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DEVELOPING AND OPTIMIZING A METHOD TO ANALYZE SUBSTRATE SPECIFICITY OF TYROSINE KINASES

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DEVELOPING AND OPTIMIZING A METHOD

TO ANALYZE SUBSTRATE SPECIFICITY OF TYROSINE KINASES

BY

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ABSTRACT

Protein Tyrosine Kinases (PTKs) are crucial enzymes which aid in cellular signal transduction pathways as well as cell cycle regulation. This is accomplished by phosphorylating downstream targets specifically on the hydroxyl group of tyrosine residues. Equally important, extensive research has established a connection between overexpression of PTKs and the development of certain cancers. One example of this is seen in Chronic Myeloid Leukemia (CML) where a chromosomal translocation occurs in patients which results in a hybrid, overexpressed BCR/Abl fusion PTK ultimately leading to tumor formation. Thus, understanding PTKs along with what substrates they act on is of utmost importance.

Along with understanding the structure and function of PTKs, identifying which substrates they phosphorylate is crucial as well. There are several methods both *in vitro* and *in vivo* which allow the study of PTK activity on their specific substrates. Each method has some setbacks. Some methods can be time-consuming to set up and use while other methods such as studying PTKs in mammalian cells can lack consistency due to other factors in the cell that can act on these enzymes and alter experimental data. A rapid and efficient system to allow characterization of PTK/substrate relationships would be highly valuable. In the following research, a bacterial system has been developed, optimized and tested which allows a researcher to screen both wild type and mutant protein tyrosine kinases for their phosphorylation activity on chosen substrates. In this screening model, PTKs Abl, Csk and Src and their respective substrates (crk l, kdSrc) were used to demonstrate the effectiveness of this method. Once demonstrated, chosen mutants of the above PTKs and substrates

were then tested to further validate this screening method. In both wild type and mutant testing using this applied *in vivo* screening system, the expected phosphorylation activity was demonstrated. This system can be used to identify substrates for a known kinase, or kinase mutants with certain substrate specificity. It is a useful platform for understanding the mechanisms of PTK substrate specificity. Once this method was developed and proven, the protocol was optimized to allow rapid colony screening of PTKs for substrate specificity. This method can be used to screen mutant PTKs generated for the substrates they phosphorylate in a rapid, effective manner in an effort to further understanding PTKs.

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DEDICATION

I would like to dedicate this Master's thesis first and foremost to my mother Mary Kfoury who has been my shining light throughout any journey I have taken in my life. Without her patience, guidance, consistent love and beautiful personality, I would never have become the person that I am today. Second, I would like to thank my brother Jimmy and my sister Joyce for being the best siblings possible and helping me in any way, shape or form throughout graduate school and in life; whether it be with provided wisdom or through action and effort. I would also like to dedicate this to my father Sami Kfoury who passed away in 2006 and whose memory lives on through my actions. I hope with this master's degree and my future accomplishments, I have made you and my family proud. This is only the beginning of what I intend on accomplishing so please be patient with me.

Lastly, I would like to dedicate this master's thesis to Dr. Gongqin Sun, who took a chance on me and trusted me to work in his laboratory. He has shaped me with his wisdom and continued effort to mold me into a great researcher. I hope I have made his choice of having me work in his lab both a rewarding and positive experience for him as it was for me.

Preface

This thesis was formatted in accordance with the manuscript format guidelines established by the graduate school of the University of Rhode Island.

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INTRODUCTION

Protein Kinases and Signal transduction

There are a multitude of enzymes that occur in nature with a wide range of functions. Some enzymes such as proteases help breakdown targeted proteins while others like restriction enzymes recognize and cleave specific recognized regions of DNA. Protein kinases function by transferring a phosphate group onto a target substrate (phosphorylation) which allows for regulation of certain biochemical processes such as cell cycle and signal transduction pathways.[1-3]

Several groups of protein kinases exist including but not limited to serine/threonine kinases, histidine kinases and protein tyrosine kinases. The main difference between these three groups of protein kinases is their method of action. Serine/threonine kinases function by phosphorylating the hydroxyl group of either serine or threonine side chains on their substrate. In contrast, histidine kinases first add a phosphate group from ATP onto their own histidine residue and then usually transfer it to an aspartate group on their substrate. Finally, protein tyrosine kinases transfer a phosphate group from ATP to the hydroxyl of tyrosine residues on their substrates.[2]Regardless of mode of action, stringent regulation of cell processes is achieved by the action of protein kinases.

Oncogenes and Protein Kinases

Cancer research and treatment has changed gradually throughout the last century. Initially, research focused on treating and preventing the proliferation of cancerous cells. Treatment consisted of radiation and chemotherapy techniques that have limited success and often cause patients to become immune to these therapies and experience drug resistance. Overall, these treatments served as the most prevalent mode of action to treat cancer patients.[4-6]

In contrast to treating the proliferation of tumor cells with the techniques listed above, a new wave of cancer treatments focuses on controlling the activity and effects of the underlying cause of a range of cancers, oncogenes. Oncogenes are the result of proto-oncogenes becoming mutated or overexpressed. Proto-oncogenes are genes that have the capacity to induce cancer development through their protein and enzymatic products, when they are either overexpressed or mutated.[5] The discovery of the first retroviral oncogene, *v-src*, in 1911 was responsible for the importance placed on understanding oncogenes and led to a shift in cancer therapy as well.[7, 8]

The discovery of the first oncogene v-src and its impact on cancer research

In 1911, Francis Peyton Rous, injected cell-free extract from a cancerous chicken tumor into healthy chickens and observed that this was promoting cancer development (sarcoma) in the chickens injected. Further research implicated the Rous Sarcoma Virus (RSV) was the driving force behind the cancer generation in the afflicted chickens.[7, 8] The Rous Sarcoma Virus genome contains three genes, *gag, pol*

and *v-src* and along with this, RSV has the capability to integrate its genes into the host chromosome (chicken) and induce overexpression of these viral genes.[9] Although overexpression of *pol* and gag is responsible for the replication of the virus, overexpression of the viral *v-src* leads to uncontrolled mitosis of the host chicken cells and ultimately tumor formation. The discovery of *v-src* was a breakthrough in science and due to this amazing discovery in cancer research; Peyton Rous was awarded the Nobel Prize in 1966.[10]

The work of Peyton Rous and research related to his discovery not only led to a better understanding of the causative agent of cancer but also promoted research on a new type of protein kinase group, protein tyrosine kinases. After the discovery of the *v-src* oncogene, research on its function elucidated a new mechanism of protein phosphorylation in which a phosphate group is attached directly to the hydroxyl group on a tyrosine residue of the targeted substrate. Due to this, these protein kinases were named protein tyrosine kinases (PTKs). The impact of the discovery of a new group of enzymes and their ability to become oncogenic fueled researchers to discover, isolate and study other PTKs.[2]

Protein Tyrosine Kinases: Structure and Function

With the discovery of *v-src's* function as a protein tyrosine kinase as well as its oncogenic potential, this led to a booming increase in research driven toward understanding the structure and functions of PTKs as well as the discovery of new PTKs. This effort was applied in hopes that by understanding these unique enzymes and their mode of action, cancer development linked to oncogenic PTKs could be further understood, and therapeutic strategies can be developed.

Since the discovery of *v-src*, many other PTKs have been discovered including Abl, Csk and Frk. These PTKs all have conserved regions as well as similar modes of action, but their specific roles in a cell tend to vary.[2] Figure 1 shows the evolutionary relationship between both receptor PTKs and nrPTKs present in nature.

Structure homology of PTKs: Receptor vs. Non-Receptor PTKs

When it comes to the protein tyrosine kinase family, there are generally two types, receptor and non-receptor PTKs. The first type, receptor tyrosine kinases (RTK) span the cellular membrane and bind extracellular ligands. There are 20 distinct families of RTKs identified which can bind extracellular large and small ligands.[11] Binding of extracellular ligands to RTKs generally causes dimerization of the receptor and trans-phosphorylation of the kinase domain thereby activating it. This activated kinase will then phosphorylate a downstream target and lead to amplification of the signal. RTKs, such as EGFR, consist of three main domains including a transmembrane

Figure 1. Evolutionary lineage of receptor and cytoplasmic PTKs.

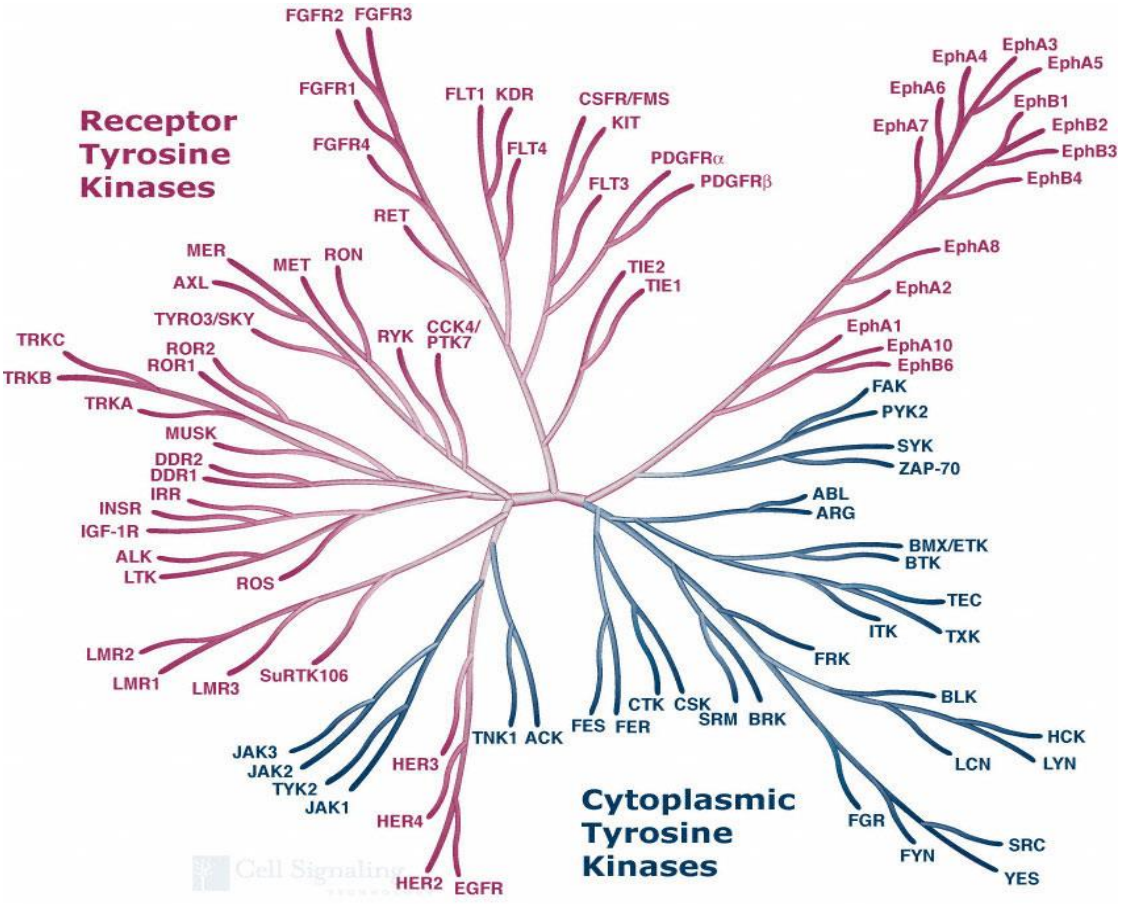

Figure 1. Evolutionary lineage of receptor and cytoplasmic PTKs. The phylogenetic tree above depicts the evolutionary lineage of PTKs, both receptor and cytoplasmic. The roots of the tree represent the most common ancestor of each species located at the end of the branches. Figure adapted from Cell and Signaling Technology.[12]

domain, extracellular ligand-binding domain, and finally an intracellular kinase domain. In contrast, non-receptor PTKs (nrPTKS) such as Src and Abl are usually found in the cytoplasm but can also be found within organelles. All nrPTKs contain a conserved kinase domain similar to that of the RTKs. In addition, each nrPTK may contain other regulatory domains. Most nrPTKs contain a conserved SH2 (Src Homology 2) domain, SH3 (Src Homology 3) domain and sometimes a PH (Plektrin Homology) domain.[3] In general, the conserved SH2 domain of nrPTKs is made up of 100 amino acids and tends to be located upstream of the catalytic domain of nrPTKs.[3, 12] The conserved SH2 domain can bind to phosphorylated tyrosine containing peptides and is also involved in localization to RTKs and other enzymes activated by tyrosine phosphorylation.

Meanwhile, the conserved SH3 domain of nrPTKs is roughly 70 amino acids long and is generally positioned upstream of the SH2 domain near the N-terminus. It has a similar localization function as the SH2 domain and can regulate kinases and other SH3-containing proteins. Finally in addition to the conserved SH3 and SH2 domains, nrPTKs can have over 25 different accessory protein sequences either at the C or N-terminus of the protein sequence.[2, 11] These domains aid in kinase regulation or function either intramolecularly or binding with other proteins that may be either substrates or regulators. These accessory protein sequences can also aid in localization and recruitment of targeted substrates

When comparing both RTKs and nrPTKs, crystallographic structures reveal generally conserved kinase and catalytic domain structures (Figure 2). The kinase domains of PTKs can be further divided into twelve sub-domains which fold together into a large and small lobe. Subdomains I-IV comprise the smaller N-terminus lobe and play a role in Mg/ATP binding. Meanwhile subdomains VI-XI make up the larger C-terminal lobe and aid in peptide/substrate recognition and phosphotransfer. Finally, the two separate lobes are linked by subdomain V.

