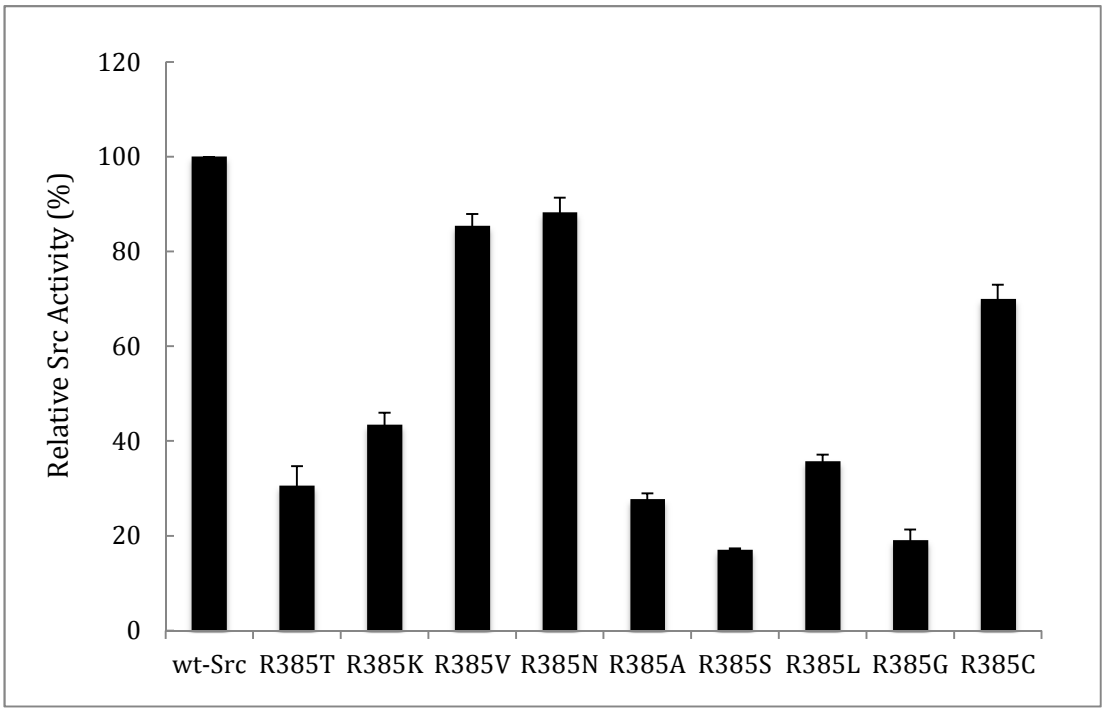

Relative Src Activity (%)
120
100
80
60
40
20
0
wt-Src
R385T
R385K
R385V
R385N
R385A
R385S
R385L
R385G
R385C

Figure 2.8 Relative catalytic activity of Src R385 mutants

The kinase activity of selected R385 mutants toward an artificial substrate, polyE_4Y was determined. WtSrc and R385 mutants at 3nM kinase concentration were incubated with 200uM ATP, 12mM $MgCl_2$ and 1mg/ml polyE_4Y in the kinase assay buffer for 30min and the relative Src catalytic activity was determined by ADP production as described in the method section.

2.3.6 Screening study of D386 residue on catalysis

A D386 library was created and screened for general catalytic activity, and representative screening results for the PTP system and non-PTP system are shown in Figure 2.9. Totally 630 random mutations were screened for each screen system and none was selected except for WtSrc from the PTP system. This result indicated that replacement by any other residue for Asp386 couldn't retain even 0.5% kinase activity based on the previous assessment of the screen method. This result supports Asp386's indispensable role in catalysis and is consistent with previous studies of the functionality of Asp386[29]. Asp386, as a signature of the catalytic core, is thought to orient the tyrosyl group of the substrate protein in a catalytically competent state. It functions as a base that abstracts a proton from tyrosine thereby facilitating its nucleophilic attacks of the γ-phosphorus atom of MgATP[29]. Mutation of the corresponding Asp386 abolishes the catalytic activity in many protein kinases[54, 55].

Figure 2.9

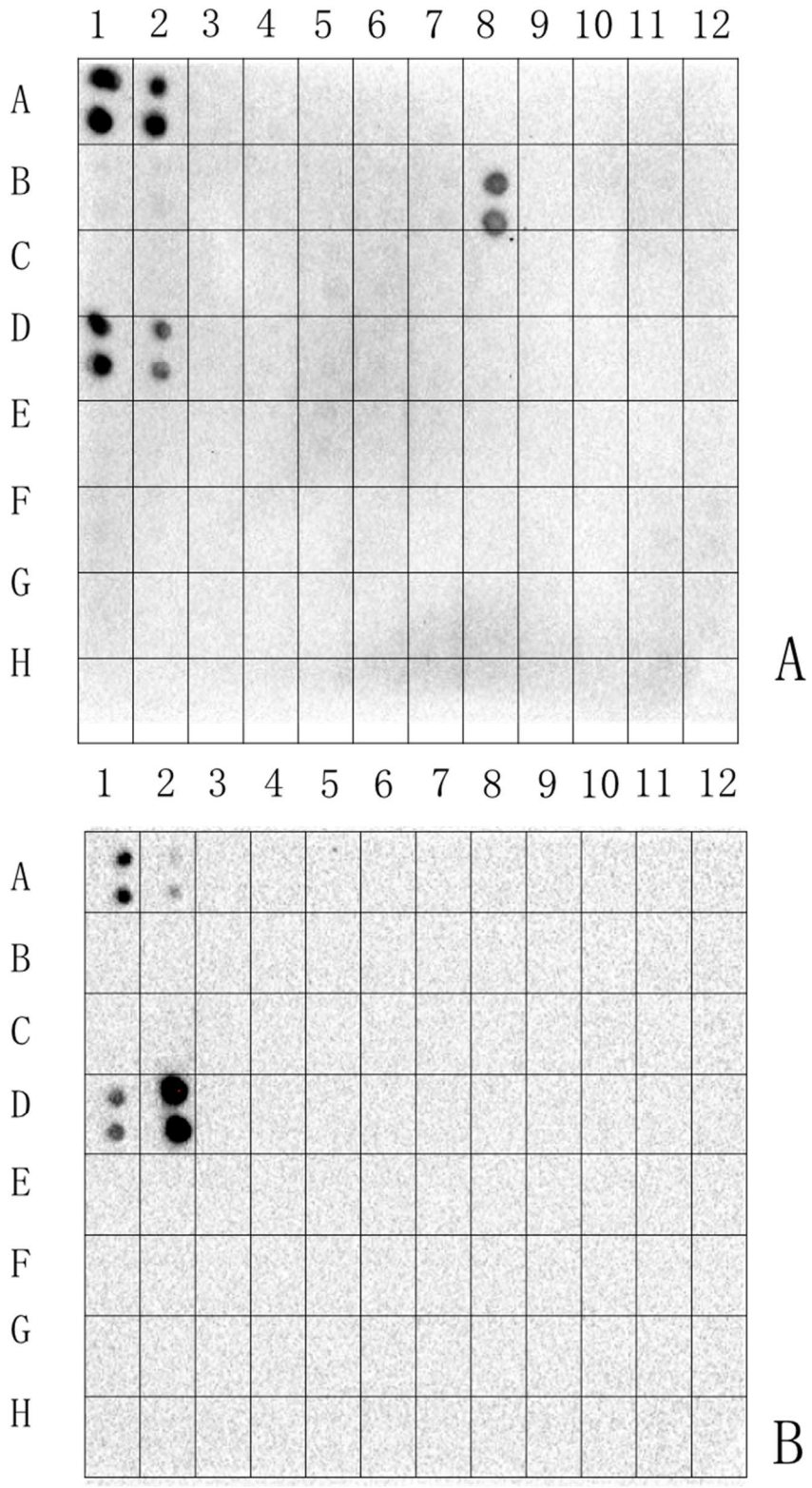


Figure 2.9 Representative catalytic activity screening results of A D386 mutation library

A D386 mutation library was constructed as described in Method section. Controls and D386 library mutants were inoculated into a 96-well plate and blotted by a monoclonal antibody specific for general tyrosine phosphorylation. Each control was inoculated repeatedly in two wells and each mutant was inoculated once into one well. The cell lysate from each well was blotted twice in the nitrocellulose membrane. The blot results were labeled in a 96-well manner. (A) A representative screening blot of D386 library in PTP system: A1, A2, D1 and D2 had WtSrc expressed as positive controls; B1, B2, E1 and E2 had KdSrc expressed as negative controls; C1, C2, F1 and F2 had SDSrc expressed as negative controls. The rest of wells had random D386 mutants expressed for screening selection. (B) A representative screening blot of D386 library in non-PTP system: A1, A2, D1 and D2 had WtSrc expressed in PTP system as positive controls; B1, B2, E1 and E2 had KdSrc expressed as negative controls; C1, C2, F1 and F2 had SDSrc expressed as negative controls. The rest of the wells had random D386 mutants expressed for screening selection.

2.4 Discussion

We have witnessed a bloom in the field of directed protein engineering in the last decade. However, this approach requires high throughput screening or selection[56]. Even though this approach has been widely applied to study different enzyme families[57-60], it has not been applied to PTKs due to the lack of high throughput screening method for functional mutant selection.

Here we developed a bacterial based high throughput screening method, to identify kinase functionalities. First, when PTKs are heterologously expressed in *E. coli*, the catalytic activity is screened by the detection of phosphorylated bacterial proteins in the cell lysate. Second, when PTKs and specific substrates are co-expressed in bacterial system, the substrate specificity is screened by the detection of specific phosphorylated substrates in cell lysate. This developed screening method was successfully adapted to study the HRD motif of Src, which resulted in a more clear understanding of the role of this motif in the kinase function. The great advantage of this proposed screening strategy is that any mutant selected from the screening is functional and informative. Thus this strategy overcomes the blindness in the traditional random mutagenesis strategy and provides a more comprehensive analysis. The developed screening method on Src catalytic activity can be directly applied to the functional study of other conserved residues or motifs of interest in Src based on the successful application of the study on the HRD motif. Also, this proposed versatile strategy could be adapted for diverse functional studies

regarding catalytic activity and substrate specificity of PTKs. To achieve this, the kinase or substrate of interest can be readily introduced into the developed screening method and functional mutants can be screened with rational design.

Characterization of HRD motif of Src with this proposed strategy led to a more comprehensive understanding of the functionality of it. First, a large group of amino acids with hydrophobic property can replace the histidine and maintain a significant fraction of the catalytic activity of Src. This finding is consistent with previous functional studies of the corresponding H384 on protein kinase and supports the proposed role of H384 as one of identified surface conserved residue forming hydrophobic structural skeletons of catalytic domain[18, 61]. Second, a wide range of structurally diverse amino acids can replace the R385 residue and maintain approaching full catalytic activity of Src. This result suggests that R385 is not essential at all in catalysis or maintaining active conformation. Finally, the D386 residue can't be replaced at all and this supports its essential role as a catalysis base.

This study of the HRD motif of Src using the developed screening method also raises an important question that remains to be answered. The HRD arginine seems unnecessary in this catalysis, but it is still chosen by evolution for a large group of protein kinases. The conservation suggests this arginine must give Src and other PTKs certain biochemical property that confers a selective advantage in evolution. The unique function of this arginine, for the group of protein kinases that choose arginine, still needs to be solved. The protein kinases, which take other amino acids

instead of arginine, may have a different regulatory mechanism underlying this position. These substantial discoveries in this study demonstrate the power of the developed high throughput screening method. This proposed screening strategy would be of great help for the selection of PTK mutants with altered properties and lead to recognition of residues important for catalysis and substrate specificity.

2.5 Materials and methods

2.5.1 Reagents and chemicals

Consumables and culture media, or media components, were purchased from Fisher Scientific. Specific antibodies were purchased from Cell Signaling Technology. All the other chemicals were purchased from Sigma.

2.5.2 Molecular cloning and protein expression

Abl, Csk, FGFR, and Lck were cloned into pGEX-4T-1 plasmid and transformed into DH5α for catalytic activity screening. Src and Src mutants were cloned into the pRSETA plasmid and transformed into *E.coli* BL21(DE3)RIL. Substrate KdSrc and Crk-L were cloned into pRSETA plasmid and PTK Abl, Csk and Src were cloned into pCDF-1B plasmid. Substrate and each PTK were co-expressed in *E.coli* BL21(DE3)RIL. Src mutants including KdSrc, and SDSrc were generated by site-directed mutagenesis(Stratagene). All constructs were verified by sequence analysis. Plasmids were introduced into the specific expression systems by electroporation.

2.5.3 Mutation Library Construction and Screening

Random mutant libraries of each residue were generated by site-directed mutagenesis using primers containing random sequences at position H384, R385 and D386, respectively. The SDSrc clone was used as the template in site-directed mutagenesis to avoid wild type Src plasmid contamination in constructed libraries. Each library was expressed in *E.coli* BL21(DE3)RIL. The mutant library was introduced into the expression systems by electroporation and each transformant

from a mutation library was inoculated into a well of 96-well plate containing 200ul LB broth with appropriate antibiotics. The plate was incubated for 24h in a 37^0C shaker. Then 0.4ul of the cell culture was blotted twice directly onto a nitrocellulose membrane and followed by western blot using anti-phosphotyrosine mouse monoclonal antibody (PY100; Cell Signaling Technology) and horseradish peroxidase (HRP)-anti-mouse secondary antibody (Stratagene).