PTK enzyme catalysis

Enzyme catalysis is similar among PTKs as well. The catalytic domains of PTKs consist of two lobes which come together to form the catalytic cleft. The N-terminal residues form the ATP binding lobe while the C-terminal residues form the substrate binding lobe. Once a substrate and ATP bind to their respective binding sites, the γ phosphate and the substrate hydroxyl group are positioned close to each other in the catalytic cleft. Next, catalysis will occur facilitating the transfer of the γ phosphate group from ATP onto the specific tyrosine residue of the substrate.[2] It has also been noted that catalytic efficiency is enhanced in the presence of divalent metal cations such as Mn^{2+} and Mg^{2+}.[13] The amino acids present around the targeted tyrosine residue of the incoming substrate play a huge role in increasing binding affinity and catalytic efficiency. Although this is true, there is no consensus on which surrounding specific amino acid residues help determine catalytic efficiency.

Figure 2. Comparison of structural domains among several nr-PTKs.

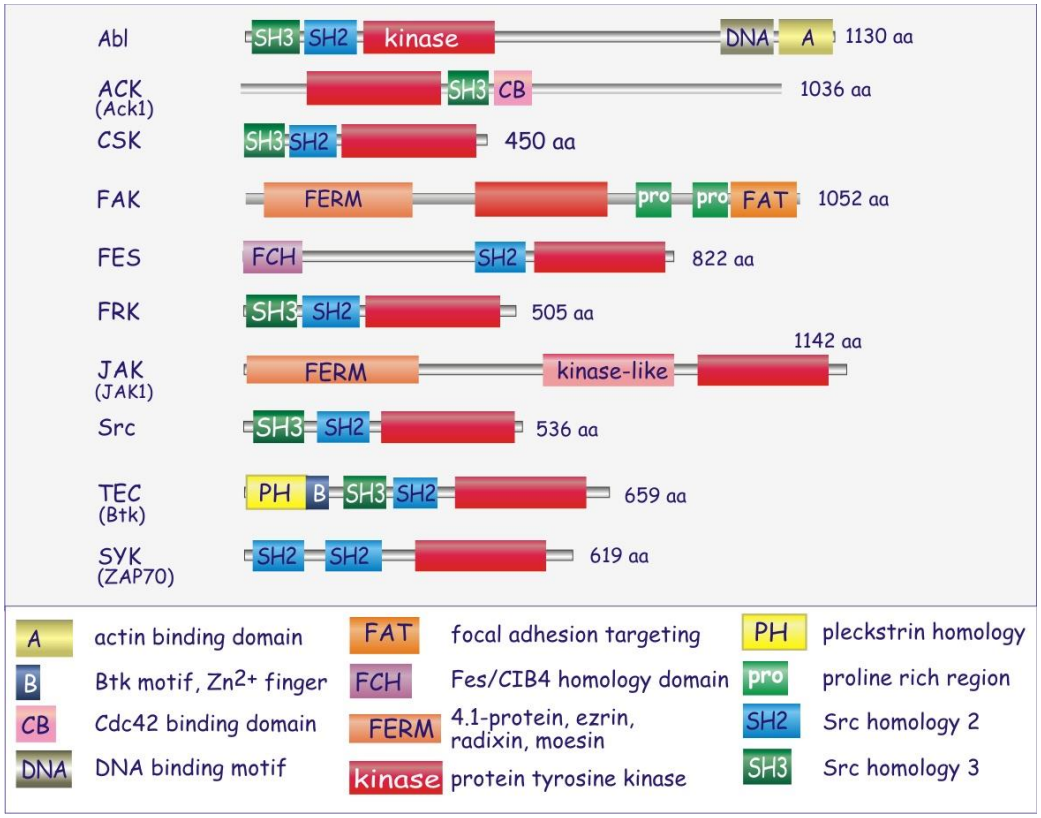

Figure 2. Comparison of structural domains among several nr-PTKs. The relative conserved and non-conserved structural domains between several nr-PTKs are shown and compared in the figure above. Figure adapted from Cell Biology.[15]

Amino acids outside the catalytic cleft can also play a huge role in increasing the efficiency of catalysis within PTKs. An example of this is when inactivation of Src is carried out by Csk on a specific C-terminal tyrosine 527. A specific stretch of amino acids on Csk's peptide binding lobe termed RSRGRS is crucial for Csk inactivation of Src. Without this region, inactivation of Src by Csk does not occur.[14] Despite similar modes of catalytic action, PTKs still achieve a high level of substrate specificity.

Model Protein Tyrosine Kinases

With the wide variety of nrPTKs that exist, it is a challenge for researchers to understand how cell signaling through PTKs is achieved. Although the catalytic domains and catalytic mode of action is generally conserved, they still manage to achieve a high level of substrate specificity. An example of this is Src PTK which demonstrates substrate specificity for its auto-phosphorylation site (Tyr416), but cannot phosphorylate its inactivation site (Tyr527), which is targeted by another PTK, Csk.[3, 12, 15] Based on this, researched have grouped nrPTKs into specific families which share similarities both structurally as well as the regulatory features they possess. Several of these model kinase families include the Src family kinases (SFKs), Csk family kinases, Abl family kinases (AFKs), Frk family kinases and finally the Tec family kinases. By grouping nrPTKs into subfamilies, it can provide an overall understanding of how signal transduction is achieved by these different nrPTKs. The three model PTK families Src, Csk and Abl that are relevant to the applied research will be discussed below.

Src family kinases (SFKs)

Being the first PTK discovered and having oncogenic potential, much is known about Src and related SFKs such as FYN and Yes. The SFK contains 9 members and tend to regulate important cell processes such as cell growth, migration, differentiation, and survival throughout various tissues in the body.[2] They directly act on growth factors and play a huge role in growth factor signaling pathways. Although

each SFK plays important roles in normal cell development and function, elevated levels of SFKs have been associated with oncogenesis in humans, specifically in colon cancers and other cancers such as lymphoma and melanoma.[2]

SFKs have a structure containing an SH3 domain, an SH2 domain, a kinase domain, an N-terminal variable site which can be modified by myristoylation or palmitoylation and finally a flexible tyrosine containing C-terminal tail. The SH2 domain plays a role in binding phosphotyrosine-containing peptide sequences while the SH3 domain has been shown to bind type II polyproline helix ligands. Meanwhile, the N-terminal variable region plays a role in membrane recruitment. Finally, the highly conserved C-terminal tail serves as a regulator of SFKs through phosphorylation or de-phosphorylation of the tyrosine located on the tail.[2, 12, 15]

Although SFKs have been shown to be oncogenic, there has to be strict regulation of SFKs to prevent this from always occurring inside the cell. This is achieved by a stringent regulation on two important residues contained in SFKs. The first residue, tyrosine 416, is the site of autophosphorylation of SFKs, which when phosphorylated activates the enzyme. This activating tyrosine is present in the autophosphorylation loop, a region highly conserved in amino acid sequence(RLIEDNE**Y**TARQGAK) among SFKs and closely related members.[3] In contrast, phosphorylation by Csk on tyrosine residue 527 on the C-terminal flexible tail of Src inactivates the enzyme and renders it inactive. When phosphorylated, the phosphorylated tyrosine residue 527 interacts with Src's SH2 domain and allows the SH3 domain and linker region to come together keeping the enzyme in an inactivated state.[2, 3, 12, 14] Because Src is a substrate for both Csk and Src, but on different tyrosine residues, it is an ideal

substrate for testing the substrate specificity for Csk and Src. The structural organization and regulation of Src is shown below in Figure 3.

Csk family kinases(CFKs)

The Csk family kinases are made up of two members, Csk (C-terminal Src Kinase) and Chk (Csk homologous Kinase). Both play a role in negatively regulating members of the Src family. Two pieces of evidence indicates that Csk and Chk belong to the same family. First, both members share a similar structural organization containing an SH3, SH2 and the SH1 domain from N-terminus to C-terminus order. Second, both members lack an auto-phosphorylation site, an SH4 myristoylation signal and regulatory phosphorylation sites common to their closest PTK family, SFKs.[1, 12, 14] Contrary to SFKs though, CFKs are considered tumor suppressors rather than having oncogenic potential. The main evidence of this was provided by performing mouse knockout studies on Csk where deletion of the Csk gene leads to early-stage embryonic lethality and several neural tube defects. Without Csk present, SFKs become consistently active and have devastating consequences. Also, Csk/Src double knockout mice develop to near parturition and show normal development which strongly suggests that CFKs are a crucial regulator and necessary for moderating SFKs in the cell.[12]

Structurally, Csk family kinases share a 40% sequence identity when compared to Src. They share a conserved SH2, SH3 and catalytic domain with Src but significant

differences do exist. Missing from their structure is an N-terminal lipid anchoring domain, an activation loop tyrosine and finally a C-terminal tyrosine-containing tail.

Figure 3. The structural organization and regulation of Src PTKs.

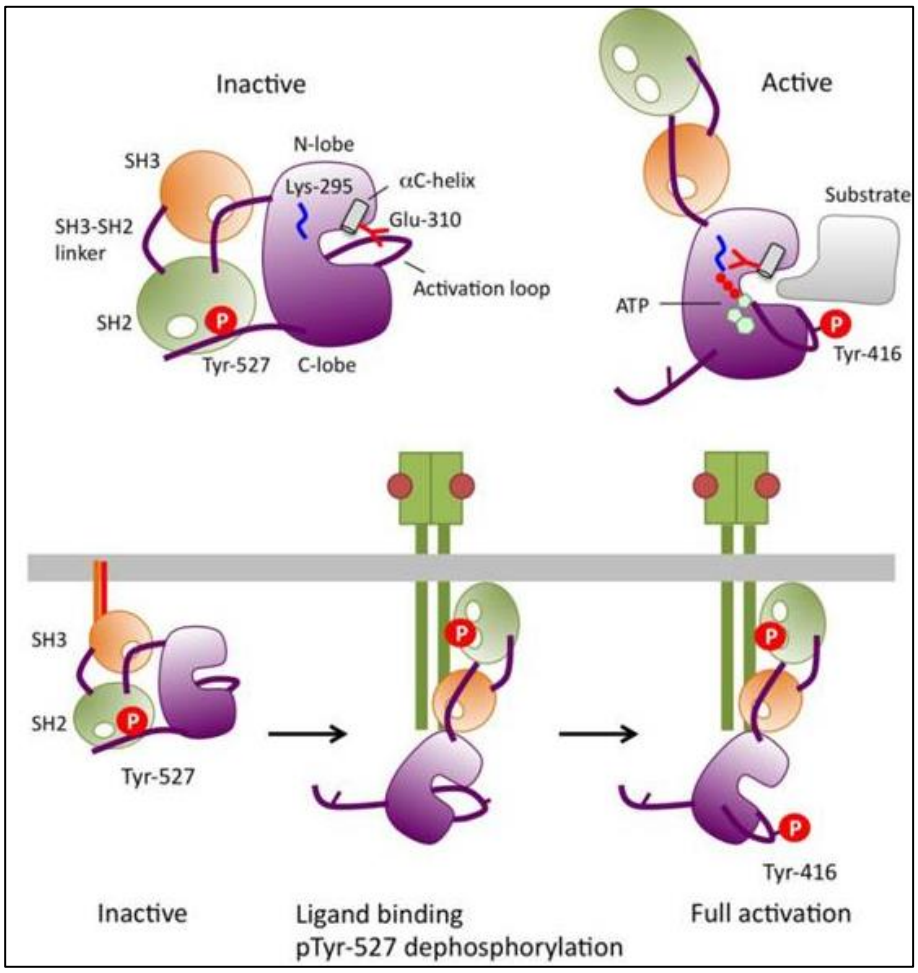

Figure 3. The structural organization and regulation of Src PTKs. The following figure depicts the events that lead to activation and inactivation of Src PTKs as well as their structural organization. Initially, phosphorylation at tyrosine residue 527 maintains the enzyme in an inactive site. Interaction with the SH2 domain keeps this enzyme in a closed conformation. Full activation is achieved when specific ligands bind to Src's SH2 or SH3 domains resulting in de-phosphorylation at tyrosine residue 527, thereby promoting formation of Src's active conformation. This is followed by auto-phosphorylation on tyrosine residue 416 of Src, leading to the full activation state of the enzyme. Figure adapted from the International Journal of Biological Sciences.[3]

This suggested to researchers that Csk was a distant relative of Src with a different mode of regulation.[3, 12]

It has been established that CFKs have limited and highly specific substrate targets, usually of the SFK. Due to such a high level of substrate specificity, this lends support as to why Csk was one of the first full-length protein tyrosine kinase to be overproduced efficiently in *E.coli* with the addition of chaperonins GroES and GroEL. By having a stringent level of substrates it targets, Csk proved to be less toxic when introduced to foreign hosts such as *E.coli*. Due to the ability to overproduce the Csk enzyme with this novel strategy, it has been widely studied and serves as a model PTK. In contrast, efforts to purify Chk enzyme have been unsuccessful in the past making it hard to study as well.[1, 3, 12] Csk's structure and regulation of Src PTKs are shown below in Figure 4.

Abl family kinases(AFKs)

The Abl family of non-receptor tyrosine kinases (AFKs) includes both c-Abl (cellular Abl) encoded by *ABL1* and its relative Arg (Abelson-Related Gene) encoded by *ABL2*. *ABL* genes are found in all metazoans, suggesting that both their structure and function were fixed early in tyrosine kinase evolution. AFKs function in such processes as cell growth, cell survival and DNA repair.[16, 17]

Structurally, both members of AFK share conserved N-termini containing an N-terminal cap, myristoylation site, SH3 and SH2 domain, and similar kinase domains.

Figure 4. Csk structure and activity on Src substrate.

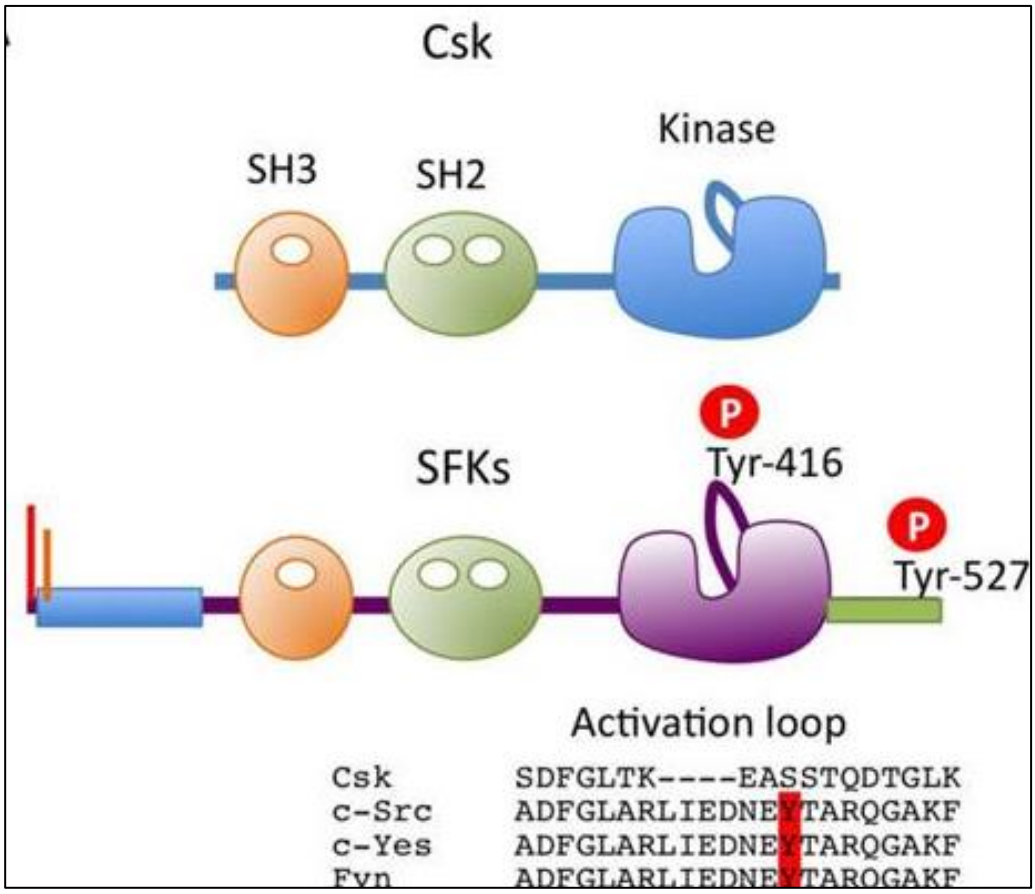

Figure 4. Csk structure and activity on Src substrate. Domain organization of Csk and SFKs is shown in the figure above. Csk inactivates SFKs through phosphorylation on tyrosine residue 527. An amino acid alignment of the activation loop of Csk and SFKs is also depicted above. Figure adapted from International Journal of Biological Sciences.[3]

In contrast, the C-terminus is quite different in that c-Abl contains nuclear localization and export signals as well as a DNA binding domain that Arg does not possess. Both members do however contain an F-actin binding domain located at their C-termini.[17, 18]

AFKs have been shown to be phosphorylated by members of the SFKs and have been shown to possess autoinhibitory mechanisms as well. These include a myristoyl group that can bind to the surface pocket in AFKs kinase domain and contribute to an autoinhibitory fold. Along with this, AFKs contain an amino-terminal cap which can stabilize the inactive conformation of these enzymes using specific surface interactions. Further experiments have demonstrated that disruption of these autoinhibitory mechanisms result in an increase in kinase activity of AFKs.[16, 18]

Another similarity to SFKs is that AFKs are proto-oncogenic and can become oncogenic due to certain mutations or overexpression events. An example of this occurs in patients with Chronic Myeloid Leukemia where a chromosomal translocation occurs between the Abl gene on chromosome 9 and the breakpoint cluster gene on chromosome 22. This fusion generates a Chimeric Chromosome called the Philadelphia chromosome (Figure 5).[16, 19] The result of this is a 210 kDa chimeric PTK called BCR/Abl which is constitutively expressed. Due to overexpression of the BCR/Abl gene, several crucial consequences occur. First, the Abl kinase domain becomes much more active thereby phosphorylating a variety of different substrate proteins. Second, BCR/Abl PTK has critical domains and sequence motifs which causes activation of downstream targets. Lastly, the fusion protein becomes strongly

Figure 5. The occurrence of the Philadelphia Chromosome in patients with Chronic Myeloid Leukemia (CML).

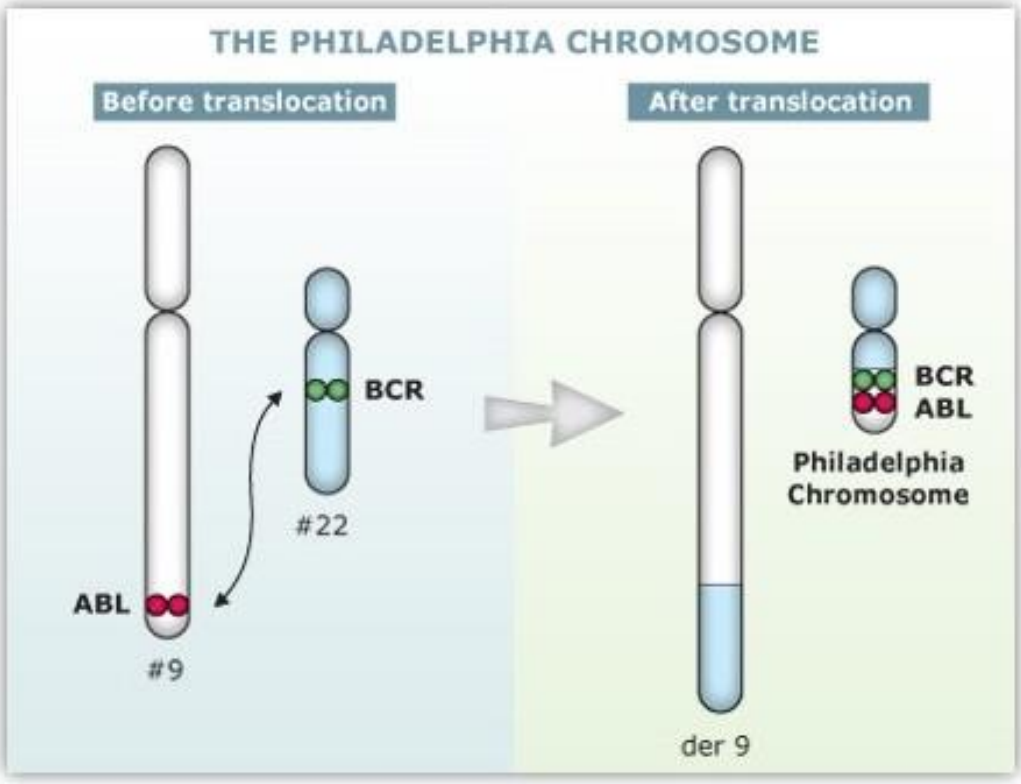

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Figure 5. The occurrence of the Philadelphia Chromosome in patients with Chronic Myeloid Leukemia (CML). In CML, a chromosomal translocation occurs between the Abl gene on chromosomes 9 and the breakpoint cluster gene (BCR) on chromosome 22. This translocation results in formation of a BCR/Abl fusion chromosome termed the Philadelphia Chromosome. This leads to production of an un-controlled and overexpressed BCR/Abl fusion PTK and development of CML. Figure adapted from the Chronic Myelogenous Society of Canada. [22]

auto-phosphorylated causing it's catalytic activity to increase heavily. Due to these three consequences, several cellular effects occur including uncontrolled cell proliferation, reduced DNA repair, and ultimately Chronic Myeloid Leukemia.[16, 19] Although many signaling pathways are activated by BCR/Abl, only a few proteins appear to be necessary for BCR-Abl-dependent transformation. These include the proteins Gab2, Myc, CrkL and STAT5. Out of these 4, CrkL is among the most preferred substrates of both wild type Abl and BCR/Abl and its binding to BCR/Abl PTK is necessary for oncogenic transformation to occur in CML patients.[16, 20] Being a substrate for both Abl and BCR/Abl, crk-l has become heavily studied. It functions in numerous biological processes including cell adhesion and migration, cell proliferation, apoptosis, and regulation of gene expression.

Studying PTKs and drawbacks of developed methods

Although much is known about PTKs, there are several drawbacks to the methods designed to study these intricate enzymes. *In vivo* methods such as studying PTKs in mammalian cells can produce complex and hard to interpret results. The main reason for this is that in a mammalian cell, there are many other factors that can play a role in mediating or altering the activity of the PTK being studied. One example of this occurs when researchers attempt to study inactivation of the SFK and FGFR families by reactive oxygen species (ROS) *in vivo*. Although studies showed that these PTKs might have been inactivated by the presence of ROS, they were unable to directly correlate it to the addition of ROS. This is due to the possibility of other

regulatory factors such as members of CFK inactivating these PTKs rather than the ROS being the direct cause of inactivation.[21] A similar problem occurs generally when studying most PTKs due to these other regulatory factors being present inside the cell and influencing the study of these enzymes by their regulatory actions.

Along with in vivo methods to study PTKs, in vitro methods such as radioactive kinase assays have their drawbacks as well. Radioactive kinase assays consist of using radioactive ATP along with both purified enzyme (PTK) and substrate in an intricate time-based reaction.[14] The drawbacks to this analysis method is that first, the researcher must be able to purify both PTK and substrate to a very high purification level; otherwise the experiment conducted will not be valid. Purification methods do work for many PTKs but some PTKs fail to express and purify correctly such as seen in attempts to purify Chk enzyme.[1] Also, some PTKs and substrates do purify but can contain contaminants when purified. Lastly, radioactive kinase assays require the use of radioactivity and its use has strict regulations and can be difficult to work with.

Focus of this thesis

The experimental data, results and discussion pertinent to this work focuses on two related projects, both of which have a similar goal of developing and optimizing systems to understand and rapidly screen PTKs for substrate specificity. The first project involves developing and optimizing an in vivo bacterial system which allows a researcher to rapidly screen PTKs for substrate specificity. The second project focuses on a colony screening method which allows for the screening of mutant PTKs in a rapid, effective manner. Both projects are aimed at allowing researchers to efficiently study PTKs and their substrates in an effort to further understand their function and roles, specifically in oncogenesis.

Developing a rapid, efficient bacterial system to analyze substrate specificity in PTKs

In an effort to overcome drawbacks experienced by researchers in analyzing PTKs as well as the substrates they target, a novel, bacterial system has been developed and optimized which allows for rapid analysis of substrate specificity in PTKs. The system developed consists of co-expressing both PTK and substrate to be analyzed in a bacterial host and following an optimized, rapid protocol which has been shown to produce expected substrate specificity when tested. By using this developed system, researchers now have another method to study PTKs with few of the drawbacks that other methods tend to present such as outside factors acting on PTKs and purification problems as seen in radioactive kinase assays.

Screening of generated PTK mutants for substrate specificity using a rapid colony screening method

In an effort to understand PTKs function and role, PTK mutants have been generated and studied using similar techniques as when studying non-mutated PTKs. Regardless of whether mutant or non-mutant, there are several problems that arise. First, the generation of mutant PTKs is time-consuming and requires prior-analysis to verify the mutations being made are relevant. Second, once established, the methods used to analyze these mutants suffer similar drawbacks as when studying wild type PTKs whether in vivo and in vitro. Recent research focuses on rapid generation of mutant PTKs with such techniques as DNA shuffling.[22, 23] Once established and proven reliable, there must be a system developed to rapidly screen these PTK mutants for their substrate specificity, which is the current goal of this study. To do this, model PTKs Abl, Csk and Src and their preferred substrates, either crk-l or kdSrc, will be used to verify this screening method. By using this optimized system to test mutant PTKs, one can rapidly screen hundreds of mutants, in a small amount of time.

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MANUSCRIPT 1

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Developing and optimizing a bacterial system for analyzing substrate specificity of protein tyrosine kinases.

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Abstract:

Protein tyrosine kinases are important enzymes whose phosphorylation activity on tyrosine residues of their substrates helps dictate cellular signal transduction as well as vital cell activities such as cell cycle regulation. These enzymes demonstrate stringent regulation on signal transduction by achieving a high level of substrate specificity. Recent research linking PTKs to certain cancers has made understanding their substrate specificity of utmost concern.

There are several in vivo and in vitro methods to analyze substrate specificity of PTKs but they tend to be time-consuming and can produce unreliable results. In vivo methods in mammalians are important but generally involve too many outside factors and influences when trying to analyze substrate specificity of a certain PTK. In contrast, in vitro methods such as radioactive kinase assays do produce accurate results but require the purification of substrate and enzyme as well as using radioactivity, a general hazard.

The following research presents a rapid and efficient bacterial system for analyzing substrate specificity of PTKs. The model PTKs and substrates to be tested are cloned into specific plasmids and co-expressed in *E.coli*. Following this, western blot and specific antibodies are used to screen the model PTKs for substrate specificity. The model PTKs Abl, Csk, Src and their preferred substrates, either crk-l or kdSrc, were used to verify the effectiveness of the proposed system. By producing the expected substrate specificity using these model PTKs, the accuracy of the system is also demonstrated.

Once the above system is proven effective, it will be optimized to allow for rapid screening of mutant PTKs using a colony screening method. Currently, one approach used to analyze PTKs and their structure is to perform site-directed mutagenesis on specific chosen sites on PTKs and analyze the effect on both structure and phosphorylation activity. Although beneficial, this process can be time-consuming and the mutations made do not always produce important results when tested. Regardless, newer approaches implemented by some researchers allow for rapid generation of mutants PTKs using such techniques as DNA shuffling. Along with the capacity to rapidly generate mutants, there must be a way to rapidly screen these mutant PTKs for substrate specificity. A colony screening method has been developed and optimized using model PTKs Abl, Csk and Src and their preferred substrates. This developed screening method can be used to rapidly screen mutant PTKs generated by the novel, fast techniques available currently.

Keywords: protein tyrosine kinase; substrate specificity; in vivo bacterial system; Abl; Csk; Src; crk-l; kdSrc; colony screening.