2.5.4 Expression and purification of Src and mutants

Src and Src mutants were constructed in the pRSETA expression plasmid. The Src constructs were expressed in *E.coli* BL21(DE3)RIL cells harboring the pREP4groESL plasmid expressing the GroES/EL chaperone and the pCDF-PTP1B plasmid expressing protein tyrosine phosphatase PTP1B. The (His)6-tagged Src constructs were purified using immobilized Ni-iminodiacetic acid-agarose as described previously[62]. Concentration and purity of each enzyme were determined by Bradford assay and SDS-PAGE, respectively. Purified enzymes were stored in 50% glycerol at -20^0C.

2.5.5 Kinase assay

Kinase activity of Src and mutants was determined using polyE4Y and ATP as the substrates. The phosphorylation reactions were performed in 50ul volumes at 25^oC in the protein kinase assay buffer: 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (pH 8.0) containing 5% glycerol, 0.005% Triton X-100, and 0.05% 2-mercaptoethanol. The standard assay

used 12 mM $MgCl_2$, 0.2 mM ATP, and 1 mg ml^{-1} $polyE_4Y$. After a 30 min reaction time, 5ul of the kinase reaction mixture was mixed with 5ul of ADP-Glo™ reagent and incubated for 40 min to stop the kinase reaction and deplete the unconsumed ATP. 10ul of Kinase Detection reagent was added and incubated for another 30 min to convert produced ADP to ATP and introduce luciferase and luciferin to detect converted ATP. Then the luminescence produced was measured by a plate-reading luminometer. Assays were performed in duplicate, and each assay was repeated three times with reproducible results.

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MANUCRIPT 2

Prepared for submission to Proceedings of the National Academy of Sciences

The Arginine in the HRD Motif of Src Plays a Crucial Role in Controlling Conformational Transitions of the Kinase Domain

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3.1 Abstract

Protein Tyrosine Kinases (PTK) play important roles in both physiological and pathological signaling pathways and have been studied extensively as potential drug targets. Conformational changes of PTKs tightly regulate their biological function and impact the affinity towards PTK inhibitors. In this study, the R385 residue on the model PTK, Src, was revealed to play a crucial role in the conformational changes that underlines Src regulation and inhibitor binding. First, R385 mutation to a number of unrelated residues can maintain catalytic activity approaching that of wild type. Second, R385 is not necessary for the activation of Src through autophosphorylation on Tyr416 in the activation loop. Third, autophosphorylated R385 mutants can still be down regulated by Csk, while autophosphorylation prevents Csk inactivation of wild type Src. Fourth, R385 mutation displays strikingly increased affinity toward type II protein kinase inhibitors compared to the wild type, which target the inactive conformation of the active site. In contrast, R385 mutants maintain similar affinity towards type I inhibitors as wild type, which target the active conformation. Altogether, these experimental results demonstrate that the R385 residue is critical for Src transitions from an active to an inactive conformation. The study of R385 defines a new functional cluster, which regulates the transition between conformational states. This study sheds light on the fundamental mechanism of regulatory conformational changes and provides more insight on design and development of PTK inhibitors.

3.2 Introduction

The human genome contains 518 protein kinase genes which constitute about 1.7% of all human genes [1]. They mediate essential signal transduction pathways in both normal and pathological states [1-3, 6, 20], which makes them useful targets for drug design and development[11, 37-39, 63]. Thus the understanding of the regulatory mechanisms of protein kinases has significant relevance.

Src is the first identified oncogene and expressed ubiquitously, which has been the subject of intense investigation for decades [9, 11, 12, 16]. Src has multi-layered and integrated regulatory mechanisms, including activation by autophosphorylation and inactivation by tail phosphorylation. Autophosphorylation on the activation loop is a common strategy to activate most protein tyrosine kinases. Upon autophosphorylation on Tyr416 in Src, the activation loop is rearranged and the active site is stabilized in an active conformation[45]. Src is also inactivated by phosphorylation of Tyr527 residue on the C terminal tail by Csk. The phosphorylated Tyr527 engages the SH2 domain in an intramolecular interaction that stabilizes the inactive conformation.

Similar to all protein kinases, the Src kinase domain has bi-lobed architecture: a small amino-terminal lobe known as the ATP-binding lobe and a large carboxyl-terminal lobe known as the peptide binding lobe. The active site is located in the cleft between these two structural lobes[17]. The catalytic loop, located in the

active site, starts with a highly conserved HRD motif, but the role of these conserved residues are not fully defined[18, 29, 33, 52, 64]. In the crystal structure of the autophosphorylated Src family kinase, Lck, the HRD arginine binds to the autophosphorylated Tyr416. It was proposed that this interaction helps stabilize the active conformation of the Src kinase domain. This proposal, however, has not been directly tested. The Arg residue has also been proposed to stabilize the inactive conformation of Src by forming a salt-bridge with conserved Glu310 from the N-lobe. Furthermore, whether the Arg residue plays any other catalytic or regulatory roles has not been studied. A comprehensive examination of the potential roles for these highly conserved residues would be necessary for understanding its contribution to the function of the kinase. Because Src is a well-recognized model of other protein tyrosine kinases, such an understanding may shed light on the structure-function understanding of other kinases.

Another important goal of structure-function studies of Src is to provide a rationale for drug design. Currently most PTK inhibitors bind to the active site as ATP-competitive inhibitors. How the conserved features in the active site of a kinase contribute to the binding affinity and specificity of inhibitors is also a critical question to answer. More specifically, for example, how the HRD motif contributes to the binding toward various inhibitors has not been examined. A comprehensive examination could provide a basis for understanding inhibitor binding and selection.

In the previous chapter, we demonstrated that Arg385 can be replaced by a

large number of structurally unrelated residues without compromising the kinase activity of Src, indicating that the Arg residue does not play a catalytic role. In this study, we demonstrate that this Arg residue controls the transition from active to inactive conformations that underlies the Src regulation by Csk and inhibition by certain small molecular inhibitors.

3.3 Results

3.3.1 Substitution of Arg385 by a wide range of amino acids still retains the catalytic activity of Src

Src has the conserved HRD motif in its catalytic loop. The understanding of Src R385 in the HRD motif is still limited to crystallographic studies that reveal its interactions. To fully understand the functionality of Arg385 of Src, a mutant library containing random replacements on Arg385 as described in the previous chapter was screened for the catalytic activity of Src. A wide range of amino acids was selected from the catalytic screening, which substituted arginine at the 385 position and maintained significant Src catalytic activity (Figure 2.7). All of the R385 mutants maintained significant catalytic activity in the standard kinase assay using polyE_4Y as a substrate (Figure 2.8). A few of the R385 mutants had almost the same catalytic activity as the wild type, including R385N (88.3% of wild type Src activity), R385V (85.4%), R385C (70.0%) and R385K (43.6%). Surprisingly, some of the substitutions didn't share any obvious similarities with Arg. This result indicates that R385 is not essential for catalysis and amino acids with very diverse functional side chains can maintain approaching full kinase activity of wild type.

To further study the functionality of the R385 residue, R385V as a hydrophobic side chain substitution, R385K as a positive charged side chain substitution, R385N as a polar side chain substitution with an amino group, and R385C as a polar side chain substitution with a sulfhydryl group were used as representative mutants for

further functional study of R385.

3.3.2 R385 mutation retained Src activation regulation associated with autophosphorylation

Previous research suggests that R385 is a highly conserved and structurally important residue. In the crystal structure of an active conformation of Src family kinase, Lck, the corresponding Arg on HRD motif is coordinating the phosphorylated tyrosine on the activation loop along with another Arg on the activation loop, R409[33]. Based on this observation, it is reasoned that the Arg385 is stabilizing the active conformation of Src when Src is autophosphorylated on Tyr416. Thus the effect of R385 mutation on Src activation associated with autophosphorylation was examined.

The autophosphorylation of WtSrc and R385 mutants was first examined to see if R385 mutants could still autophosphorylate on the activation loop. Autophosphorylation can be achieved by incubating the enzyme with ATP and Mg^{2+} in the kinase assay buffer. WtSrc and R385 mutants at the same concentration of 450nM were incubated and fractions were removed at indicated period of time for western blot detecting the level of phosphorylated Tyr416 (Figure 3.1). Results showed that WtSrc and R385 mutants reached saturated phosphorylation after 30 min incubation. It was difficult to determine the rate difference between WtSrc and R385 mutants because when enzymes were purified as described above, they were all autophosphorylated to slightly different basal levels (data not shown). From the time-course autophosphorylation detection, we can conclude that R385 mutants are

able to autophosphorylate as wild type within 30min.

We then determined if the R385 mutants were still activated by autophosphorylation. To compare kinase activity of WtSrc and R385 mutants with or without autophosphorylation, enzymes were pre-incubated in kinase assay buffer in the presence or absence of Mg^{2+} for 30min. Based on the observation that the autophosphorylation of WtSrc and R385 reached a plateau within 30min, we chose a 30-min pre-incubation to allow full phosphorylation on Tyr416. The relative kinase activities with or without autophosphorylation were determined for WtSrc and R385 mutants (Figure 3.2). Src Y416F and Src Y527F mutants were included in the assay, serving as controls that cannot be phosphorylated on either Tyr416 or Tyr527. When Tyr416 was replaced by phenylalanine, there was no difference in kinase activity with the presence or absence of Mg^{2+} in the pre-incubation because the lack of the autophosphorylation site. WtSrc had ~2.3 fold of original activity with autophosphorylation, and all R385 mutants and Src Y527F had similar patterns of activation.

These results demonstrate that R385 is not necessary for Src activation associated with autophosphorylation on Tyr416. This observation contradicts the proposed function of R385 stabilizing the super active conformation of the kinase domain when Tyr416 is phosphorylated. To this point, R385 is found not necessary for either catalysis or activation through autophosphorylation, which is surprising. This discovery led us to explore the role of R385 in other aspects of Src regulation.

Figure 3.1

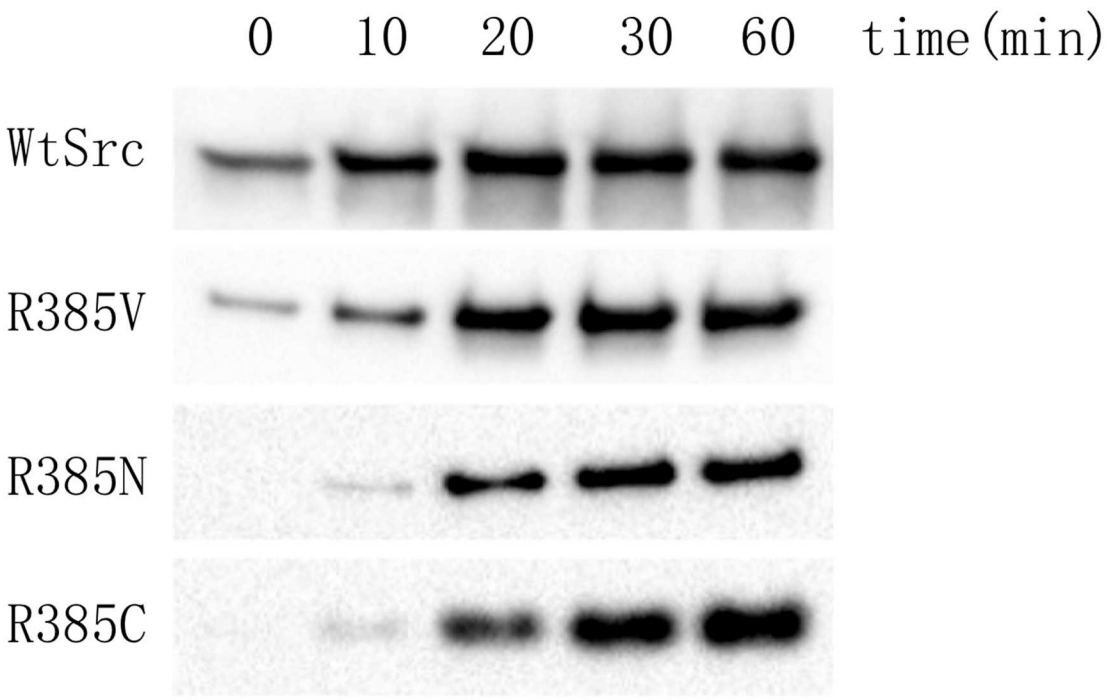

Figure 3.1 Time course of the autophosphorylation of Src and R385 mutants

WtSrc and selected R385 mutants at 450nM kinase concentration were incubated in kinase assay buffer. Aliquots were taken at indicated period of time and the autophosphorylation was detected by a monoclonal antibody specific for phosphorylated Tyr416.

Figure 3.2

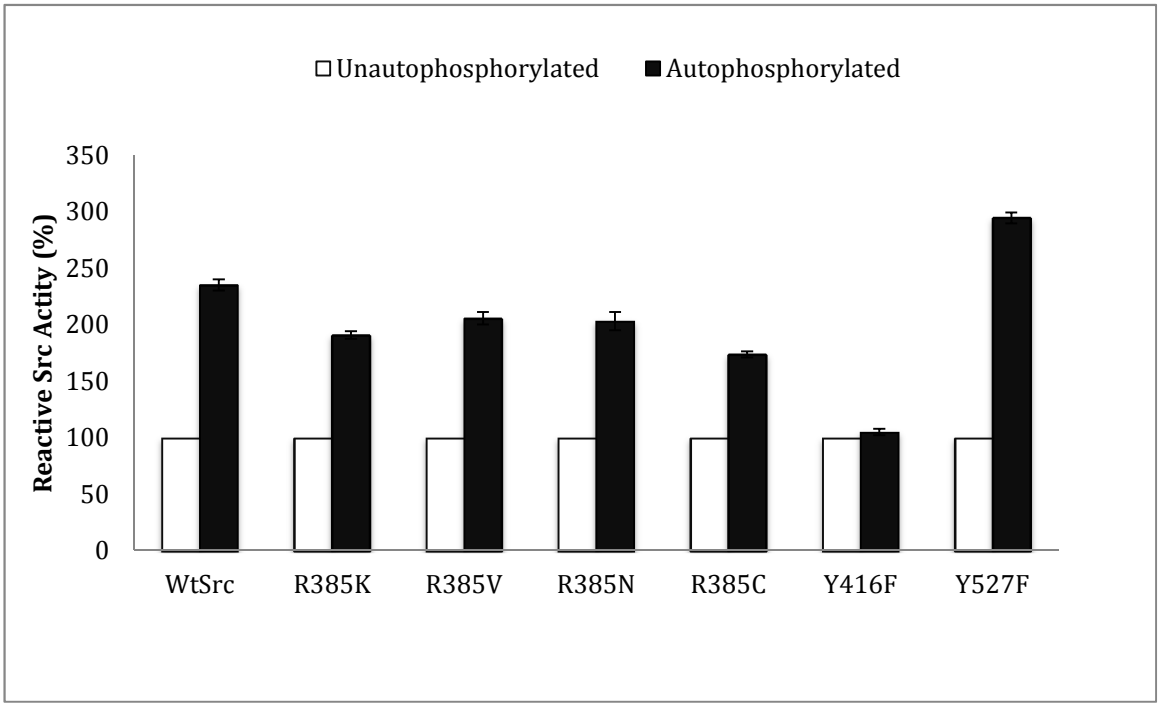
Unautophosphorylated
Autophosphorylated
Reactive Src Actity (%)
350
300
250
200
150
100
50
0
WtSrc
R385K
R385V
R385N
R385C
Y416F
Y527F

Figure 3.2 Effect of R385 mutation on Src activation regulation associated with autophosphorylation

WtSrc and R385 mutants at 450nM concentration were pre-incubated in the presence or absence of Mg^{2+} in the kinase assay buffer for 30min and the kinase activity of the wild type and the mutants at 3nM kinase concentration (diluted from pre-incubation) was determined using polyE_4Y as a substrate by the standard kinase assay: white for the relative activity of unautophosphorylated enzyme (with the absence of Mg^{2+} in the pre-incubation) and black for the relative activity of autophosphorylated enzyme (with the presence of Mg^{2+} in the pre-incubation). The Mg^{2+} concentration was compensated for in the final kinase assay.

3.3.3 Effect of R385 mutation on dephosphorylation of phosphorylated Tyr416 by PTP1B

Phosphoprotein phosphatase reverses the action of protein kinases by catalyzing the removal of the phosphate. PTP1B is universally expressed and has the potential of playing a role in Src regulation by catalyzing the dephosphorylation of Src[8, 30]. A protection mechanism has been observed in the dephosphorylation of the Tyr172 in the AMP-activated protein kinase (AMPK), which belongs to the family of serine/threonine protein kinases[65]. Activation of AMPK requires the phosphorylation of Tyr172 in the activation loop of the kinase domain and the bound AMP not only stimulates AMPK but also protects the Tyr172 from dephosphorylation. Based on the observation of the direct contact of the R385 with phosphorylated tyrosine on the activation loop in the structure, it is plausible that R385 protects the phosphorylated tyrosine from dephosphorylation by a phosphatase, which is a similar mechanism as AMPK has.

Dephosphorylation of the phosphorylated Tyr416 on Src was examined for WtSrc and R385 mutants. WtSrc, R385K and R385C mutants at 450nM kinase concentration were first pre-incubated for 1 hour to achieve full autophosphorylation. Then the autophosphorylated WtSrc and R385 mutants were treated with same amount of PTP1B in the presence of Na_2EDTA and fractions were removed at indicated period of time for western blot analysis of the remaining phosphorylation level of Tyr416 (Figure 3.3). The presence of Na_2EDTA should block autophosphorylation by

chelating Mg^{2+}. The amount of PTP1B added in the dephosphorylation assay was optimized so that, the phosphorylation level of Tyr416 for WtSrc decreased gradually in the time period tested. To ensure accurate determination of the rate difference in dephosphorylation between WtSrc and R385 mutants, each mutant was tested side by side with WtSrc and each experiment was repeated. To ensure the intensity of the signal stayed in a linear range, the western blot results were processed using Image Lab™ Software and none of the signal used for calculation was saturated for exposure. As shown in Figure 3.3(A), Tyr416 phosphorylation level of WtSrc and R385K before PTP1B added was indicated as 0min time point in the dephosphorylation assay. For each indicated time point after PTP1B was added, the remaining Tyr416 phosphorylation level was determined and the relative remaining phosphorylation compared with before PTP1B added was visualized on histogram. The dephosphorylation of R385K was obviously faster than WtSrc. In the side-by-side experiment between R385C and WtSrc, R385C also had a higher dephosphorylation rate than WtSrc (Figure 3.3(B)). This result suggests that the interaction between R385 and phosphorylated Y416 protects Tyr416 from dephosphorylation, a similar regulatory mechanism as observed in AMPK.

Figure 3.3 (A)

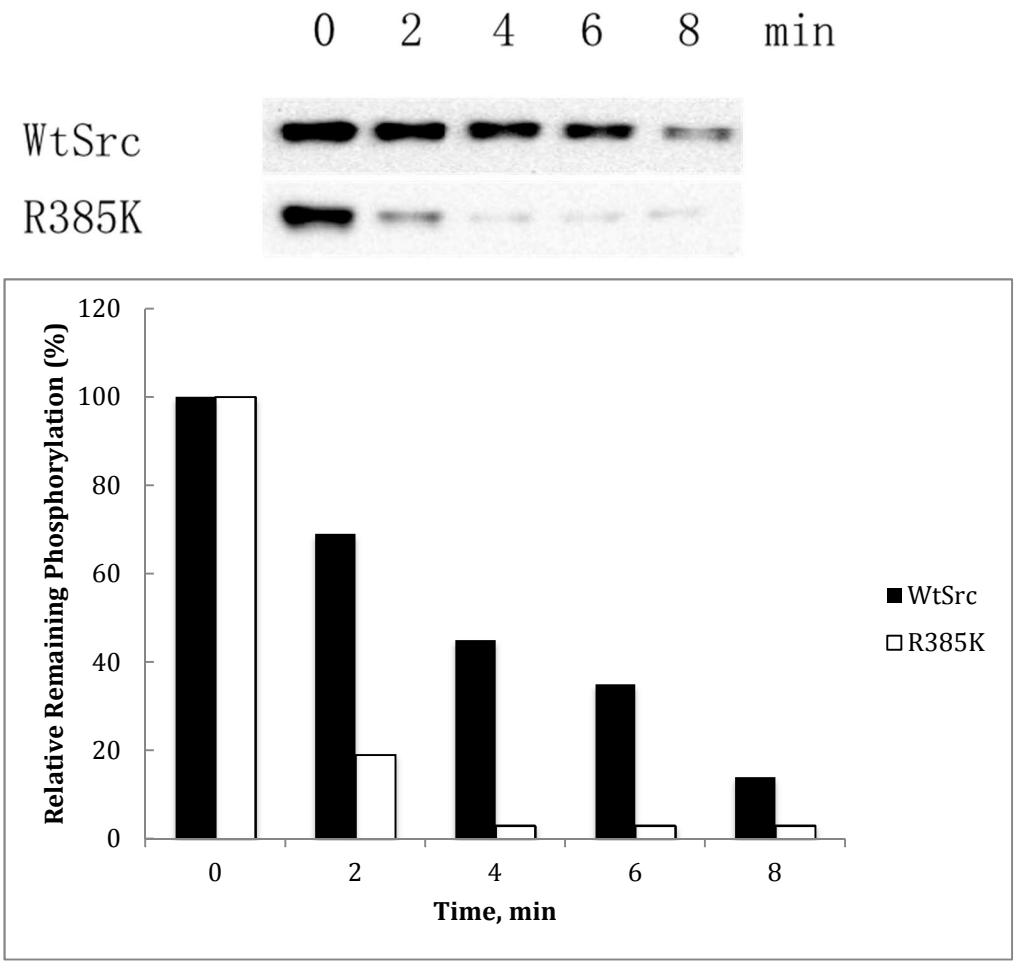
0 2 4 6 8 min
WtSrc
R385K
Relative Remaining Phosphorylation (%)
120
100
80
60
40
20
0
0
2
4
6
8
Time, min
WtSrc
R385K

Figure 3.3 (B)

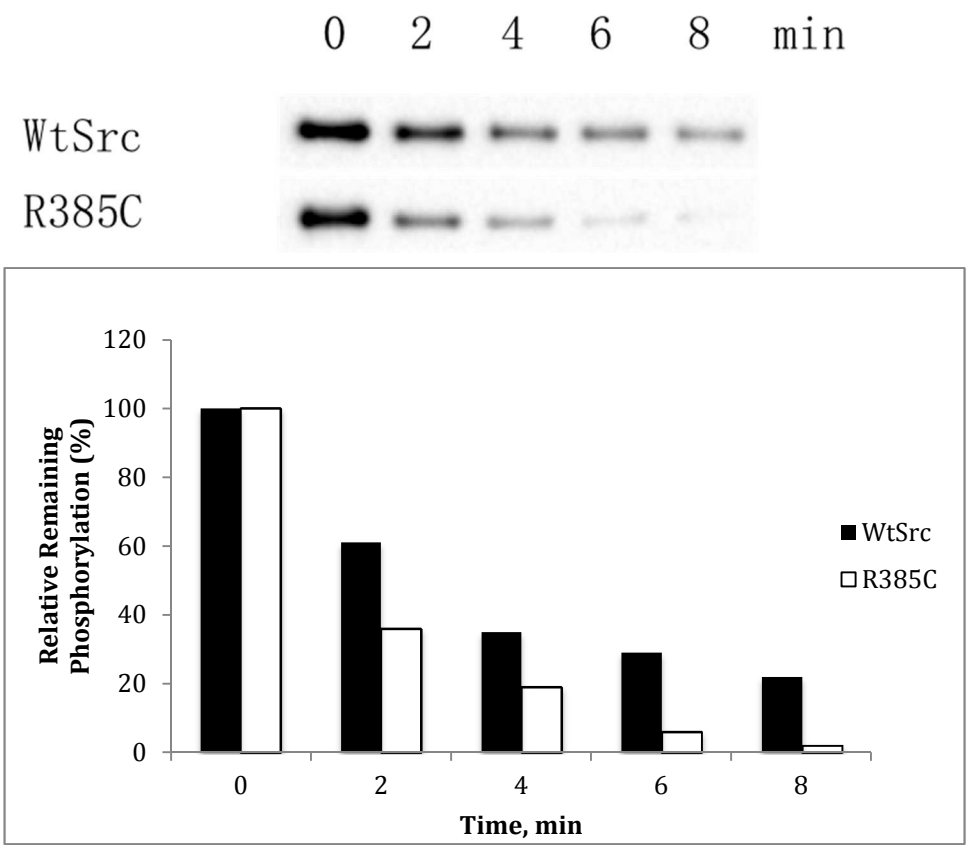
0 2 4 6 8 min
WtSrc
R385C
120
100
80
60
40
20
0
Relative Remaining Phosphorylation (%)
0
2
4
6
8
Time, min
WtSrc
R385C

Figure 3.3 Effect of R385 mutation on the dephosphorylation of the phosphorylated Tyr416 by PTP1B

WtSrc and R385 mutants at 450nM kinase concentration were incubated in the kinase assay buffer for 60min allowing autophosphorylation. Then the same amount of PTP1B and Na_2EDTA were added and aliquots were taken at indicated period of time. The remaining phosphorylation level was detected by a monoclonal antibody specific for phosphorylated Tyr416. Each R385 mutant was tested side by side with WtSrc and the phosphorylation intensity was visualized in histogram: (A) dephosphorylation examination of WtSrc and R385K; (B) dephosphorylation examination of WtSrc and R385C.

3.3.4 Effect of R385 mutation on Src regulation by Csk phosphorylation of the tail Tyr527

Csk phosphorylating Tyr527 on the C terminal tail of Src is another major mechanism of Src regulation. To test the effect of R385 mutation on Csk inactivation of Src, WtSrc and R385 mutants at 450nM were first pre-incubated with Csk at a molar ratio of 1:1 for 30 min, and then enzymes from the pre-incubation were diluted to 30nM or appropriate concentration for the standard kinase assay using Src optimal peptide as a substrate (Figure 3.4). Csk dosen't recognize Src optimal peptide as a substrate. The original activity of unphosphorylated Src and mutants was determined by pre-incubation in kinase assay buffer without $Mg^{2+}$and the absence of Mg^{2+} prevented autophosphorylation in the pre-incubation. Src Y416F and Src Y527F were included in the assays as controls. As seen in Figure 3.4, without tail tyrosine phosphorylation by Csk, Src Y527F kinase activity wasn't inhibited under this described condition. Indeed, the activity of Y527F increased by 250% of the original activity, which resulted from autophosphorylation in the pre-incubation. Activity of all R385 mutants and Src Y416F decreased to ~30% of the original activity when pre-incubated with Csk. This was in direct contrast with the WtSrc, which was not inactivated by the incubation with Csk. This result isn't consistent with what is known about WtSrc. Csk inactivation of Src has been reported widely both in vivo and in vitro [66, 67].