Introduction:

Protein tyrosine kinases are important enzymes in mammalian signal transduction pathways as well as many other important cellular processes such as cell cycle regulation. They phosphorylate specific substrates on their tyrosine residues using a phosphate group derived from ATP.[1-3] By doing this, these enzymes regulate specific cellular activities and cell function. Although generally sharing several conserved structures, PTKs still manage to exhibit very specific substrate specificity even when comparing homologous PTKs such as Src and Csk.[1-3]

Recent breakthrough research has linked several PTKs to the development of certain cancers. An example of this is in Chronic Myeloid Leukemia (CML) where a chromosomal translocation produces a mutated, overexpressed BCR/Abl PTK which leads to unregulated cell proliferation and subsequent tumor formation in patients with CML.[4] In contrast, PTKs such as Csk have been shown to be tumor suppressors, inactivating PTKs of the Src family.[3] Regardless of whether oncogenic or tumor suppressing, this recent research has placed utmost importance on understanding substrate specificity of these enzymes. The hopes are that by discovering and understanding the targets of PTKs, this information can be used to develop drugs that target PTKs or their substrates and regulate the effects of these mutated or overexpressed enzymes in afflicted cancer patients.

There are several developed methods to test substrate specificity of PTKs but regardless of the methods used, all remain time-consuming and can produce erroneous results. Using in vivo mammalian cells to analyze substrate specificity of PTKs can be

performed, but the results can be misleading due to the amount of other proteins and enzymes in a cell that can alter substrate specificity of PTKs.[5] Relevant to this, in vitro methods such as radioactive kinase assays require the purification of both PTKs and substrates and involve using radioactive compounds.[5,6] Regardless whether in vivo or in vitro, both ways tend to present a myriad of problems to a researcher.

In the following research, we sought to develop and optimize a bacterial system which can be used to analyze substrate specificity of PTKs in a rapid, accurate manner. Using this developed system, three specific model nrPTKs (Abl, Csk and Src) were expressed in combination with their preferred substrates, either crk-l or kdSrc, in an effort to achieve the predicted substrate specificity demonstrated by these protein tyrosine kinases. Once predicted substrate specificity by these PTKs was verified, both mutant PTKs and substrates were also tested to further validate the system developed. By demonstrating proper substrate specificity using both wild-type and mutant PTKs with their substrates, a new *in vivo* method for analyzing substrate specificity of respective PTKs has been developed which is both rapid and accurate. Once this method was established, it was optimized in an effort to develop a colony screening method that can detect substrate specificity of rapidly generated mutant PTKs contained in colonies on a bacterial plate. Model PTK Abl, Csk and Src and their preferred substrates were once again used to demonstrate the effectiveness of this rapid, novel colony screening method.

Materials and Methods:

Reagents and Chemicals

Consumables and culture media, or media components were purchased from Fischer Scientific. The PY20 anti-phosphotyrosine antibody was purchased from Santa Cruz Laboratories. The P-Src (Y527), P-Src (Y416) and Anti-Rabbit IgG was purchased from Cell Signaling Technology. The p-Tyr antibody(PY20) antibody was purchased from Santa Cruz laboratories. The Phospho-Crkl (207) antibody was purchased from Abcam and the anti-mouse IgG secondary antibody from Sigma-Aldrich. All other chemical reagents used were purchased from Sigma-Aldrich.

Cloning of model PTKs and Substrates into selected plasmids

After optimization by Yixin Cui, it was shown that expression of selected PTK coding regions of wild-type Abl, Csk(full-length), Src(aa 83-533) and mutants into low copy number plasmid pCDF-1b was shown to work best in achieving proper substrate specificity in this system. The substrates tested kdSrc and its mutants were previously cloned into low copy number plasmid pRSET-A. [5,6] Substrate crk-l was previously cloned into pGEX-4t-1.[4] To clone the selected coding regions of the PTKs Abl, Csk, Src and mutants into pCDF-1b, a general sub-cloning procedure was followed. Generally, the plasmids that contained the selected PTK coding regions and the pCDF-1b plasmids to be cloned into were digested in 50 µl Eppendorf tubes with specific restriction enzymes at 37° Celsius for 1 hour. The digestion products were loaded onto a 1% agarose DNA gel and the proper-sized bands gel-extracted using

Qiagen's QIAquick gel extraction kit and quantified with Thermo-Scientific's Nanodrop 8000. Once quantified, specific quantities of both digested insert and vector were used to set up calculated insert to vector ratios and these were ligated overnight @ 16°C. Next, these ligation products were transformed into electro-competent *E.coli Bl21-Gold(DE3)* cells using electroporation(2mm cuvettes) and plated on LB plates containing appropriate antibiotics and incubated overnight in a 37°C non-shaking incubator. The following day, colony-PCR was performed on the colonies produced using a combination of primers specific for pCDF-1b plasmid (pCDF-1b +) and the insert cloned (ex. Src Y454A -). Restriction enzyme digest analysis was also performed using the same restriction enzymes initially used to clone in the insert to further verify correct sub-cloning of desired insert into pCDF-1b. Finally, the newly made insert-containing plasmid products were sent for sequencing to the University of Rhode Island Genomics and Sequencing Center and subsequently verified by sequence analysis.

Co-expression of PTK-containing and substrate-containing plasmids in E.coli BL21-Gold(DE3)

Through cloning as described above, the PTK-containing plasmids were already present in the strain of choice, *E.coli BL21-Gold(DE3)* and these stocks were then separately made electro-competent using a general high-efficiency electro-competent cell protocol. The substrate-containing plasmids, either pRSET-A or pGEX-4t-1, were isolated from previously made stock cultures using Qiagen's

miniprep kit, and transformed into the appropriate strains using electroporation in 2mm cuvettes. The transformed products were recovered in 500µl of LB media for 1 hour in a 37°C shaking incubator(225 rpm) and this recovery product was then plated on LB plates with appropriate antibiotics added. The following day, the transformant colonies were tested for presence of both PTK and substrate-containing plasmids using colony PCR with primers specific for each plasmid and insert. Table 1 indicates exactly which PTK/Substrate combinations were made to be tested using the developed screening system. Table 2 indicates the PTK/Substrate combinations tested using the colony screening method. Once tested, the expression and activity of the PTK/substrate strains were verified by performing a western blot using P-tyr antibody(PY20,) which detects phospho-tyrosine containing proteins.

TABLE 1. PTK and substrate combinations generated for testing with developed method.

PTK-containing plasmid	**Co-expressed with***	**Substrate-containing plasmids**
pCDF-1b-Abl	+	1.) pGEX-4t-1-crk-l
		2.) pRSET-A-kdSrc(wt)
		3.) pRSET-A-kdSrc-Y416F
		4.) pRSET-A-kdSrcY527F
pCDF-1b-Csk(wt)	+	1.) pGEX-4t-1-crk-l
		2.) pRSET-A-kdSrc(wt)
		3.) pRSET-A-kdSrc-Y416F
		4.) pRSET-A-kdSrcY527F
pCDF-1b-Csk-R281A + R283A	+	1.) pGEX-4t-1-crk-l
		2.) pRSET-A-kdSrc(wt)
		3.) pRSET-A-kdSrc-Y416F
		4.) pRSET-A-kdSrcY527F
pCDF-1b-Src(wt)	+	1.) pGEX-4t-1-crk-l
		2.) pRSET-A-kdSrc(wt)
		3.) pRSET-A-kdSrc-Y416F
		4.) pRSET-A-kdSrcY527F
pCDF-1b-Src-RSRGRS + Y458F	+	1.) pGEX-4t-1-crk-l
		2.) pRSET-A-kdSrc(wt)
		3.) pRSET-A-kdSrc-Y416F
		4.) pRSET-A-kdSrcY527F

**Escherichia coli BL21(DE3)* was used as the host strain in all co-expressions.

TABLE 2. PTK and substrate co-expressions used in developed colony screening method.

PTK-Containing Plasmid	**Co-expressed with***	**Substrate-containing Plasmid**
pCDF-1b-Abl	+	1.) pGEX-4t-1-crkl
		2.) pRSET-A-kdSrc
pCDF-1b-Csk	+	1.) pGEX-4t-1-crkl
		2.)pRSET-A-kdSrc
pCDF-1b-Src	+	1.)pGEX-4t-1-crkl
		2.)pRSET-A-kdSrc

**Escherichia coli BL21(DE3)* was used as the host strain in all co-expressions.

Preparation of samples to be tested

Once the appropriate strains were made, each PTK/substrate combination strain to be tested was grown in 5 ml of LB media with appropriate antibiotics added for approximately 13 hours in a 37°C shaking incubator(225 rpm) until an OD_{600} value between .7 and 1.4 was reached. To normalize the amount of cells in each sample to be tested, an equation (200µl/OD_{600} value= amount aliquoted) was used. Once the appropriate amount of each culture was aliquoted into Eppendorf tubes, samples were spun at 13,000 rpm for 5 minutes in a mini-spin Eppendorf centrifuge then resuspended in 25µl of 1X SDS sample buffer with β-Mercaptoethanol and heated in a 95°C heating block for 10 minutes. After heating, samples were once again spun at 13,000 rpm for 5 minutes and approximately 9µl of each sample to be tested was loaded appropriately into Biorad 15 well any-kD protein gel and run for 1 hour at 100 volts in an SDS-PAGE buffer system. Once complete, the gel produced was used for western blotting purposes as described below. Duplicate gels were also produced and subsequently Coomassie stained for 30 minutes to vefiry the quantitation of samples. Following de-staining and equilibration in water, gels were analyzed with the Bio-Rad GelDoc system.

Western Blotting of Samples and Exposure

For western analysis, samples were first subjected to SDS-PAGE as described above. The resolved protein gels produced were incubated in transfer buffer along with the appropriate filter paper and membranes used. The protein gels were then

blotted to a 0.45 µm nitrocellulose membrane at 15 volts for 15 minutes using Bio-Rad's semi-dry transfer apparatus. Once complete, the membranes are then quickly rinsed in 20 ml TBST buffer for 2 minutes and blocked in 20ml, 5% non-fat milk for approximately 1 hour with gentle shaking. Upon completion of blocking, membranes were washed 3 times for 3 minutes with TBST buffer and then incubated in the appropriate 1° antibody concentration in TBST for 1 hour at room temperature with gentle agitation. After 1 hour incubation with 1° antibody, the membranes were rinsed 3 times for 5 minutes in TBST buffer. After washing, the membranes were then incubated in the appropriate secondary antibody diluted in 5% non-fat milk for 1 hour at room temperature with gentle shaking. Subsequently, the membranes are then washed 3 times for 5 minutes each with TBST buffer. Membranes are then dried and incubated for 1 minute in Bio-Rad's clarity reagent (250 µl of each solution A,B). After incubation in the clarity reagent, the membranes are placed in between laminating sheets and exposed for multiple time points using high resolution chemi-blot protocol in Bio-Rad's Universal Hood II GelDoc imager system.

Optimized colony screening sample preparation

To prepare samples, the strains to be tested are used to inoculate 5 ml of LB broth contained in a 15 ml culturing tube with appropriate antibiotics added. These culture tubes are then grown for approximately 13 h (till logarithmic phase) in a 37° shaking incubator. After approximately 13 h of growth, the OD_{600} value is measured and the cultures are then induced with 0.45 µm IPTG for 1 hour at room temperature

with gentle shaking. After 1 hour of induction, approximately 0.5µl of each culture to be tested is pipetted directly onto a sterile 0.45µm nitrocellulose membrane. The number of samples that can be tested with this method is only limited to size and spacing that can be accomplished on the membrane to be used. For optimization purposes, approximately 5, 0.5µl samples of each PTK/Substrate combination to be tested was pipetted from left to right onto the membrane with at least ½ inch separation between samples. This spacing ensures that the signals produced by each sample do not counteract with signals from other samples after western blot. Once the membrane is prepared with samples, the membrane is then placed in a petri dish with approximately 15ml of 10% SDS buffer added and then placed inside a 95°C oven for 10 minutes to lyse cells and release proteins. After heating of membrane, membrane is quickly rinsed in TBST and followed up by an optimized western blot protocol specific for this developed method.

Optimized Colony Screening Western Blot protocol

Upon quickly rinsing membrane with 15 ml TBST buffer, membrane is blocked in 5% non-fat milk with gentle shaking for 30 minutes. After blocking, membrane is washed 3 times (2mins/wash) with 15ml TBST buffer by gentle agitation. Next, the membrane is incubated for 1 hour at room temperature in the appropriate primary antibody concentration diluted in TBST buffer with shaking. After incubation with the primary antibody, membrane is washed 3 times (3mins/wash) with 15 ml TBST buffer per wash. Following, membrane is incubated in

the appropriate concentration of secondary antibody diluted in 5% non-fat milk for 1 hour at room temperature. Next, membrane is washed 3 times (3mins/wash) in TBST buffer than membrane is dried and exposed to BioRad's clarity reagent for 2 minutes. After incubation in the clarity reagent, the membranes are placed in between laminating sheets and exposed for multiple time points using high resolution chemi-blot protocol in Bio-Rad's GelDoc imager system.

Results and Discussion:

Demonstrating substrate specificity of model kinases Abl, Csk and Src

To demonstrate the effectiveness of the developed and optimized screening method, we tested the substrate specificity of model PTKs Abl, Csk and Src toward their known substrates. First, Abl should show a high level of phosphorylation of substrate crk-l on tyrosine residue 207 when compared to both Csk and Src which have low phosphorylation activity on this substrate. Second, Src should demonstrate greater substrate specificity for its preferred substrate kdSrc on tyrosine residue 416 in comparison to both Abl and Csk respectively. Finally, Csk should demonstrate a high level of substrate specificity on tyrosine residue 527 of kdSrc, when compared to both Abl and Src.

The first substrate tested using the developed screening method in combination with the model PTKs above was crk-l, Abl's preferred substrate. Abl demonstrated high substrate specificity toward crk-l on tyrosine residue 207 in comparison to both Csk and Src as expected. The result of this can be seen in Figure 1.