It has been demonstrated the Src inactivation requires stoichiometric amounts of

Csk[24, 68]. To rule out the possibility that Csk used was not as active as expected, higher Csk : Src molar ratios of 5 : 1, 10:1 or even 50: 1 were used in pre-incubation. But still no inactivation by Csk for wt-Src was observed under these conditions (data not shown).

It has been reported that autophosphorylation of Src and Yes, another Src family member, blocks their inactivation by Csk[30]. We wondered if wild type Src underwent autophosphorylation in the pre-incubation, which prevented its inactivation by Csk. The fact Y416 was inactivated by Csk supports this possibility. To test this possibility, autophosphorylation and tail phosphorylation were assessed when Src was incubated in the kinase assay buffer with the presence of Csk. Src incubated in the absence of Mg^{2+} or Csk served as controls. As seen in Figure 3.5, WtSrc was purified with a basal level of autophosphorylation on Tyr416 and didn't autophosphorylate further more in the pre-incubation with the absence of Mg^{2+}. Significant autophosphorylation on Tyr416 was detected for WtSrc after pre-incubation in kinase assay buffer regardless the presence or absence of Csk (Figure 3.5B and 3.5C). When Src was incubated in the presence of Csk, Tyr527 was significantly phosphorylated as seen in figure 3.5D. R385 mutants had the same patterns in the pre-incubation as wild type Src (data not shown). This demonstrated autophosphorylation and phosphorylation on the tail by Csk both occurred in the pre-incubation.

The double phosphorylated form of Src has been examined and activation of Src

depends on phosphorylation on Tyr416 regardless of the phosphorylation status of Tyr527[30]. Thus, autophosphorylation is likely the reason why WtSrc was not inactivated by Csk under the described condition above. Autophosphorylation of Src is by an intermolecular mechanism and thus concentration dependent[69]. To minimize autophosphorylation during pre-incubation with Csk, WtSrc at 10nM kinase concentration was pre-incubated with Csk at 5:1 or 10:1 Csk:Src molar ratio for 30min and the phosphorylation status of Tyr416 and Tyr527 was examined at the end of the pre-incubation. Under this condition described, the phosphorylation level of Tyr527 was dominant, while autophosphorylation was minimized (Figure 3.6A). The activity of Src was inactivated to ~50% of the original activity when pre-incubated with Csk (Figure 3.6B). These observations demonstrate Src gets inactivated by Csk only when it isn't autophosphorylated, which is consistent with the previous research.

So far, our results show that autophosphorylated R385 mutants can be regulated by Csk inactivation, while autophosphorylation prevents Csk inactivation of wild type Src. This raises the question why autophosphorylation does not seem to prevent inactivation by Csk for R385 mutants. We tested this possibility directly.

Figure 3.4

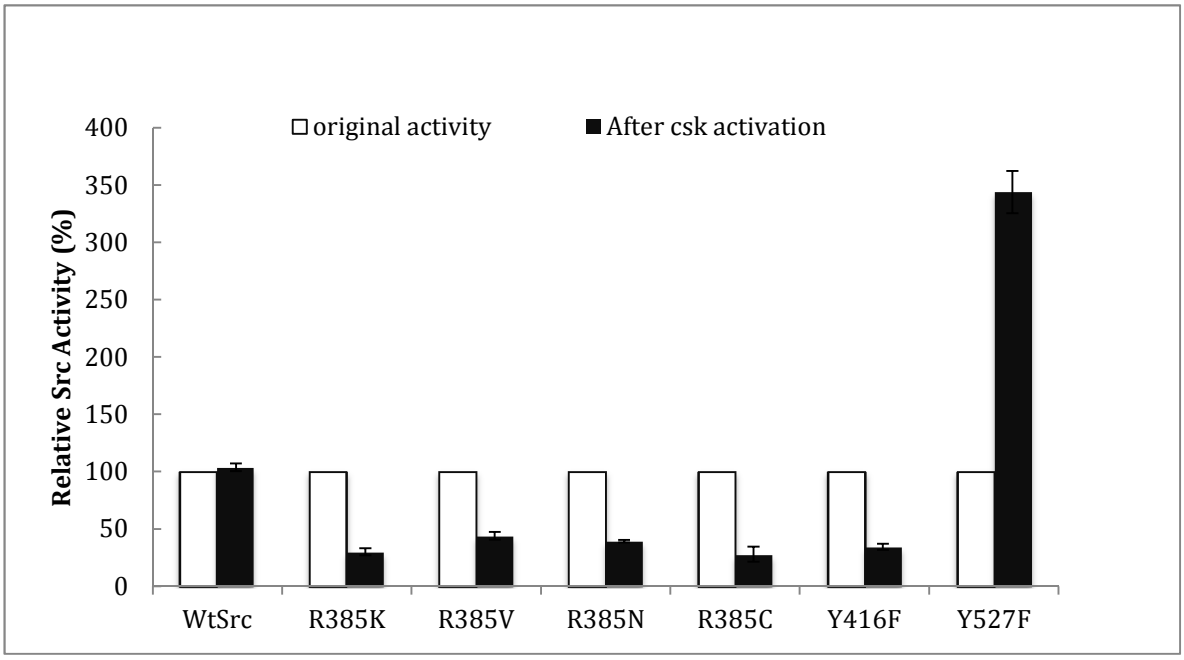
original activity
After csk activation
Relative Src Activity (%)
400
350
300
250
200
150
100
50
0
WtSrc
R385K
R385V
R385N
R385C
Y416F
Y527F

Figure 3.4 Effect of R385 mutation on Src inactivation by Csk regulation

WtSrc, R385 mutants, Src Y416F and Src Y527F at 450nM kinase concentration were incubated with Csk at 1:1 ratio in kinase assay buffer for 30min and the kinase activity of the wild type and the mutants at 30nM kinase concentration (diluted from pre-incubation) was determined using Src optimal peptide as a substrate by the standard kinase assay. Original activity was determined by pre-incubation in kinase assay without Mg^{2+}. The Mg^{2+} concentration was compensated at the final kinase assay. The relative activity after Csk inactivation was presented.

Figure 3.5

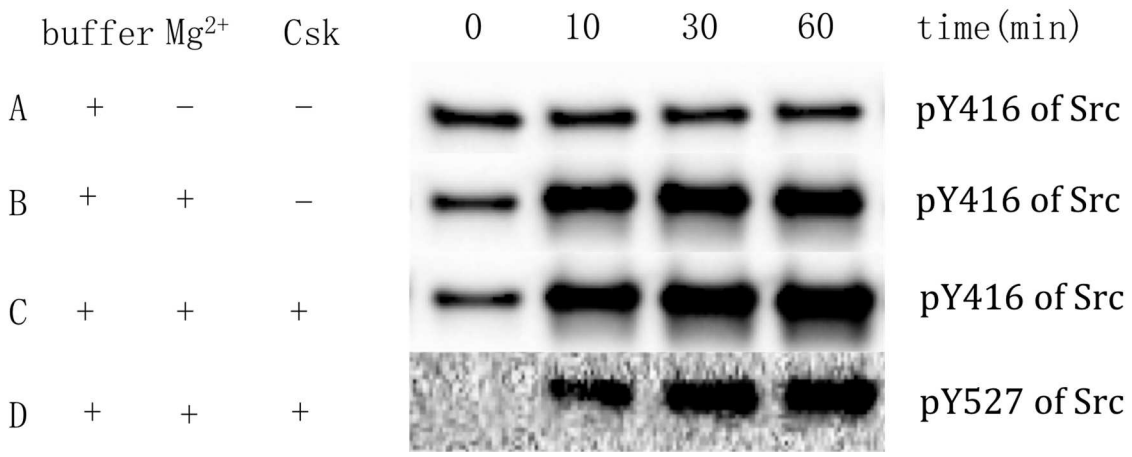
buffer
Mg2+
Csk
0
10
30
60
time(min)
A + − −
B + + −
C + + +
D + + +
pY416 of Src
pY416 of Src
pY416 of Src
pY527 of Src

Figure 3.5 Phosphorylation status of Src when it was incubated with Csk

(A) WtSrc at 450nM kinase concentration was incubated in kinase assay buffer with the absence of Mg^{2+}. (B) WtSrc at 450nM kinase concentration was incubated in kinase assay buffer with the presence of Mg^{2}. (C) WtSrc at 450nM kinase concentration was incubated with Csk at 1:1 ratio in kinase assay buffer. Aliquots were taken at indicated period of time and the autophosphorylation was detected by phosphorylated Tyr416 antibody for (A), (B) and (C). (D) WtSrc at 450nM kinase concentration was incubated with Csk at 1:1 ratio in kinase assay buffer and the tail phosphorylation was detected by phosphorylated Tyr527 antibody.

Figure 3.6(A)

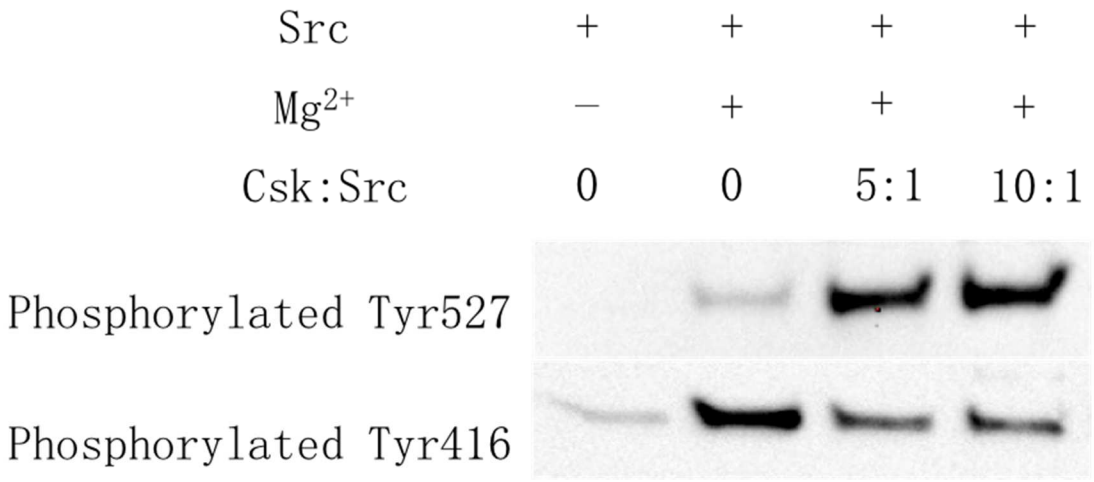
Src + + + +
Mg^{2+} − + + +
Csk:Src 0 0 5:1 10:1
Phosphorylated Tyr527
Phosphorylated Tyr416

Figure 3.6(B)

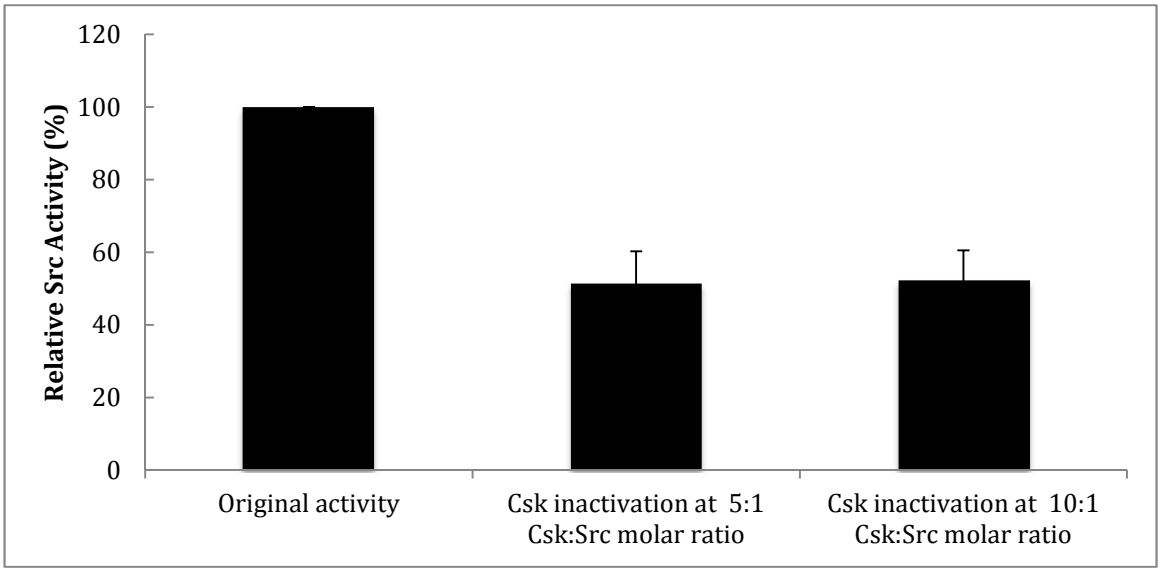
120
100
80
60
40
20
0
Relative Src Activity (%)
Original activity
Csk inactivation at 5:1 Csk:Src molar ratio
Csk inactivation at 10:1 Csk:Src molar ratio

Figure 3.6 WtSrc was inactivated by Csk only when WtSrc was not autophosphorylated

(A) Phosphorylation status of WtSrc on Tyr416 and Tyr527 when WtSrc at 10nM kinase concentration was pre-incubated with Csk at 5:1 or 10:1 Csk:Src ratio in kinase assay buffer for 30min. The phosphorylation level was determined by western blot with specific antibody at the end of the pre-incubation. (B) WtSrc at 10nM were pre-incubated with Csk at 5:1 or 10:1 Csk:Src molar ratio in kinase assay buffer for 30min and the kinase activity of WtSrc activity at 30nM kinase concentration (diluted from pre-incubation) was determined using Src optimal peptide as a substrate by the standard kinase assay. Original activity was determined by pre-incubation in kinase assay buffer with the absence of Mg^{2+}. The Mg^{2+} concentration was compensated at the final kinase assay. The relative activity after Csk inactivation was presented compared with original activity.

3.3.5 Effect of R385 mutation on Csk inactivation when Src was first autophosphorylated

R385 mutants can be inactivated by Csk when autophosphorylated based on the previous observations (Figure 3.4). However, it was not clear in the above experiments whether R385 mutants were first inactivated by Csk and then not affected by autophosphorylation or they are first autophosphorylated and then still able to be inactivated by Csk. To test this question, we had the enzymes autophosphorylated to maximum first and then incubated with Csk to see if the activity was inactivated. WtSrc and R385K were first incubated at 450nM in kinase assay buffer for 30 min allowing autophosphorylation and then Csk was added into the reaction at 1:1 ratio for another 30 min. After the first 30min pre-incubation, autophosphorylation should be saturated for WtSrc and R385K mutant based on previous observation. WtSrc got significant autophosphorylation on both Tyr416 and Tyr527 after the treatment (data not shown) and WtSrc was activated to ~238% of the original catalytic activity (Figure 3.7). This result demonstrates that double phosphorylated form of WtSrc maintains the active conformation, which is consistent with what is known. R385K mutant with the same treatment got phosphorylated significantly on both Tyr416 and Tyr527 similarly as WtSrc (data not shown). However, R385 mutant in the double phosphorylated form was inactivated to ~35% of the original activity. This result demonstrates that R385K can be inactivated by Csk even when it is first autophosphorylated. The R385 mutation makes the auto

phosphorylated kinase still subject to inactivation by phosphorylation on C terminal tail. This result indicates that phosphorylation on the activation loop prevents the transition from super active conformation to inactive conformation for Src even when the tail tyrosine is phosphorylated; whereas, R385 mutation makes Src transition from super active conformation to inactive conformation directly when the tail was phosphorylated. This result suggests that R385 plays a crucial role in controlling the conformational transition underlying the Src regulation by Csk phosphorylation.

Figure 3.7

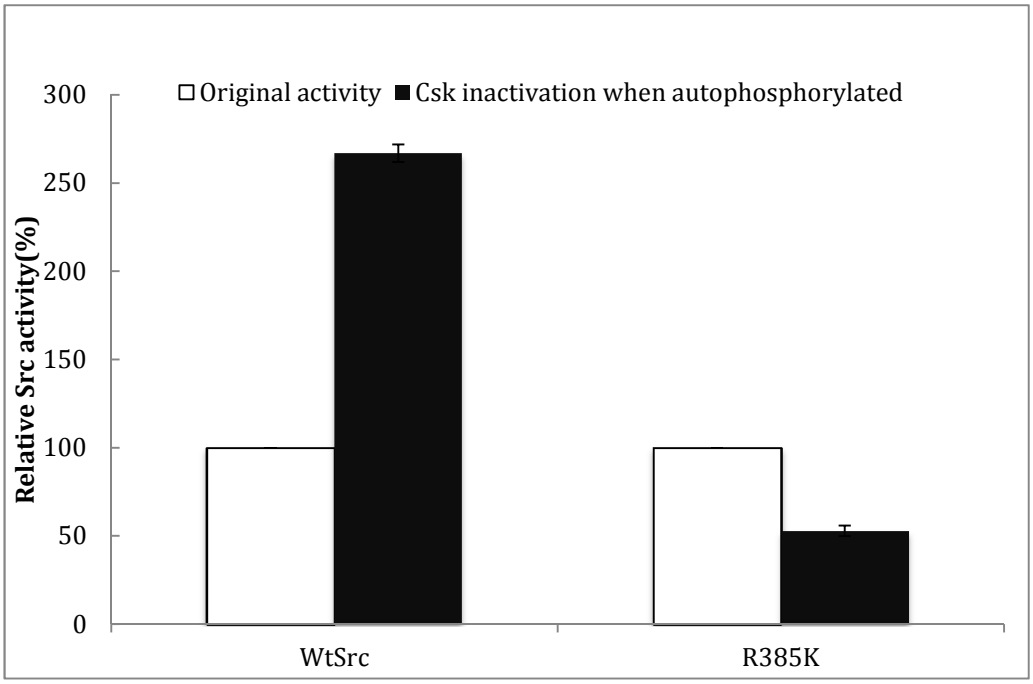
Original activity
Csk inactivation when autophosphorylated
Relative Src activity(%)
300
250
200
150
100
50
0
WtSrc
R385K

Figure 3.7 Inactivation by Csk when Src was first autophosphorylated

WtSrc and R385K were first pre-incubated at 450nM in kinase assay buffer for 30min allowing autophosphorylation and then incubated with Csk at 1:1 ratio for another 30min. The kinase activity of the WtSrc and R385K at 30nM kinase concentration (diluted from pre-incubation) was determined using Src optimal peptide as a substrate by the standard kinase assay. WtSrc and R385K were pre-incubated at 450nM in kinase assay buffer for 60min in the absence of Csk or Mg^{2+} and then kinase activity was determined as original activity. The Mg^{2+} concentration was compensated for in the final kinase assay. The relative activity of Csk inactivation when first autophosphorylated is presented compared with original activity.

3.3.6 Effect of R385 mutation on Src affinity towards Type II inhibitors

Most of the current kinase inhibitors are ATP competitive and form one to three hydrogen bonds with the kinase that are normally formed by the adenine ring of ATP[38]. Based on their interaction with target kinases, PTK inhibitors are generally classified into three types. Type III inhibitors are allosteric and target outside the active site of kinases, which will not be discussed in this study. Type I inhibitors constitute a majority of the ATP competitive inhibitors and bind to the active conformation of protein kinases, while type II inhibitors bind to the inactive conformation. A variety of inactive conformations have been observed even for a single kinase, thus type II inhibitors can potentially achieve more selectivity by exploiting energetic differences among the conformations of different kinases[63]. Results in the previous sections suggested that R385 plays an important role on conformational transition of Src underlying its inactivation by Csk. In that case, it would be expected that R385 might contribute to the conformational transition of Src to an inactive conformation important for the binding of type II PTK inhibitors.

Most of the characterized type II inhibitors do not inhibit Src or have a relatively low potency. Notably, imatinib is known to be highly selective and inhibit Abl well by targeting the inactive conformation [44, 70]. While sharing strikingly high homology with Abl, Src is not inhibited by imatinib. Ponatinib, another type II inhibitor, has high potency toward Abl (IC50=0.37nM) and very low potency towards Src(IC50=5.4nM)[71-73]. Sorafenib[74] is a multikinase inhibitor and its potency

towards Abl or Src is not clear yet. Thus, Imatinib, ponatinib, and sorafenib were selected as representative type II inhibitors in this study. They are all FDA-approved cancer therapeutics and bind to the inactive conformation of protein kinases, as demonstrated by crystallized structures[38].

IC50 of each inhibitor towards WtSrc and R385K mutant was determined as described in the methods section. WtSrc and R385K at the same concentration were assayed in the presence of a series of concentrations of each inhibitor. IC50 towards each inhibitor was compared between WtSrc and R385K: R385K bound to imatinib with an IC50 ~25 fold lower than to WtSrc (Figure 3.8); R385K mutation lowered the IC50 of ponatinib toward Src by ~9 fold (Figure 3.9); R385K lowered the IC50 of Src for sorefenib by ~72 fold (Figure 3.10). These data indicated that R385 mutation improves the affinity of Src towards the type II PTK inhibitors. These results demonstrated that wild type Src was not able to readily transition into the inactive conformation, thus preventing the binding of the these type II inhibitors. The mutation of R385K removed the energy barrier and enabled the mutation to readily transition to the inactive conformation, which favored the binding of the type II inhibitors. This conclusion is consistent with the finding in the previous section that mutation of R385 enables active Src to be inactivated by Csk.

Figure 3.8

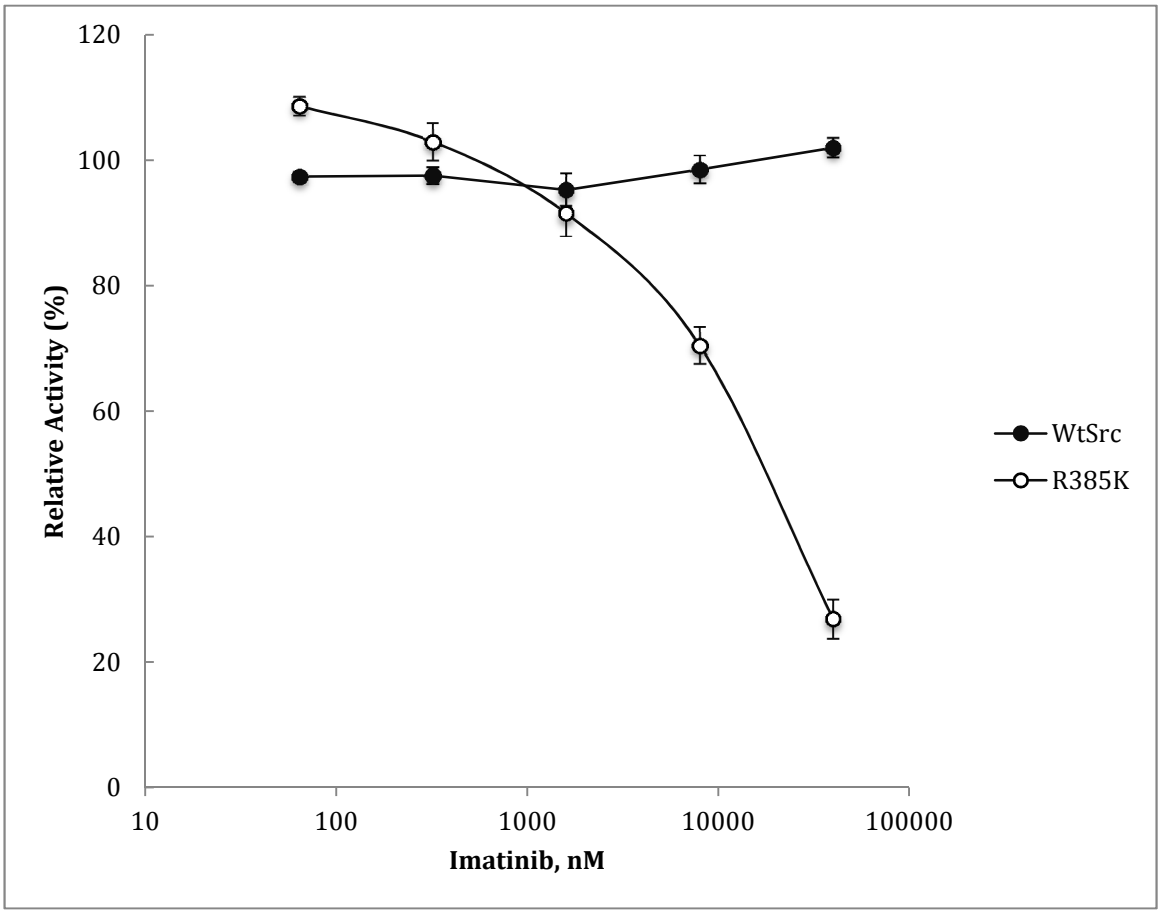
120
100
80
60
40
20
0
Relative Activity (%)
10
100
1000
10000
100000
Imatinib, nM
WtSrc
R385K

Figure 3.8 Imatinib inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and R385K was measured in the standard kinase assay with the presence of 0.064, 0.32, 1.6, 8, and 40uM imatinib and graphed as a relative percentage of the uninhibited activity to determine imatinib 's IC50: WtSrc=432.76uM; R385K=17.38uM.