The next substrate tested, kdSrc, is phosphorylated by both Csk and Src but on different tyrosine residues (Tyr 416(Src), Tyr 527(Csk)). This modified kdSrc substrate has a lysine to methionine mutation in its ATP binding region which heavily reduces its autophosphorylation ability. The first residue, tyrosine 416, is the preferred auto-phosphorylation site of Src and proper substrate specificity should be demonstrated when compared to Abl and Csk which both show minimal phosphorylation activity on this residue. Once again, the expected high

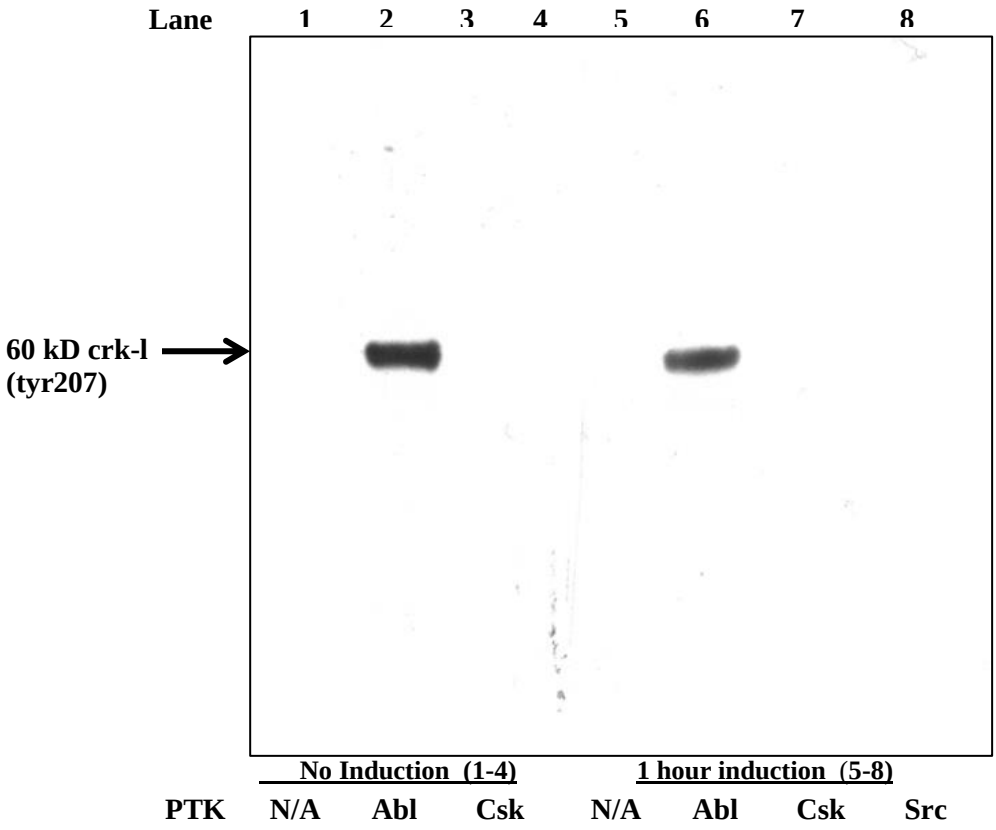
Lane 1 2 3 4 5 6 7 8
60 kD crk-l
(tyr207)
No Induction (1-4)
1 hour induction (5-8)
PTK N/A Abl Csk N/A Abl Csk Src

Figure 1. Abl, Csk and Src phosphorylation of substrate crk-l at tyrosine residue 207. All samples tested contained an identical substrate, crk-l. Lanes 1 and 5 serve as control lane samples containing substrate crk-l only with no PTK co-expressed. Lanes 2- 4 represent samples of Abl, Csk and Src separately co-expressed with substrate crk-l before induction with IPTG. Lanes 6-8 represent samples 2-4 after 1 h of induction with 0.4mM IPTG. The approximate size of the crk-l band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-crk-l (tyr207) at a 1:50,000 concentration and 2° anti-mouse IgG HRP-linked antibody at a 1:15,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

phosphorylation activity of Src toward this residue was observed in comparison to Abl and Csk which showed minimal activity as shown by the western blot in Figure 2 below.

Finally, Csk must demonstrate high substrate specificity toward kdSrc on tyrosine residue 527 when compared to Abl and Src. As expected, substrate specificity of Csk toward kdSrc on tyrosine residue 527 was in fact observed in comparison to both Abl and Src as shown in Figure 3.

Overall, demonstrating the expected substrate specificity of the three chosen model PTKs using the developed screening method relied heavily on optimization of the system. Initially, each strain tested was induced for 1 hour with 0.4mM IPTG upon reaching a logarithmic phase OD_{600} value and cell lysates were produced at both 0 hour and 1 hour induction time periods as in Figure 1 above. Initially, this worked great when testing substrate specificity of the PTKs tested toward crk-l substrate. In contrast, when testing substrate specificity of the various chosen PTKs toward kdSrc, it was seen that 1 hour induction samples tend to produce results not consistent with the expected substrate specificity of the PTKs tested. In detail, phosphorylation of the kdSrc substrate by both Csk and Src was becoming too saturated in samples taken after 1 hour induction with 0.4mM IPTG and not producing the expected difference in substrate specificity. Due to this, future cell lysate samples used in the developed screening method were not induced and taken at the appropriate OD_{600} value. This led to the conclusion that leaky-expression of both PTK and substrate-containing

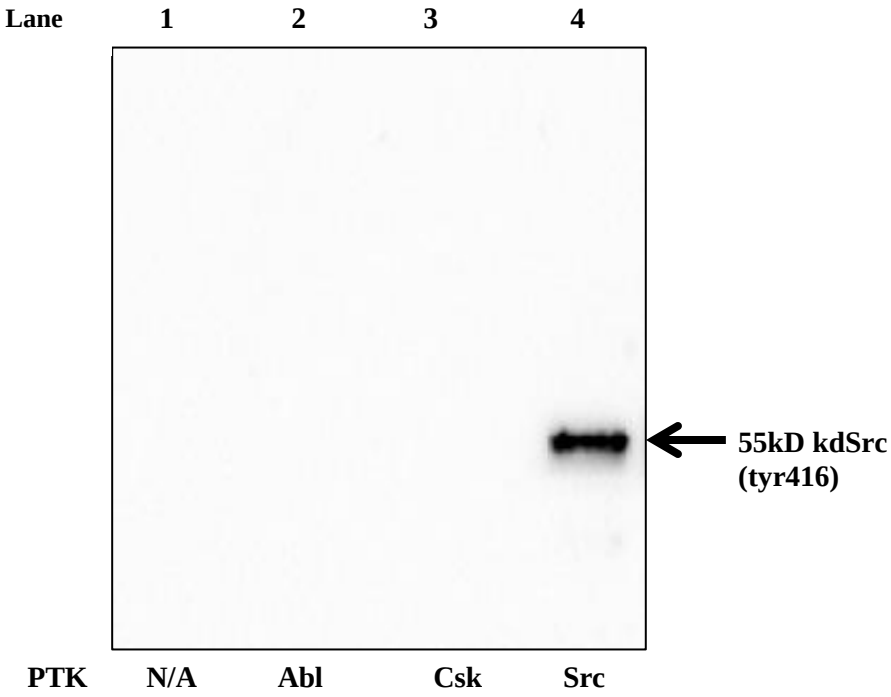
Lane 1 2 3 4
55kD kdSrc
(tyr416)
PTK N/A Abl Csk Src

Figure 2. Abl, Csk and Src phosphorylation of substrate kdSrc at tyrosine residue 416. All samples tested contained an identical substrate, kdSrc. Lane 1 serves as a control and contains substrate kdSrc only with no PTK co-expression. Lanes 2-4 are samples tested containing substrate kdSrc co-expressed individually with Abl, Csk or Src PTK. The approximate kdSrc band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-Src (Y416) at a 1:5,000 and 2° anti-rabbit IgG at a 1:10,000. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

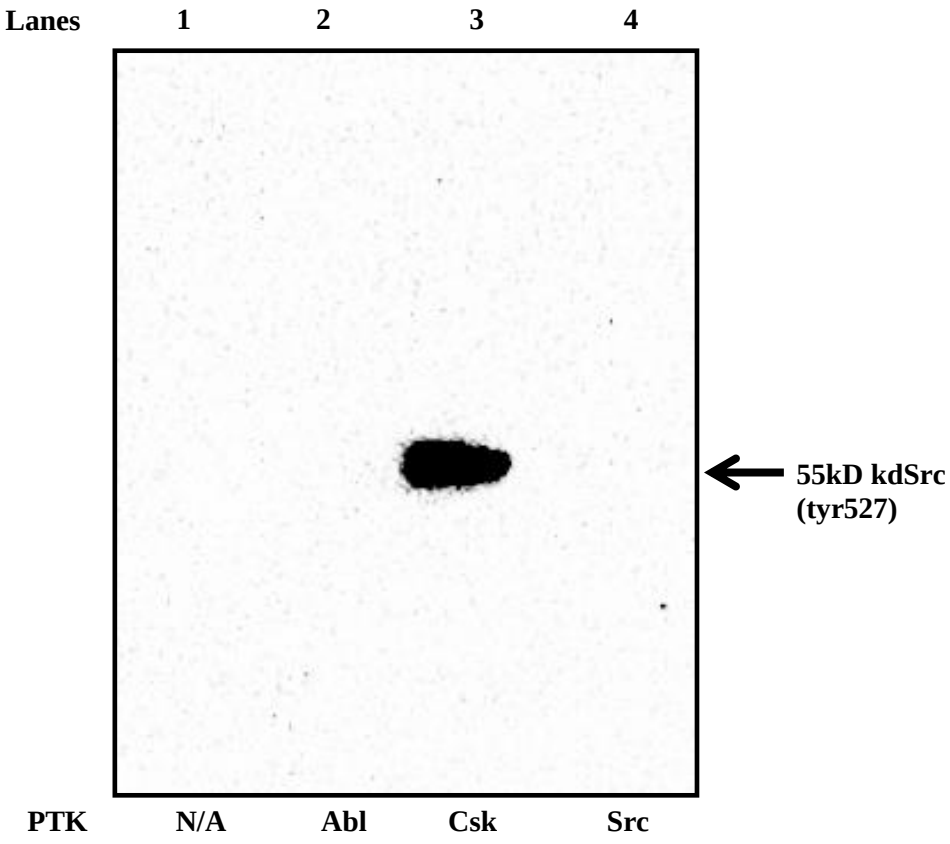
Lanes 1 2 3 4
55kD kdSrc
(tyr527)
PTK N/A Abl Csk Src

Figure 3. Abl, Csk and Src phosphorylation of substrate kdSrc at tyrosine residue 527. All samples tested contained an identical substrate, kdSrc. Lane 1 serves as a control and contains substrate kdSrc only with no PTK co-expression. Lanes 2-4 are samples tested containing substrate kdSrc in combination separately with the three PTKs Abl, Csk and Src. The approximate size of the kdSrc band is indicated by arrow. Antibodies used to perform blot were 1° Phospho-Src (Y527) at a 1:5,000 concentration and 2° anti-rabbit IgG at a 1:10,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

plasmid in this system is sufficient for demonstration of correct substrate specificity of these model PTKs toward their respective substrates. Once substrate specificity of Abl, Csk and Src toward their known substrates was demonstrated using this system, the next step was to validate the system further using both mutant PTKs and mutated substrates.

Testing of mutant PTKs to further validate system

The next step to further validate the developed method to analyze PTKs for substrate specificity was to test two specific mutant PTKs generated; Csk R281A, + R283A and Src RSRGRS+ K458F. Both of these mutants have had amino acids mutated which have previously been shown to have altered phosphorylation activity on the substrate kdSrc when compared to their wild-type forms.(6)

The first mutant PTK tested, Csk double mutant (DM) R281A + R283A, has had arginine residues replaced with alanine residues at amino acids 281 and 283. This has been shown in vitro, using radioactive kinase assays, to cause this mutant to dramatically lose its ability to phosphorylate kdSrc at its inactivation site, tyrosine residue 527, when compared to wild type Csk.[6] Therefore, it is expected, once tested using the proposed screening method, that this mutant should demonstrate a loss of phosphorylation activity on kdSrc at tyrosine residue 527, when compared to wild type.

In fact, this is what was observed as shown in Figure 4. Once this Csk R281A + R283A mutant demonstrated the expected phosphorylation activity when tested, the next mutant analyzed in this system was Src RSRGRS + K458F.

The Src RSRGRS + K458F mutant has had amino acids 354-359 (KGETGK) replaced with the amino acid sequence RSRGRS as well as a K458F mutation. These seven residues are collectively referred to as the substrate-docking site of Csk and have been shown to be crucial for Csk phosphorylation of Src at tyrosine residue 527.[6] To figure out which amino acids of Src to replace with the residues crucial for Csk's recognition of Src at tyrosine residue 527 (RSRGRS), a sequence alignment was performed between Csk and Src's peptide binding lobe (figure 5). Once determined which amino acids to mutate, this specific Src mutant was made and tested, in vitro; showing much higher phosphorylation activity at tyrosine residue 527 of kdSrc when compared to wild type Src, which phosphorylates this residue minimally.(6) Therefore, this altered phosphorylation activity should be demonstrated when tested in this in vivo optimized system to further establish its accuracy. It was shown that this Src mutant does indeed phosphorylate substrate kdSrc on tyrosine residue 527 at a much greater level when compared to wild type form as seen in Figure 6.

To show that the Src RSRGRS +K458F, mutant still retained its auto-phosphorylation ability at tyrosine residue 416 of kdSrc despites the mutations, a western blot was performed using antibodies specific for this residue. As seen in Figure 7 below, this mutant still retained its phosphorylation activity at tyrosine residue 416 of kdSrc, although it is less active, when compared to Src (wt).