Figure 3.9

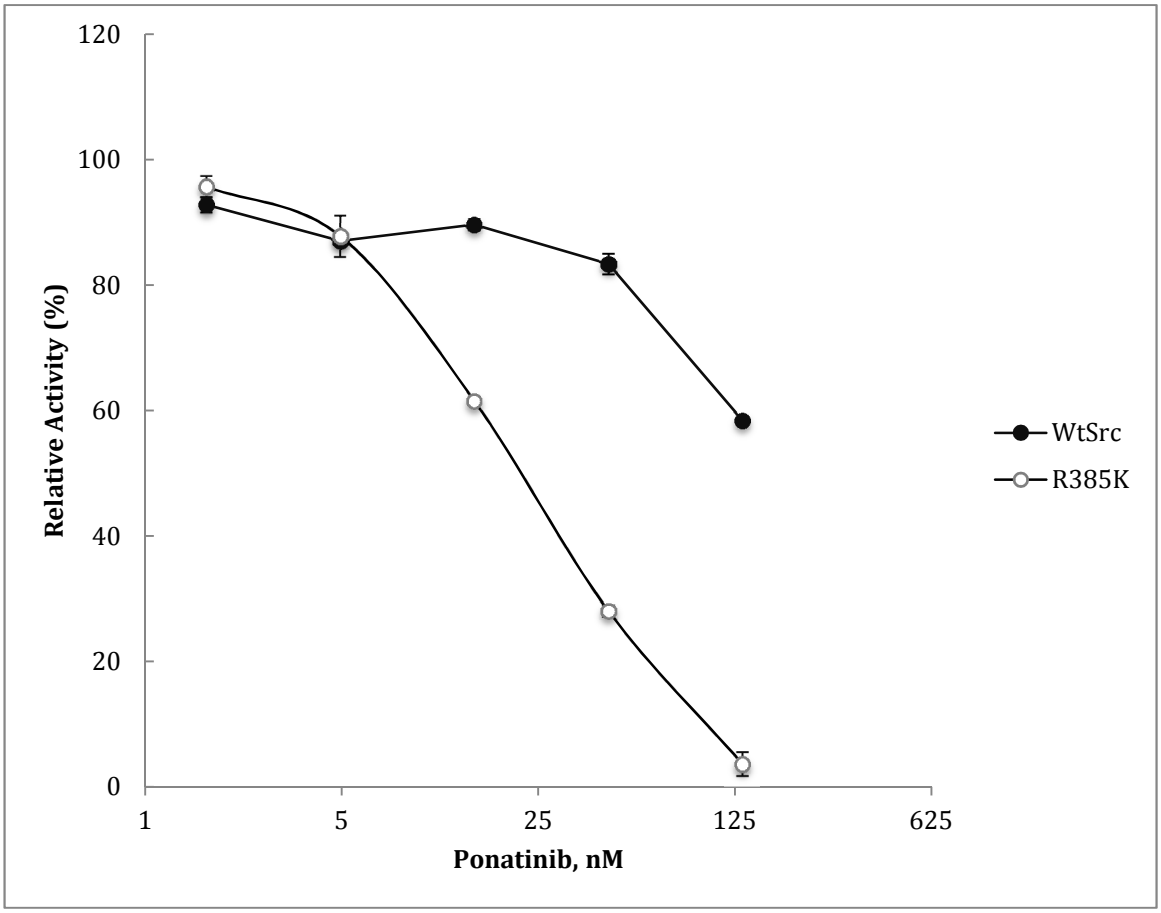
120
100
80
60
40
20
0
Relative Activity (%)
1
5
25
125
625
Ponatinib, nM
WtSrc
R385K

Figure 3.9 Ponatinib inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and R385K was measured in the standard kinase assay with the presence of 1.646, 4.938, 14.8, 44.4, and 133.2nM ponatinib and graphed as a relative percentage of the uninhibited activity to determine ponatinib's IC50: WtSrc=182.00nM; R385K=20.61nM.

Figure 3.10

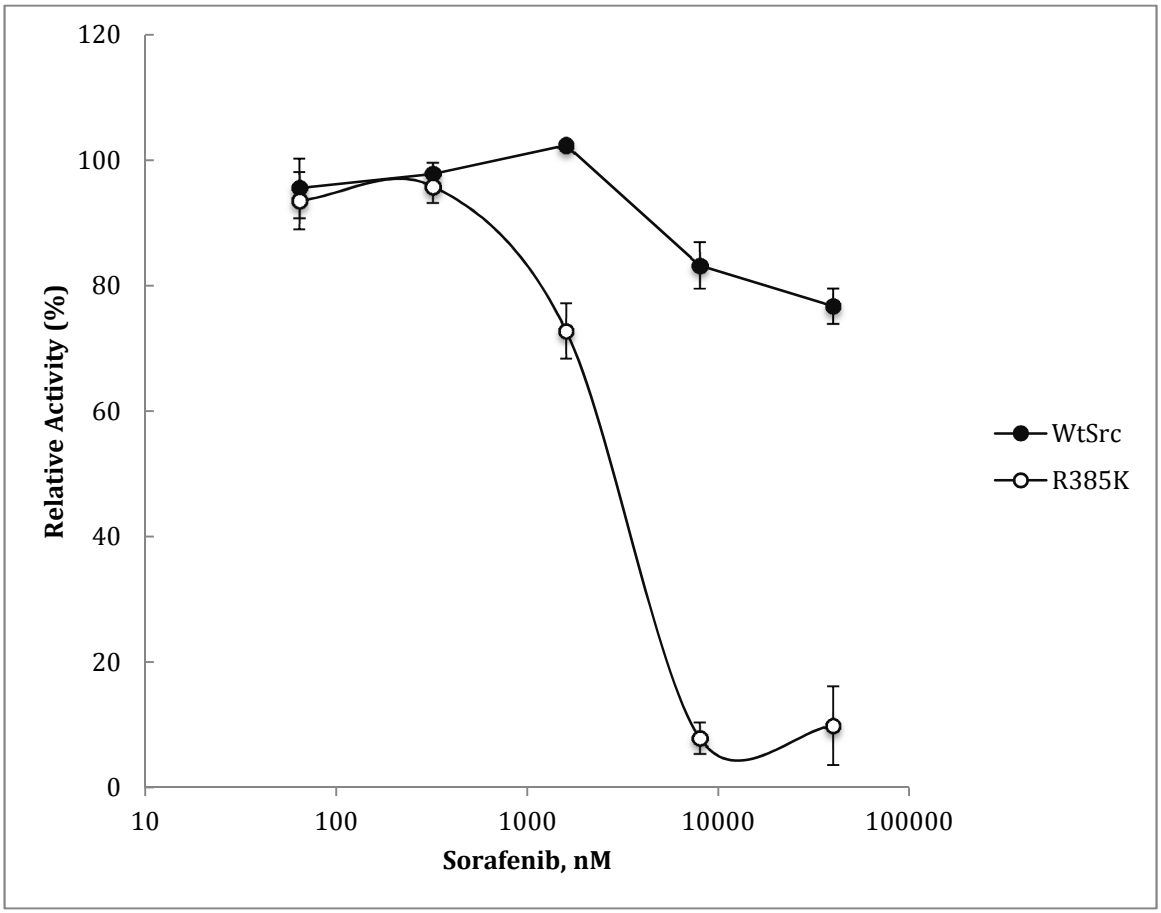
120
100
80
60
40
20
0
Relative Activity (%)
10
100
1000
10000
100000
Sorafenib, nM
WtSrc
R385K

Figure 3.10 Sorafenib inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and Src R385K was measured in the standard kinase assay with the presence of 0.064, 0.32, 1.6, 8, and 40uM sorafenib and graphed as a relative percentage of the uninhibited activity to determine sorafenib's IC50: WtSrc=117.27uM; R385K=1.62uM

3.3.7 Effect of R385 mutation on Src affinity towards type I inhibitors

With the observation of improved affinity towards type II inhibitors as a result of Arg385Lys mutation, the affinity of R385K toward type I PTK inhibitors was explored. Because the Type I inhibitors bind to the active conformation of kinases, the mutation was not expected to affect the binding of these inhibitors.

Both dasatinib[75] and bosutinib[76] are dual Src / Abl inhibitors, which are known to bind to active conformation of Src family kinase and belong to type I inhibitor[77]. The IC50 of WtSrc and R385K mutant towards each inhibitor was determined as described above. As shown, WtSrc (3.12nM) and R385K (2.16nM) had just the same IC50 towards dasatinib (Figure 3. 11). Consistent with this pattern, the difference between WtSrc (4.88nM) and R385K (2.70nM) towards bosutinib was almost negligible (Figure 3.12).

To ensure not just R385K mutant behave this way, the drug sensitivity of dasatinib towards WtSrc and representative R385 mutants including R385V, R385K, R385A, R385C and R385N was probed at a set concentration of Dasatinib, 4nM. As shown in Figure 3.13, the kinase activity of WtSrc and R385 mutants was all inhibited to a similar level, ~ 10-30% of the original activity. This result showed that WtSrc and all R385 mutants had similar drug sensitivity towards dasatinib. It demonstrates that R385 mutation is able to maintain the active site of kinases, instead of destabilizing it. This is consistent with the previous finding that R385 mutation doesn't destabilize the active conformation and the super active conformation caused by

autophosphorylation. Finally, it can be reasoned that R385 mutation may not stabilize either the inactive or active conformation, but makes the transition from active conformation to inactivation conformation easier.

All together, study of the R385 mutation towards PTK inhibitors demonstrates that R385 plays an important role in controlling the conformational plasticity that provides the basis of Src inactivation and Type II inhibitor binding.

Figure 3.11

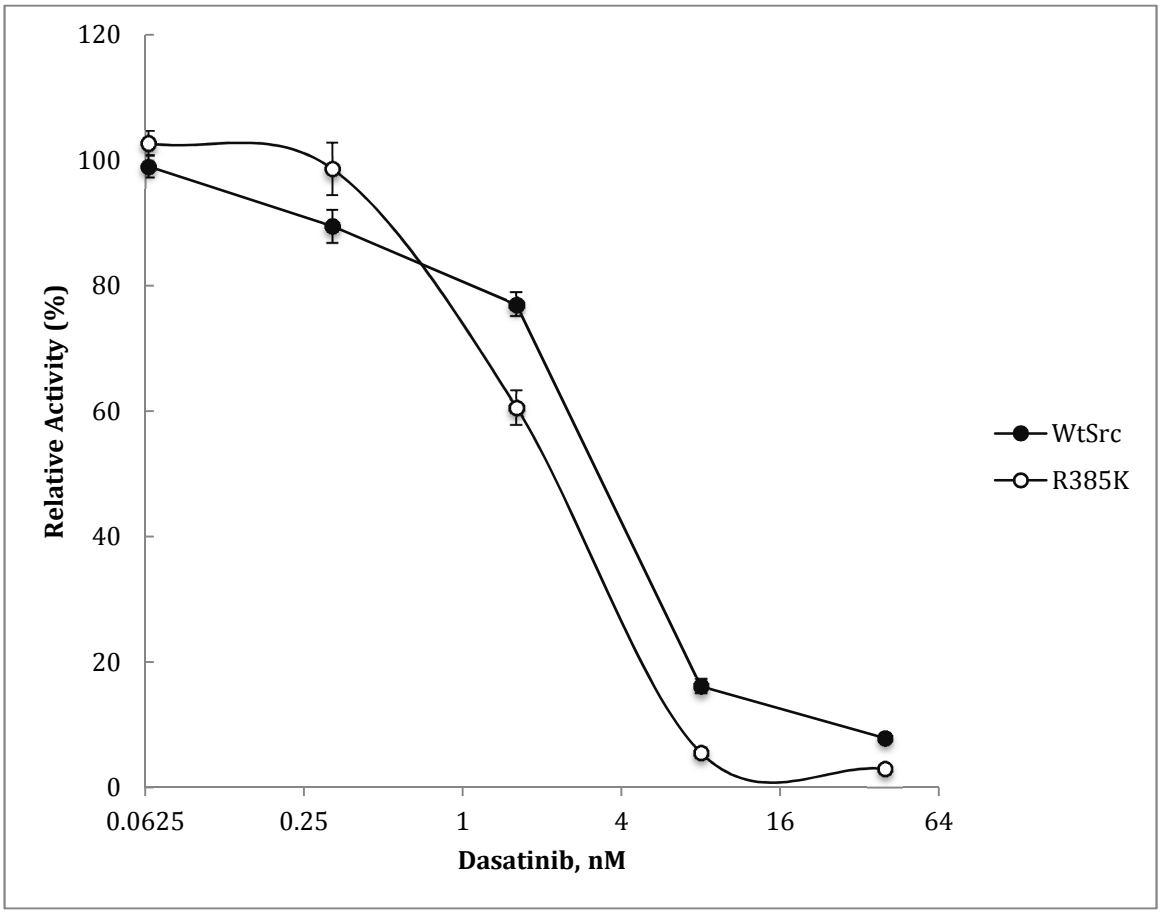
120
100
80
60
40
20
0
Relative Activity (%)
0.0625
0.25
1
4
16
64
Dasatinib, nM
WtSrc
R385K

Figure 3.11 Dasatinib inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and Src R385K was measured in the presence of 0.064, 0.32, 1.6, 8, and 40nM dasatinib and graphed as a relative percentage of the uninhibited activity to determine dasatinib's IC50: WtSrc=3.12nM; R385K=2.16nM.

Figure 3.12

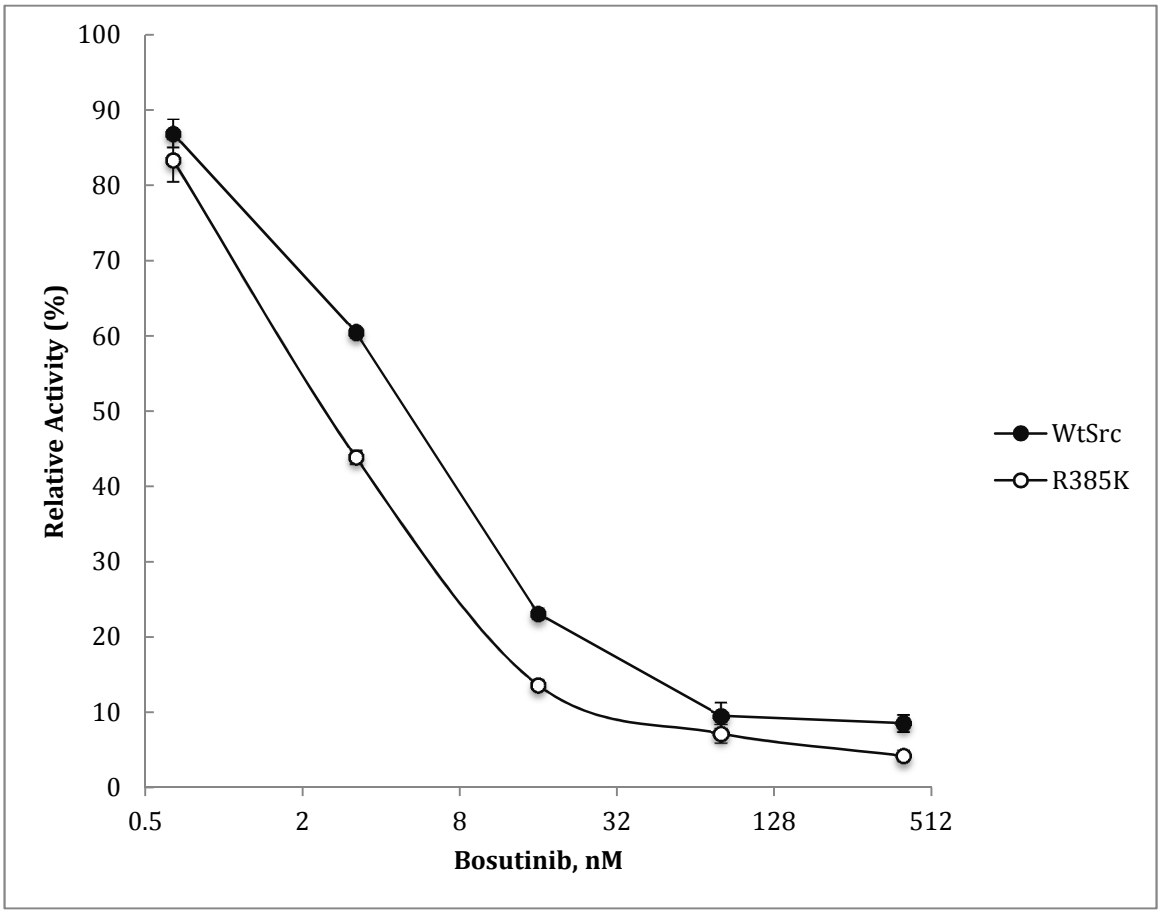
100
90
80
70
60
50
40
30
20
10
0
Relative Activity (%)
0.5
2
8
32
128
512
Bosutinib, nM
WtSrc
R385K

Figure 3.12 Bosutinib inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and Src R385K was measured in the presence of 0.64, 3.2, 16, 80, and 400nM bosutinib and graphed as a percentage of the uninhibited activity to determine bosutinib's IC50: WtSrc=4.88nM; R385K=2.70nM.

Figure 3.13

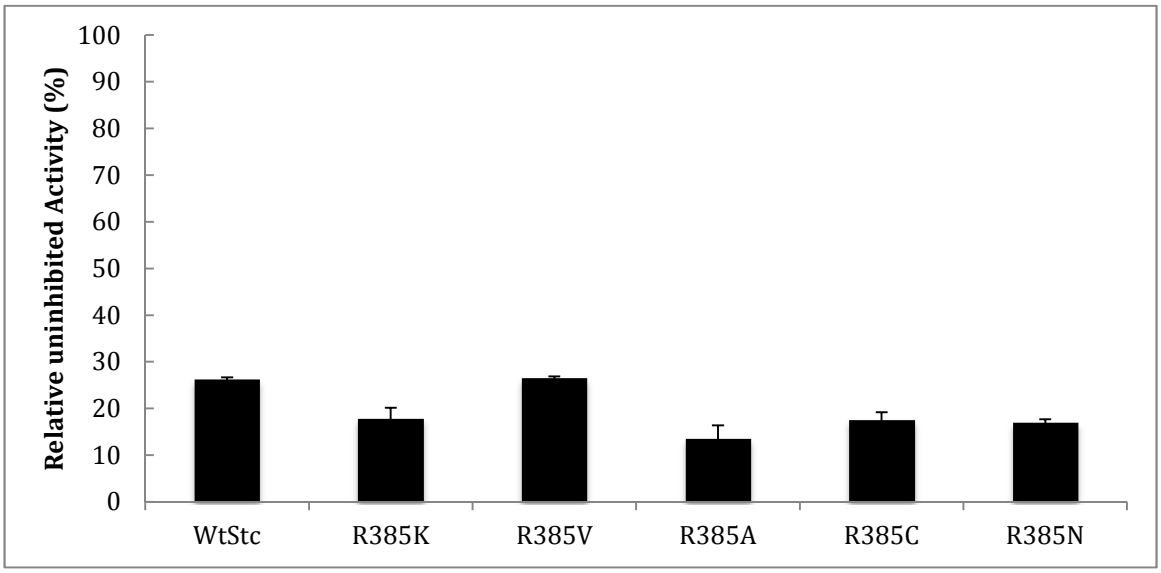
Relative uninhibited Activity (%)
100
90
80
70
60
50
40
30
20
10
0
WtStc
R385K
R385V
R385A
R385C
R385N

Figure 3.13 Drug sensitivity of dasatinib for WtSrc and selected R385 mutants

Relative kinase activity of WtSrc and R385 mutants at 3nM was determined by the standard kinase assay in the presence of 4nM dasatinib compared with original activity in the absence of dasatinib.

3.3.8 R385 mutants can be used to study the property of novel PTK inhibitors

With the understanding of the R385 mutation functioning in transition between different conformational states, affinity differences between WtSrc and R385 mutation towards a given inhibitor could be used to study the binding property of the inhibitor towards Src in turn.

A-770041 and WH-4-023 are novel Lck inhibitors, and both are potent and selective. A-770041 was found to be 300 fold selective for Lck over Fyn, another Src family kinase [78]. WH-4-023 displays selectivity over a range of closely related kinases, exhibiting >300-fold selectivity against p38 α and KDR [79]. However, so far there is still little known about the mechanism of the selectivity. Thus, we decided to test the sensitivity of A-770041 and WH-4-023 towards WtSrc and R385K, respectively. As shown (Figure 3.14 and 3.15), WtSrc (14.57uM) and R385K (1.08uM) had distinct IC50 towards A-770041 and WtSrc (93.68nM) and R385K (20.70nM) had 3-fold different IC50 towards WH-4-023. The affinity difference between WtSrc and R385K toward these inhibitors indicates that these inhibitors may act as type II inhibitors and that the selectivity of A-770041 and WH-4-023 over closely related kinases may result from the energetic differences among kinases. This discovery helps the understanding of the selectivity of the two potent inhibitors against other closely related kinases.

Figure 3.14

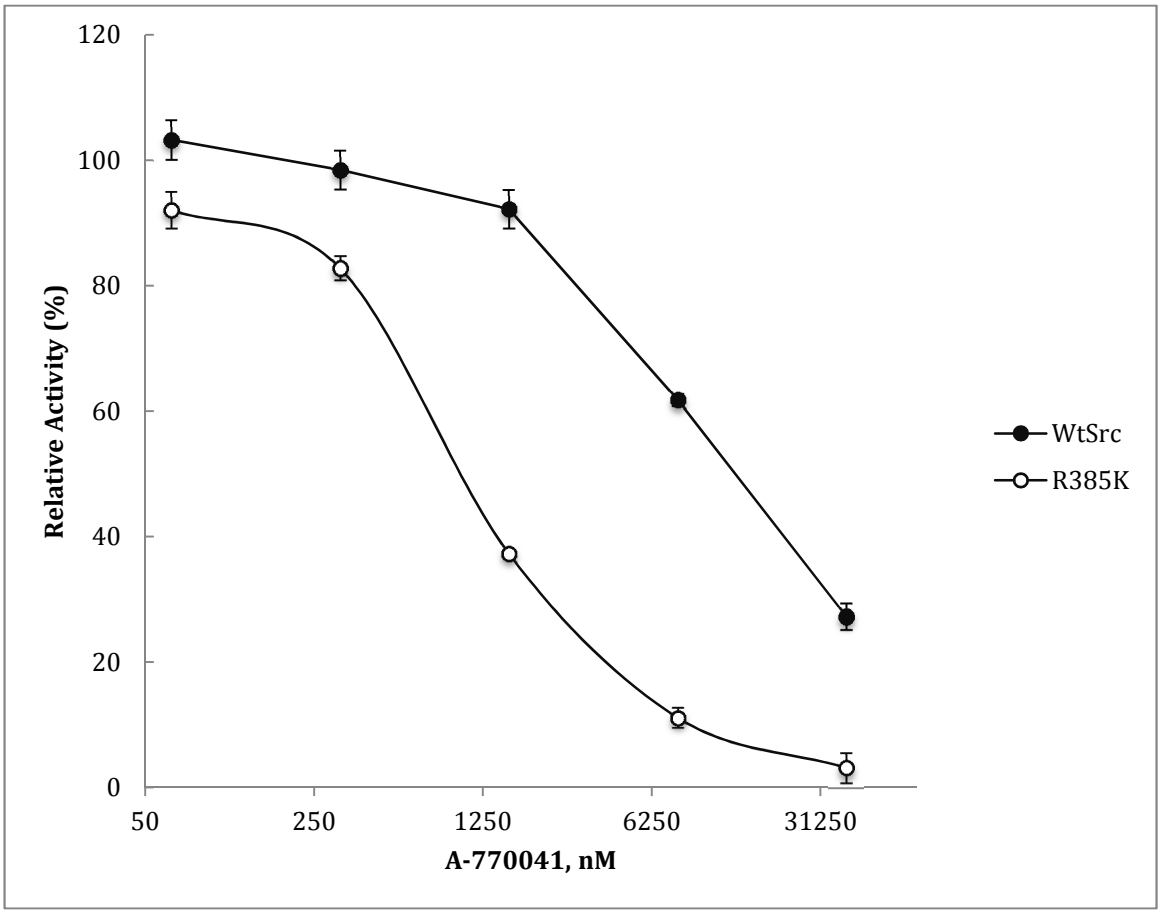
120
100
80
60
40
20
0
Relative Activity (%)
50
250
1250
6250
31250
A-770041, nM
WtSrc
R385K

Figure 3.14 A-770041 inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and Src R385K was measured in the presence of 0.064, 0.32, 1.6, 8, and 40uM A-770041 and graphed as a relative percentage of the uninhibited activity to determine A-770041's IC50: WtSrc=14.57uM; R385K=1.08uM.

Figure 3.15

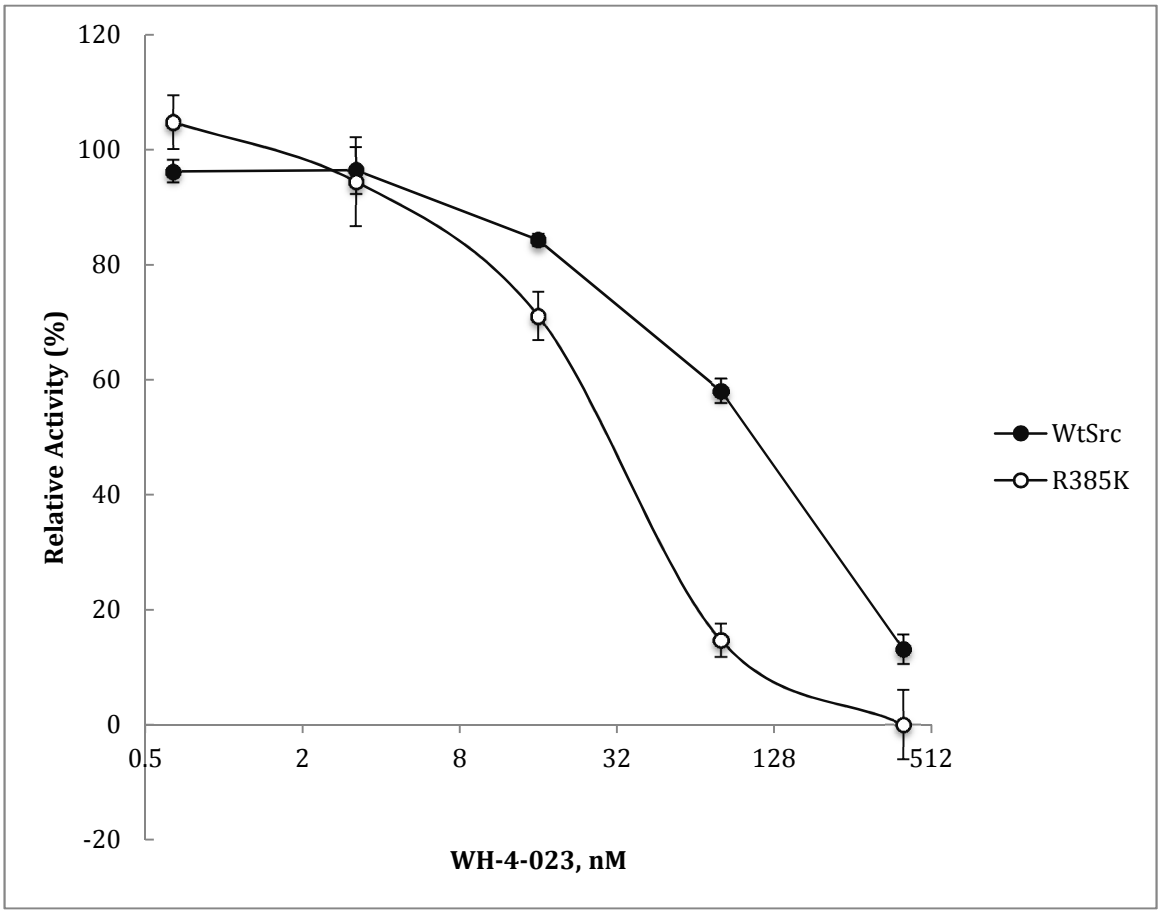
120
100
80
60
40
20
0
-20
Relative Activity (%)
0.5
2
8
32
128
512
WH-4-023, nM
WtSrc
R385K

Figure 3.15 WH-4-023 inhibition comparison between WtSrc and Src R385K

The activity of WtSrc and Src R385K was measured in the presence of 0.64, 3.2, 16, 80, and 400nM WH-4-023 and graphed as a relative percentage of the uninhibited activity to determine WH-4-023's IC50: WtSrc=93.68nM; R385K=20.70nM.