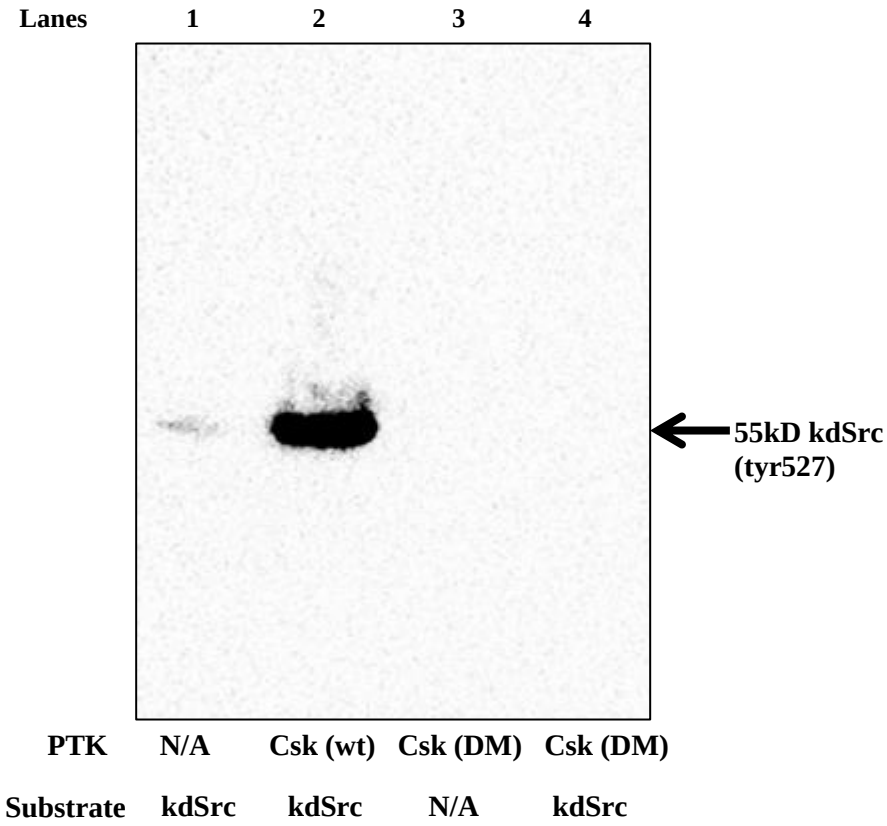
Lanes 1 2 3 4
55kD kdSrc
(tyr527)
PTK N/A Csk (wt) Csk (DM) Csk (DM)
Substrate kdSrc kdSrc N/A kdSrc

Figure 4. Csk (wt) vs. Csk (DM) R281A + R283A, phosphorylation of substrate kdSrc at tyrosine residue 527. All samples tested contained an identical substrate, kdSrc. Lane 1 serves as a control containing substrate kdSrc only with no PTK co-expression. Lanes 2 and 4 contain the substrate kdSrc co-expressed with either Csk (wt) or Csk (DM) R281A + R283A. The approximate kdSrc band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-Src (Y527) at a 1:5,000 concentration and 2° anti-rabbit IgG at a 1:10,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

Figure 5. Alignment of the amino acid sequences of Csk, Chk, Src, and Hck in the peptide-binding lobe.

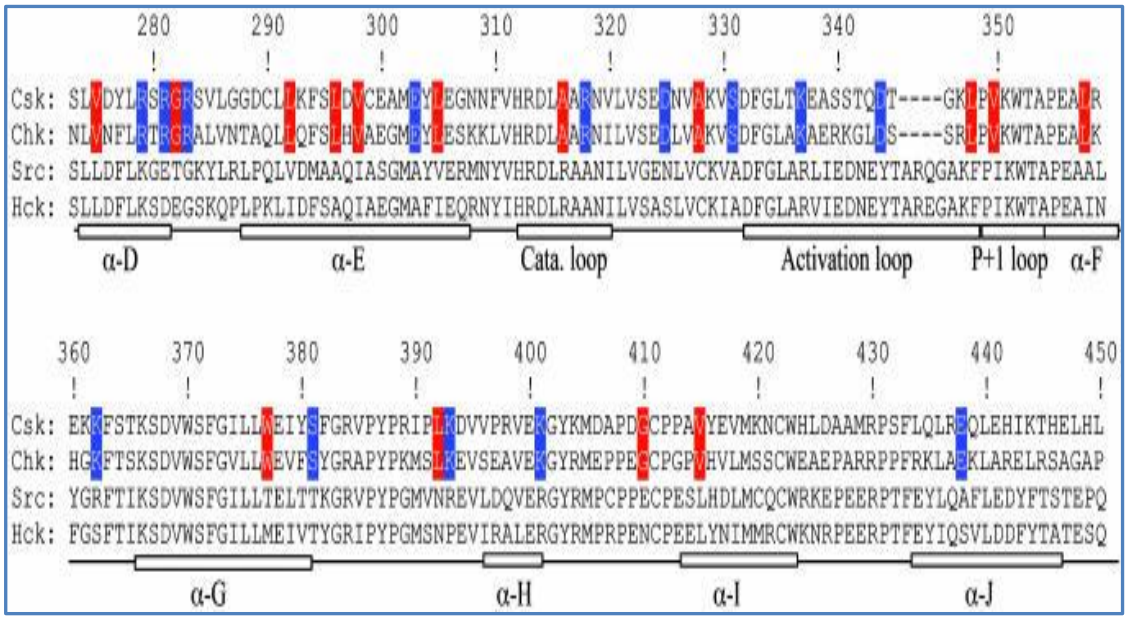

Figure 5. Alignment of the amino acid sequences of Csk, Chk, Src, and Hck in the peptide-binding lobe. Residues that are uniquely conserved in the Csk family are highlighted blue (polar or charged) or red (hydrophobic or Gly). Figure adapted from Lee et al. [6]

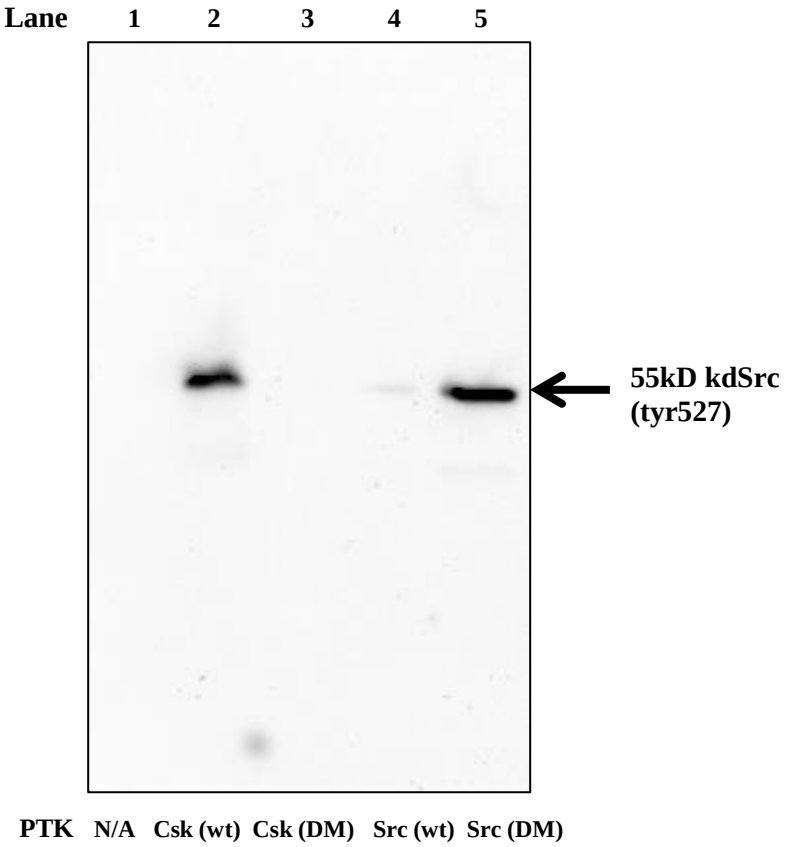
Lane 1 2 3 4 5
55kD kdSrc
(tyr527)
PTK N/A Csk (wt) Csk (DM) Src (wt) Src (DM)

Figure 6. Src (wt) vs. Src (DM) RSRGRS + K458F phosphorylation activity of substrate kdSrc at tyrosine residue 527. All samples tested contained an identical substrate, kdSrc. Lane 1 serves as a control containing substrate kdSrc only and no PTK co-expression. Lane 2 and 4 represent both wild type and mutant Csk and Src PTKs co-expressed separately with substrate kdSrc. The approximate kdSrc band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-Src (Y527) at a 1:5,000 concentration and 2° anti-rabbit IgG at a 1:10,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

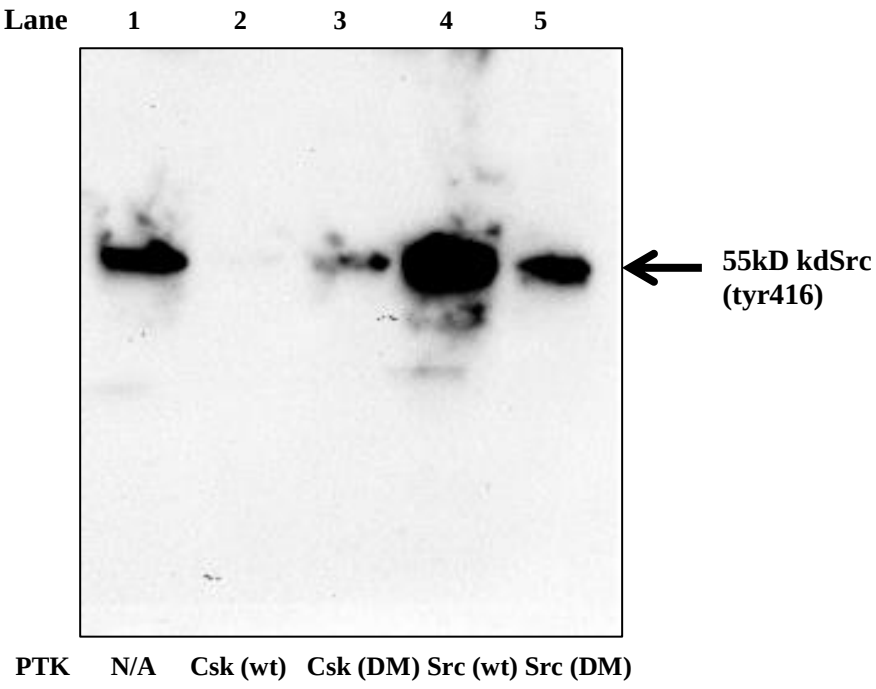
Lane 1 2 3 4 5
55kD kdSrc (tyr416)
PTK N/A Csk (wt) Csk (DM) Src (wt) Src (DM)

Figure 7. Src (wt) vs. Src (DM) RSRGRS + K458F phosphorylation activity of substrate kdSrc at tyrosine residue 416. All samples tested contained an identical substrate, kdSrc. Lane 1 serves as a control containing substrate kdSrc only and no PTK co-expression. Lane 2 and 4 represent both wild type and mutant Csk and Src PTKs co-expressed separately with substrate kdSrc. The approximate kdSrc band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-Src (Y527) at a 1:5,000 concentration and 2° anti-rabbit IgG at a 1:10,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

Testing of mutant substrates

The final step to validating the established system for screening PTKs for substrate specificity was to test two specific mutants of substrate kdSrc. The first mutant, kdSrc Y416F, has had the autophosphorylation tyrosine residue 416 replaced with a phenylalanine residue. The purpose of this generated mutation was to greatly reduce autophosphorylation by Src PTKs on this specific residue. In fact, this mutant did demonstrate a much lower level of phosphorylation activity by Src at this residue when tested in vitro. The second mutant, kdSrc Y527F, has the tyrosine 527 crucial for its inactivation replaced with a phenylalanine residue. In consequence, in vitro assays demonstrated a much lower level of phosphorylation by Csk on this altered residue then when compared versus kdSrc. Therefore, these two specific mutants of kdSrc were chosen as good candidates to validate the developed system.

The first mutant tested, kdSrc Y416F, should demonstrate a greatly reduced phosphorylation activity by Src at tyrosine residue 416 when compared with Src activity on kdSrc. As expected, when tested, the kdSrc Y416F mutant showed reduced levels of phosphorylation by Src compared to kdSrc. Having tyrosine residue 416 replaced with a phenylalanine greatly reduced Src's ability to phosphorylate this generated mutant as seen in Figure 8 below.

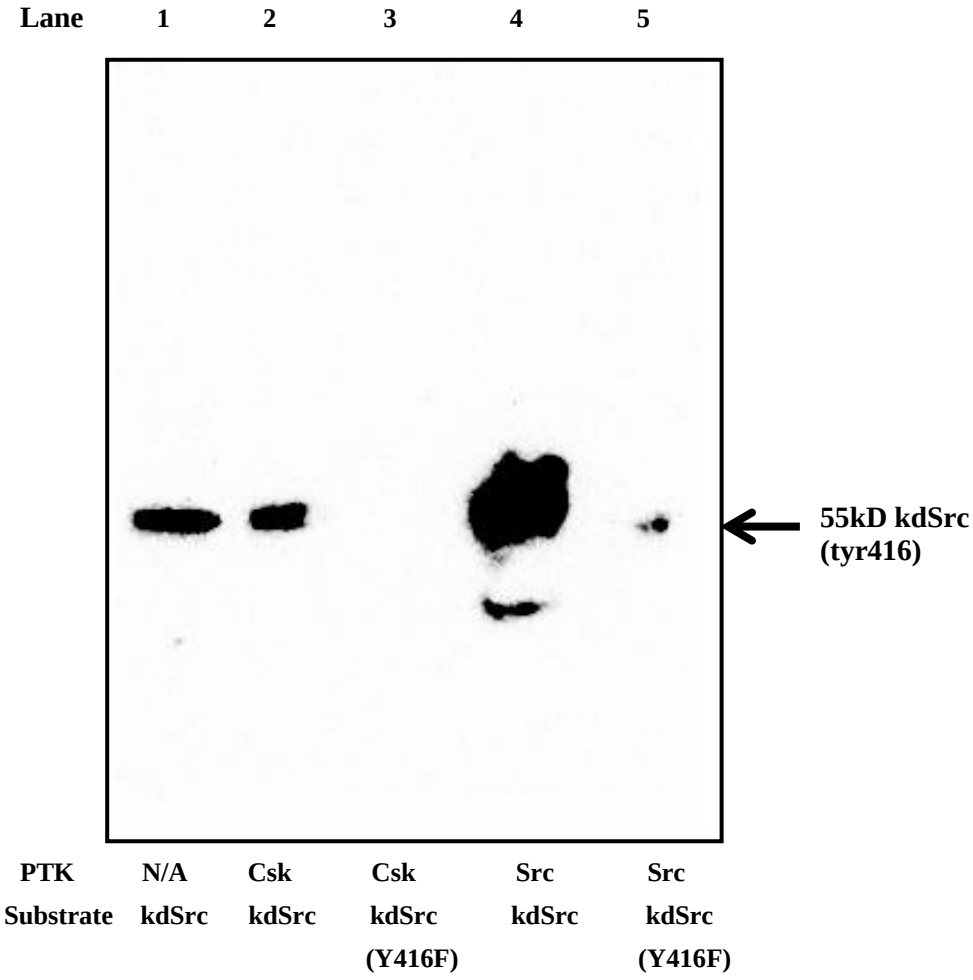
Lane 1 2 3 4 5
55kD kdSrc
(tyr416)
PTK N/A Csk Csk Src Src
Substrate kdSrc kdSrc kdSrc kdSrc kdSrc
(Y416F) (Y416F)

FIGURE 8. Csk and Src phosphorylation of kdSrc vs kdSrc (Y416F) substrates on tyrosine residue 416. Lane 1 represents a control with substrate kdSrc (Y416) only without co-expression of PTK. Lanes 2 and 4 represent Csk and Src co-expressed separately with kdSrc. Lanes 3 and 5 represent Csk and Src co-expressed separately with mutant substrate kdSrc (Y416F). The approximate kdSrc band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-Src (Y416) at a 1:5,000 concentration and 2° anti-rabbit IgG at a 1:10,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

The second mutant tested within this developed system was kdSrc (Y527F), a mutant lacking the crucial tyrosine necessary for its inactivation at this site. As expected, when co-expressed with Csk, the kdSrc (Y527F) mutant substrate demonstrated a greatly reduced phosphorylation level at tyrosine 527 when compared to kdSrc in the same setup, as shown in figure 9. This is a further indication of the effectiveness of the optimized method for analyzing substrate specificity in PTKs.

Optimization of developed colony screening method

Initially, the first attempt when establishing the colony screening method was to use the strains to be tested directly from an isolation streak plate with isolated colonies. These individual colonies were aseptically spotted with toothpicks directly onto nitrocellulose membranes, and these membranes were placed in a petri dish containing 20ml 0.4mM IPTG for 1 hour to promote induction of cells contained on membranes. Following this, membranes were transferred to another petri dish containing approximately 15ml of 10% SDS and heated in a 95°C oven for 10 minutes, to promote lysing of cells and release of proteins to be blotted. After lysing, membranes were dried and blotted using the optimized western blot protocol described in the materials and methods section.

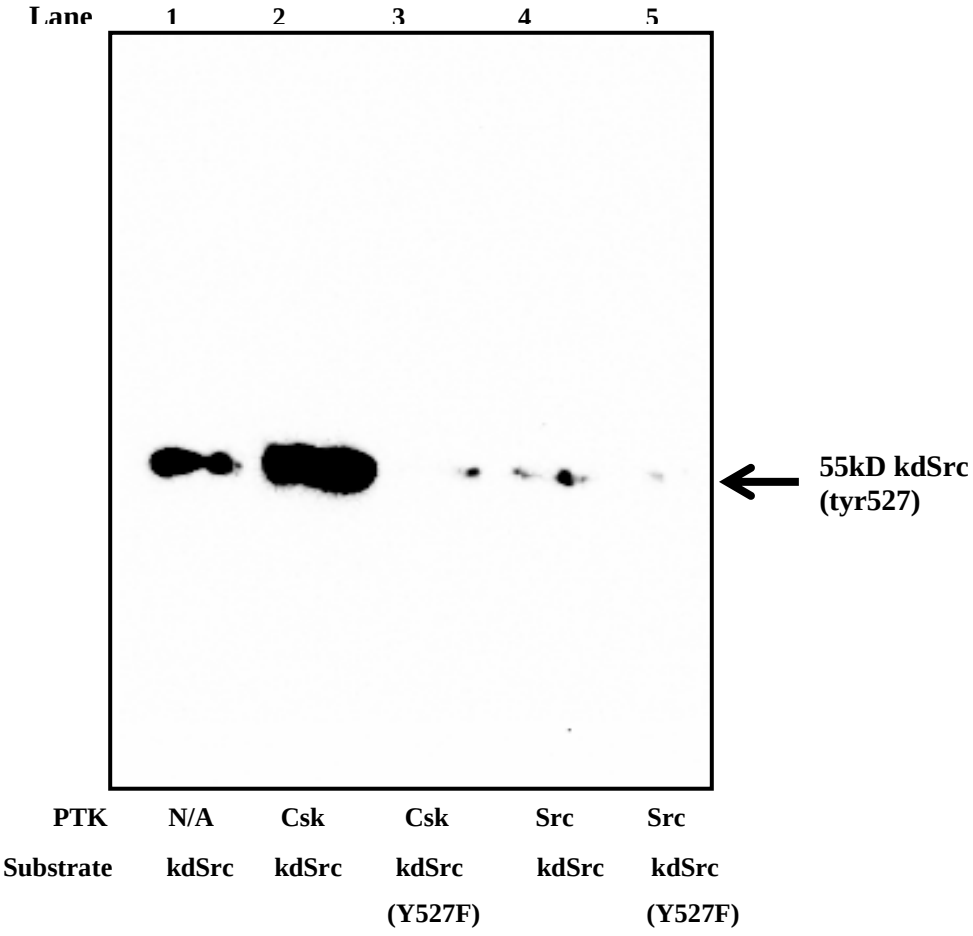
Lane 1 2 3 4 5
55kD kdSrc
(tyr527)
PTK N/A Csk Csk Src Src
Substrate kdSrc kdSrc kdSrc kdSrc kdSrc
(Y527F) (Y527F)

FIGURE 9. Csk and Src phosphorylation of kdSrc vs KdSrc (Y527F) substrates on tyrosine residue 527. Lane 1 represents a control with substrate kdSrc (Y527F) only without co-expression of PTK. Lanes 2 and 4 represent Csk and Src co-expressed separately with kdSrc. Lanes 3 and 5 represent Csk and Src co-expressed separately with mutant substrate kdSrc (Y527F). The approximate kdSrc band is indicated by the arrow. Antibodies used to perform blot were 1° Phospho-Src (Y527) at a 1:5,000 concentration and 2° anti-rabbit IgG at a 1:10,000 concentration. All samples were produced and tested according to the developed screening method protocol as described in materials and methods section.

Using this method, the three strains containing Abl, Csk and Src co-expressed individually with crk-l substrate were used to prepare a membrane as shown in figure 10a. Once prepared, the membrane was blotted using the Phospho-crk-l (Tyr207) antibody which detects phosphorylation on tyrosine residue 207 of crk-l, Abl's preferred substrate. In contrast, Src minimally phosphorylates this residue while Csk has not been shown to act on this residue. Once the prepared membrane was blotted, Abl did display a much higher phosphorylation activity when compared to both Csk and Src as shown in figure 10b.

Although the correct substrate specificity was demonstrated with the protocol used to generate samples used in figure 10b, the signals tended to overlay and transfer to other sections of the membrane while exposing films. Therefore, double sets of identical signals would be observed either below or above where the colonies were toothpicked onto the membrane. Another issue with this protocol was that colonies would wash off occasionally during the induction and lysing process and therefore, no signal would be observed. In an effort to overcome this, the protocol was altered so that the strains tested were to be grown and induced in liquid culture, rather than grown on an LB plate and induced on the sample-containing membrane as used to generate samples in figure 10b. Samples would then be pipetted directly onto them membrane, lysed and blotted. This optimization was done in hopes to limit the problems of transferred colonies washing off the membrane, or signals being transferred over to nearby sections of membrane.

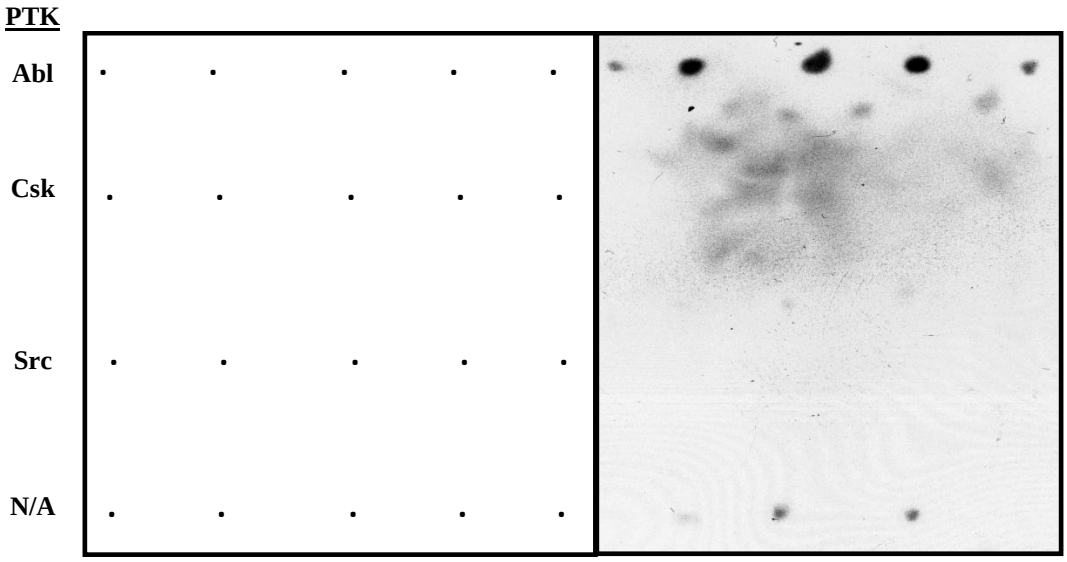
PTK
Abl
Csk
Src
N/A
(A)
(B)

Figure 10. Optimization of colony screening method by testing substrate specificity of PTKs Abl, Csk and Src at tyrosine residue 207 of crk-l substrate. **A.** Shown is the setup of colonies transferred directly onto a nitrocellulose membrane to be tested and blotted. All samples tested contained a similar crk-l substrate. **B.** Either of three PTKs Abl, Csk and Src was co-expressed with substrate crk-l and 5 samples of each were toothpicked as indicated in figure. After induction and lysing, membrane was blotted with primary antibody Phospho-crk-l (tyr207) at a concentration of 1:50,000 and secondary anti-mouse IgG at a 1:15,000 concentration.

Although the prior protocol did demonstrate the ability to screen PTKs for substrate specificity when phosphorylation activity of PTKs Abl, Csk, and Src was tested at tyrosine residue 207 of crk-l substrate, its optimization did indeed lead to better and cleaner results.

Use of optimized sample preparation protocol for colony screening substrate specificity of kdSrc

To optimize this colony screening protocol, along with changes to sample and membrane preparation, it was concluded that growing the colonies in liquid culture and then pipetting calculated volumes of these cultures directly onto the membrane would remove some of the setbacks of the prior protocol. To determine appropriate volume of sample to be pipetted onto the nitrocellulose membrane, several test runs had to be performed. Initially, 1µl of sample tested was spotted directly onto the membrane. Although this seems like a small amount of sample, it was determined that this amount used was actually causing the samples tested to produce results that are not indicative of the expected substrate specificity. For example, when testing phosphorylation activity of kdSrc substrate at tyrosine residue 416 by PTKs Abl, Csk and Src, both Abl and Src were showing similar phosphorylation activity on this residue, contrary to expected. This blot is shown below in Figure 11. Therefore, the amount of each culture pipetted directly onto the membrane was reduced to approximately 0.5µl. At this low volume, samples were shown to demonstrate proper substrate specificity.

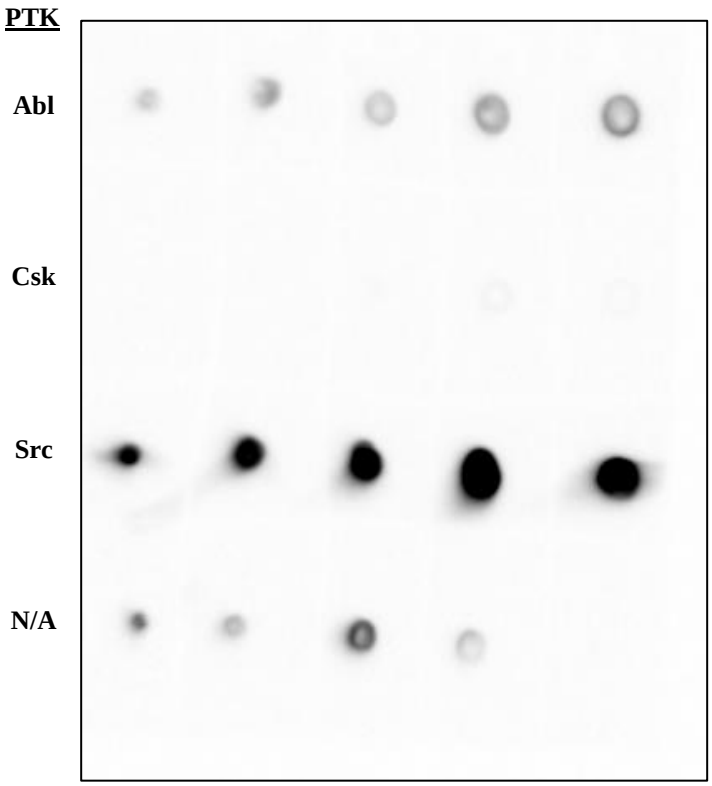
PTK
Abl
Csk
Src
N/A

Figure 11. Phosphorylation activity of PTKs Abl, Csk, and Src at tyrosine residue 416 of substrate kdSrc using an optimized protocol. All PTKs tested were co-transformed with the same kdSrc substrate. Amount of samples pipetted onto membrane and blotted was 1µl per spot. Either of three PTKs Abl, Csk and Src was co-expressed with substrate kdSrc and 5 samples of each were spotted as indicated in figure 2. The final set of samples (bottom row) were substrate kdSrc samples with no PTK co-expression used as a control (autophosphorylation). Membrane was blotted with primary antibody Phospho-Src (Tyr416) at a 1:5,000 concentration and secondary anti-rabbit IgG antibody at a 1:10,000 concentration.