3.4 Discussion

Although different conformations of the active site have been well demonstrated in numerous PTKs[80], what controls different conformational status has not been demonstrated in any. This current study demonstrates that the arginine in the HRD motif plays a crucial role in the control of conformational transition in the protein tyrosine kinase, Src.

R385 doesn't play a role in catalysis

It has been demonstrated in the previous chapter that a quite wide range of structurally diverse amino acids can replace the HRD arginine of Src and maintain approaching full catalytic activity, consistent with previous reports of R385 mutation. Mutation of the arginine to alanine in yeast PKA reduces the kinase activity to 10.5% of the wild type activity, but still maintains the viability of the yeast[54]. The embryo extracts of the HRD arginine to cysteine mutant of Drosophila Src64 has 90% of wild type kinase activity[52]. Regarding the biological function during the development of Src64, this mutant has no effect on microfilament contraction, ring canal growth or hatching, but has defects in egg production[52]. The arginine in the HRD motif is conserved in most protein kinases but not all, and some authors even identify the HRD motif as an "HXD motif"[46]. All together, it suggests that the arginine is not essential for catalysis of Src.

R385 is not required for Src autophosphorylation or activation by autophosphorylation

All R385 mutants still get autophosphorylated on Tyr416 and activated through autophosphorylation as wild type Src, which is in direct contrast to the previous understanding of the role of R385. In the leading model of Src activation, phosphorylation of Tyr416 triggers an electrostatic switch and shifts the conformational equilibrium to the stabilized active conformation. Upon Tyr416 phosphorylation, the activation loop adopts an active configuration, which has been well-defined in the active forms of Lck [33]and insulin receptor tyrosine kinase[35]. The phosphorylated activation loop forms two strands that pack against the N lobe of the kinase, pinned in place by coordination of the phosphorylated Tyr416 with two arginines, Arg385 in the catalytic loop and Arg409 in the activation loop. Thus, the role of the R385 was suggested to stabilize the active configuration of the activation loop when it is phosphorylated[29]. Indeed, R385 mutation to unrelated amino acids in this study can maintain not only the active conformation of the catalytic site, but also the active configuration of the activation loop when it is phosphorylated for Src activation.

R385 protects phosphorylated Tyr416 in the activation loop from dephosphorylation

The HRD arginine protects the phosphorylated Tyr416 in the activation loop from dephosphorylation by PTP1B as demonstrated in this study. It is consistent with the directed contact between the arginine and the phosphorylated tyrosine displayed in the crystallized structure. This suggests that the R385 plays a role in preventing the inactivation of Src by phosphatase. In other words, R385 prevents the disruption of the active configuration of the activation loop when it is phosphorylated and thus prevents the conformational transition to the inactivation conformation. Notably, even the closely related lysine can't replace the arginine to prevent the dephosphorylation or maintain the interaction with the phosphorylated tyrosine. It suggests some other kind of interaction between the phosphorylated tyrosine and the arginine in the structural environment, instead of the proposed salt-bridge formed between the negative charged tyrosine and the positive charged arginine in the previous research[29]. A similar regulatory mechanism has been observed in AMPK that the small amphipathic □G-helix of the kinase domain is involved in AMPK inactivation by protein phosphatase[65].

R385 is required for autophosphorylation to override the Src inactivation by Csk

Src activity can be inactivated by Csk through an intramolecular interaction, but the autophosphorylation on Tyr416 overrides this inactivation[30]. Upon autophosphorylation, the activation loop is rearranged into the active configuration,

which prevents the inactivation of Src. Phosphorylated Tyr416 and the rearranged activation loop together play a kinetic proofreading role. This kind of phenomenon has been well recognized in protein phosphorylation reactions as a way of error correction[31]. However, it was observed in this study that all of the R385 mutants can be readily inactivated by Csk even when it is first autophosphorylated on Tyr416. This suggests that R385 prevents the disruption of the active configuration of the activation loop induced by Csk phosphorylation and thus prevents the conformational transition to the inactivation conformation. Furthermore, this result contradicts the current understanding of the R385 function on the inactive conformation. In the crystal structure of the inactive conformation of the Src kinase domain[29], R385 forms salt-bridge with the conserved Glu310 located in the helix αC and thus stabilizes the inactive configuration of the helix αC. However, all tested R385 mutants can be inactivated by Csk readily and maintain the inactive conformation. Indeed, R385 acts as an energy barrier and is only responsible in preventing the disruption of the active configuration of the activation loop when phosphorylated based on the observation in this study.

R385 is a key residue in differentiating Src and Abl in type II inhibitor binding

Ever since the discover of imatinib, a highly successful cancer therapeutic commercially known as Gleevec, the selectivity of it towards Abl over other highly homologous PTKs like Src has been intensely investigated[49, 81-84], but ultimately

without a decisive success. Recently, the different selectivity is proposed to be due to the thermodynamic penalty caused during conformational transition[84-86]. This current study of the R385 mutation towards both type I and II inhibitors reveals HRD arginine as a structural basis for the thermodynamic penalty caused during conformational transition from active to inactive conformation proposed in the previous research. First, R385 mutants with structurally diverse substitutions have almost identical affinity towards type I PTK inhibitors as wild type, which target the active conformation of the active site. This suggests that R385 is not essential in maintaining the active conformation targeted by type I inhibitors. Second, strikingly increased affinity toward type II PTK inhibitors is observed for R385 mutations with structurally diverse substitutions compared to the wild type Src. Type II inhibitors target the inactivate conformation of the active site. Upon binding with type II inhibitors, the activation loop undergoes conformational change that flips the aspartate in the DFG motif away from the active site and the phenylalanine into the ATP binding site, the so called DFG-out conformation[44]. Thus, it suggests that R385 mutation removes the energetic barrier caused by the transition to the inactive conformation of Src induced by the type II inhibitors. Also, R385 mutations with structurally diverse substitutions can maintain the inactive conformation, which binds well with the type II inhibitors. It suggests that R385 is not responsible in stabilizing the inactive conformation of the active site. All together, evidence support that the HRD arginine acts as energetic barrier and prevents the conformational

transition from active conformation to inactive conformation when Src binds to a type II inhibitor. The role of R385 as energetic barrier in small molecular inhibitors binding is consistent with the role of it in Src inactivation by Csk.

R385 represents a control of the conformational transition of catalytic domin from active to inactive

This current study on R385 provides a structural basis for the control of the conformational transition of Src. Several lines of evidence presented above support this. Src R385 acts as an energetic barrier in the transition from active configuration to the inactive configuration and makes it easier when induced. This similar regulation mechanism also has been observed in Abl. The interaction of the type II inhibitors with Abl depends on the configuration of the activation loop[81, 84, 87]. Abl with phosphorylated activation loop is less sensitive to the type II inhibitors. Abl pays an additional energetic penalty in adopting the DFG-out inactive conformation. These observations make this energetic regulation in conformational transition well recognized and R385 presents as a control of it in Src.

The unique function of R385 can also be used to study the binding property of small molecular inhibitors in return, which is an essential research area in drug design and development. The binding affinity of two novel inhibitors, A-770041 and WH-4-023, towards wild type Src and R385K mutation was determined in this study.

This characterization reveals that the thermodynamic penalty caused by conformational transition might also contribute to the selectivity of these two inhibitors.

This study leads to the identification of the crucial role of R385 in transition between different conformational states. One question remains to be answered. What is the role of the HRD arginine in Abl and other protein kinases, that have an arginine in the HXD motif. Since both Abl and Src are conserved in this HRD motif, the arginine must have a different regulatory roles compared with Src due to the very different affinity towards imatinib. This implies that the HRD arginine doesn't function alone, but under the overall structural environment. This leads to more questions as to what are these residues and how do they function synergistically with the regulatory HRD arginine for different kinases. The Src R385 mutation could serve as a model for the further study.

3.5 Materials and methods

3.5.1 Reagents and chemicals

Consumables and culture media, or media components, were purchased from Fisher Scientific. Inhibitors used were purchased from Selleckchem. Specific antibodies were purchased from Cell Signaling Technology. All the other chemicals were purchased from Sigma.

3.5.2 Expression and purification of Src and mutants

Src and Src mutants were constructed in pRSETA expression plasmid. The Src constructs were expressed in *Escherichia coli* BL21(DE3)RIL cells harboring the pREP4groESL expressing the GroES/EL chaperone and the pCDF-1B expressing protein tyrosine phosphatase PTP1B. The (His)6-tagged Src constructs were purified using immobilized Ni -iminodiacetic acid-agarose as described previously[62]. Concentration and purity of each enzyme were determined by Bradford assay and SDS-PAGE, respectively. Purified enzymes were stored in 50% glycerol at -20^{0}C.

3.5.3 Expression and purification of PTP-1B, CSK and Src optimal peptide

Constructs of Csk tyrosine kinase, PTP-1B phosphatase, and Src optimal peptide were expressed in DH5α cells using pGEX-4T-1 plasmid. As described previously, the glutathione-S-transferase (GST)-fusion proteins were purified using glutathione-agarose resin[88]. Concentration purify of each enzyme were determined by Bradford assay and SDS-PAGE, respectively. Purified enzymes were stored in 40% glycerol at -20^{0}C. Peptide substrate were stored in -80^{0}C directly without glycerol.

3.5.4 Kinase assay

Kinase activity of Src and mutants was determined by using polyE4Y, or Src optimal peptide, and ATP as the substrates. The phosphorylation reactions were performed in 50ul volumes at 25°C in the protein kinase assay buffer: 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (pH 8.0) containing 5% glycerol, 0.005% Triton X-100, and 0.05% 2-mercaptoethanol. The standard assay used 12mM $MgCl_2$, 0.2mM ATP, and 1mg ml^{-1} $polyE_4Y$, or 10μM Src optimal peptide. After a 30-min reaction time, 5ul of the kinase reaction mixture was mixed with 5ul ADP-Glo™ reagent and incubated for 40 min to stop the kinase reaction and deplete the unconsumed ATP. 10ul Kinase Detection reagent was added and incubated for another 30 min to convert produced ADP to ATP and introduce luciferase and luciferin to detect converted ATP. Then the luminescence produced was measured by a plate-reading luminometer. Assays were performed in duplicate, and each assay was repeated three times with reproducible results. For kinase assays designed to determine inhibitor's IC50, inhibitors used in this study were dissolved in 10% DMSO, diluted in series, and included within the kinase reaction.

3.5.5 Autophosphorylation assay of Src and mutants

To determine the effect of autophosphorylation on Src or a mutant, it was allowed to autophosphorylate at 450 nM in kinase assay buffer described for 30 min. At the end of the autophosphorylation reaction, the enzyme was diluted to

appropriate concentration (3nM for wild type Src and usually a few fold higher for mutants depends on the activity of the specific mutant) for kinase assay. To determine the level of autophosphorylation of WtSrc or a mutant, the autophosphorylation was carried out as above for 5min, 10min, 20min, 30min and up to 1 hour. At indicated time, aliquots were removed and prepared for SDS-PAGE. The autophosphorylation on Tyr416 was identified with specific phosphorylated Tyr416 antibody.

3.5.6 Phosphatase assay with phosphorylated Src on Tyr416 or mutants by PTP-1B

Src and mutants were first autophosphorylated as described above for 1 hour to achieve fully autophosphorylation on Ty416. The dephosphorylation of autophosphorylated Src and mutants at 337.5nM was performed by incubation with the same amount of PTP-1B in the presence of 10mM Na_2EDTA for 8 min. At indicated time, aliquots were removed and prepared for SDS-PAGE. The phosphorylation levels of Src and mutants on Tyr416 were visualized by western blot with a monoclonal antibody specific for phosphorylated Tyr416 and quantified by band intensity. To ensure precisely quantification, signal was captured at linear range and dephosphorylation of Src and each mutant were done synchronously.

3.5.7 Csk inactivation assay of Src and mutants

To determine the inactivation of Src and mutants by Csk, the kinase activity of Src

and mutants was assayed using Src optimal peptide as substrate in the presence of Csk. Since Src optimal peptide is a far superior substrate for Src than Csk, kinase assay for Src and mutants can be precisely performed without removing Csk in the reaction. Src and mutants were first incubated with Csk for 30min and the phosphorylation level on Try527 was determined by western blot with specific phosphorylated Tyr527 antibody. After 30 min incubation with Csk, the enzyme was diluted to appropriate concentration (1nM for Src and usually a few fold higher for mutants depends on the activity of mutant) for kinase assaay.

3.5.8 Csk inactivation assay of Src and mutants when autophosphorylated

To determine the effect of Tyr416 phosphorylation on Csk inactivation, wild type Src and mutants were first autophosphorylated at 450nM for 30 min, and then incubated with Csk for another 30 min. Then enzymes were diluted to appropriate concentration for the kinase assay as described above.

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