Once this appropriate sample volume was established, a membrane was setup to demonstrate substrate specificity of PTKs Abl, Csk and Src at tyrosine residue 416 of substrate kdSrc. If proper substrate specificity is observed using this new protocol, then Src when co-expressed with kdSrc should show the greatest phosphorylation activity on tyrosine residue 416 when compared to both Abl and Csk co-expressed with similar substrate. In fact, this is what was demonstrated as shown in figure 11 below.

As Figure 12 demonstrates, proper substrate specificity by the tested PTKs was achieved in relation to phosphorylation activity at tyrosine residue 416 using this lesser volume of sample. Therefore, this identical 0.5µl sample volume was used in the last blot due to its success.

Upon establishing the expected substrate specificity of PTKs Abl, Csk and Src toward substrate kdSrc on tyrosine residue 416, the next step was to test for phosphorylation activity on tyrosine residue 527 of kdSrc by these same three enzymes. If this method is accurate, Csk should demonstrate high phosphorylation activity on tyrosine residue 527 of kdSrc when compared to both Abl and Src which have demonstrated minimal activity at this site. Once tested using the developed protocol, the expected result was observed and can be seen in figure 13.

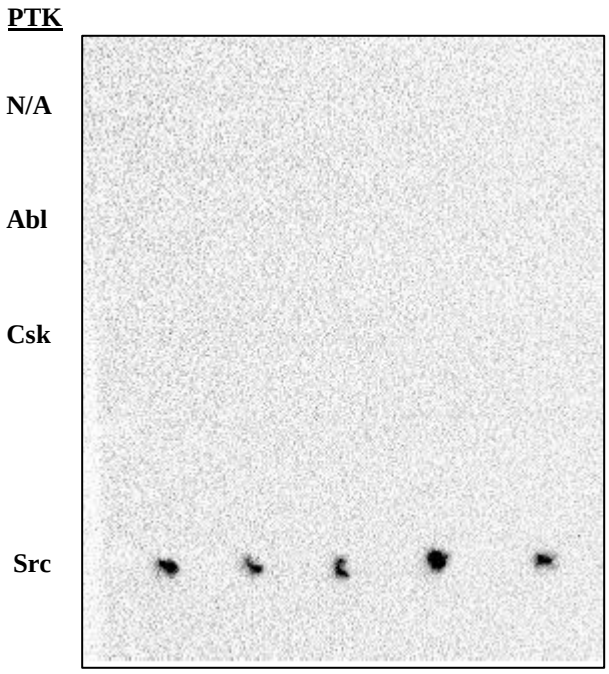
PTK
N/A
Abl
Csk
Src

Figure 12. Phosphorylation activity of PTKs Abl, Csk, and Src at tyrosine residue 416 of substrate kdSrc using lesser volume of sample. All PTKs tested were co-transformed with the same kdSrc substrate. Amount of samples pipetted onto membrane and blotted was 0.5µl per spot. Either of three PTKs Abl, Csk and Src was co-expressed with substrate kdSrc and 5 samples of each were spotted as indicated in Figure 10. The final set of samples (top row) contains substrate kdSrc with no PTK co-expression as a control. Membrane was blotted with primary antibody Phospho-Src (Tyr416) at a 1:5,000 concentration and secondary anti-rabbit IgG antibody at a 1:10,000 concentration. Blot was carried out by Yixin Cui.

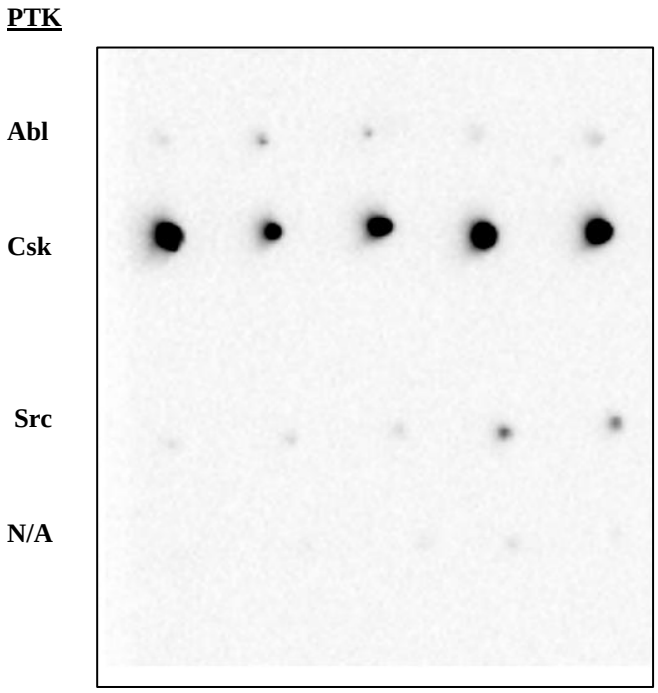
PTK
Abl
Csk
Src
N/A

Figure 13. Phosphorylation activity of PTKs Abl, Csk, and Src at tyrosine residue 527 of substrate kdSrc using lesser volume of sample. All samples tested contained similar kdSrc substrate. Amount of samples pipetted onto membrane and blotted was 0.5µl per spot. Either of three PTKs Abl, Csk and Src was co-expressed with substrate kdSrc and 5 samples of each were spotted as indicated in figure 9a. The final set of samples (bottom row) contains substrate kdSrc with no PTK co-expression as control. Membrane was blotted with primary antibody Phospho-Src (Tyr527) at a 1:5,000 concentration and secondary anti-rabbit IgG antibody at a 1:10,000 concentration.

Discussion

Overall, the goal when developing this novel system to screen PTKs for substrate specificity was to first optimize the protocol to make it very easy to screen for substrate specificity when testing different PTKs. The second goal was to use model PTKs Abl, Csk and Src along with a few of their preferred substrates to demonstrate the effectiveness of using this system to screen PTKs for substrate specificity. Finally, the third goal was to use a set of generated mutant PTKs and mutant substrates in an effort to further verify that this system can screen PTKs for substrate specificity, even mutant samples.

Initially, several optimizations were made in an effort to demonstrate the proper expected substrate specificity. First, it was determined that cultures used to produce cell lysates in the system had to be grown to a specific OD_{600} value generally between 0.7-1.2(logarithmic phase). If cultures were grown too little, the signals produced by western blot were hard to detect, and if the cultures are grown too much, the substrates tested would generally become too phosphorylated regardless of which PTK was tested. Therefore if cultures are grown to an OD_{600} value that is too high, and testing of substrate kdSrc on tyrosine 416 is performed with different PTKs, both Csk and Src would both show high phosphorylation activity on the substrate tested even though Csk phosphorylates this residue minimally. Therefore, growth of the cultures must be limited to the logarithmic phase OD_{600} value.

Another optimization made was in choosing which plasmids to use for expression of the genes of substrates and PTKs tested. Initially the genes of substrates were cloned

into low-copy number plasmid pCDF-1b and genes of PTKs into high-copy number plasmids pRSET-A or pGEX-4t-1 as carried out by Yixin Cui. After further testing, this proved to be unsuccessful. The reason for this is that if the amount of substrate expressed is limited compared to the amount of PTK expressed, substrate specificity of any PTK tested with a specific substrate will generally be masked. For example, if 100 substrate kdSrc proteins are produced and 1000 Csk and Src PTKs are each produced, and these samples are tested and blotted for phosphorylation of kdSrc at tyrosine residue 416, then a difference in substrate specificity will not be seen. The researcher will observe that kdSrc at tyrosine residue 416 has been phosphorylated equally by both Csk and Src even though this is not what occurs in nature. Therefore, to overcome this problem, the genes of the substrates tested were cloned and expressed in high-copy number plasmids pRSET-A (kdSrc) or pGEX-4t-1(crk-l) and genes of PTKs in low-copy number plasmid pCDF-1b. By limiting the amount of PTKs expressed in contrast to the number of substrate being produced, proper difference in substrate specificity could be observed and achieved with this developed system. These were the two biggest optimizations made to the developed system in an effort to make it as accurate as possible when analyzing substrate specificity. The second goal of this research was to use this optimized system in combination with testing three important PTKs Abl, Csk and Src along with two substrates, crk-l and kdSrc, in an effort to demonstrate that it can achieve proper substrate specificity. With the optimizations made, the results seen did indeed show that the system could demonstrate a difference in substrate specificity by different PTKs when tested against chosen substrates.

The first important result demonstrated was when testing phosphorylation activity at tyrosine residue 207 of substrate crk-l when co-expressed individually with Abl, Csk and Src. Since crk-l is one of Abl's preferred substrates and is minimally phosphorylated by both Csk and Src, this difference in substrate specificity should be and was demonstrated by this system. The second result was testing phosphorylation activity of kdSrc at tyrosine residue 416 when co-expressed individually with the same three PTKs as above. Since Src phosphorylates this residue heavily in comparison to both Abl and Csk, this should be demonstrated when tested using the developed system. When tested, this expected result was produced and repeated. Finally, phosphorylation activity of kdSrc at tyrosine 527 by Abl, Csk and Src was tested using this optimized system. Csk shows much greater phosphorylation activity at this residue when compared to Abl and Src. It was indeed shown that Csk phosphorylation activity on tyrosine residue 527 on kdSrc was much greater in comparison to the Abl and Src as expected. Once substrate specificity was demonstrated using the three chosen model PTKs Abl, Csk, and Src, the final step taken was to validate the use of this developed method with mutant PTKs and substrates.

The first mutant tested, Csk (DM) R281A + R283A, has specific amino acids arginine residues replaced with alanine in its peptide binding lobe region. These residues, when mutated, cause a dramatic loss in this mutant's phosphorylation activity on tyrosine residue 527 of kdSrc.[6] When tested using the developed system, this loss of activity on tyrosine 527 of kdSrc was demonstrated and highly reduced when compared to Csk's (wt) activity on this same substrate.

The second mutant tested, Src RSRGRS +K458F, has had amino acids 354-359 (KGETGK) replaced with RSRGRS amino acid sequence and a K458F replacement, giving this mutant the ability to phosphorylate kdSrc substrate on tyrosine residue 527.[6] This mutant should demonstrate this altered phosphorylation activity when implemented in the developed system if the system is to be further validated. It was shown that the Src RSRGRS + K458F mutant phosphorylated kdSrc on tyrosine residue 527 much more when compared to Src (wt)'s activity on this residue as expected. Once this was accomplished, the final validation for this developed method was to test two specific mutant substrates, kdSrc (Y416F) and kdSrc (Y527F). The first mutant, kdSrc (Y416F) has had the tyrosine residue at its auto-phosphorylation site replaced with a phenylalanine residue. The result of this mutation is that this site can no longer be phosphorylated by PTKs and therefore this should be demonstrated when tested using the method developed to screen PTKs for substrate specificity. Once tested, the kdSrc (Y416F) mutant when co-expressed with either Csk or Src shows little to no phosphorylation activity. In contrast, kdSrc (wt) is phosphorylated heavily by Src as shown by testing. Finally, mutant kdSrc (Y527F) which has had the tyrosine residue 527 at its inactivation site replaced with a phenylalanine residue should demonstrate a lack of phosphorylation activity by both Csk and Src when tested on this residue. As expected, Csk which heavily phosphorylates kdSrc (wt) on tyrosine 527 showed a lack of phosphorylation activity when co-expressed with kdSrc (Y527F) in this system. Src which minimally phosphorylated kdSrc(wt) at tyrosine residue 527 also showed a loss of activity when co-expressed with this mutant.

Overall, after optimization and testing of both wild type and mutant PTKs/substrates using this developed system, its effectiveness at displaying substrate specificity of different wild type and mutant model PTKs and substrates was demonstrated through repeated testing. Concluding, there are many beneficial reasons to use this developed method to screen protein tyrosine kinases for substrate specificity. For one, it negates most of the drawbacks that other in vivo and in vitro methods used for analyzing PTK substrate specificity, present. Second to this, once setup of system is established, results can be produced within 1-2 days, which is rapid compared to other methods. Finally, with the established protocol, using this system is fairly convenient and employs basic laboratory techniques in which any researcher can execute.

Once the method above was established in being effective for screening PTKs for substrate specificity, the protocol was optimized to allow a researcher to rapidly screen a large number of PTK mutants generated through current techniques such as DNA shuffling.[7,8] After a few changes, a developed colony screening method for rapidly screening PTKs was established and proven effective by using model PTKs Abl, Csk and Src and the preferred substrates. Although no mutant PTKs were tested during this study, by demonstrating the proper substrate specificity of model PTKs, the goal was to provide convincing evidence that this method can be used to screen mutant PTKs that can be generated through DNA shuffling or other methods. Concluding, this novel colony screening method provides a rapid way to screen mutant PTKs, once generated, for chosen substrate specificity in a cost-effective, replicative manner.

One drawback of both systems is that the observed specificity is not quantitative and thus once substrate specificity is demonstrated using either method, it needs to be further quantified by other methods, such as radioactive analysis.

Acknowledgments:

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