Progress towards mechanistic studies of Src tyrosine kinase using ligated protein constructs

Master’s Thesis

Presented to

The Faculty of the Graduate School of Arts and Sciences
Brandeis University
Department of Biochemistry
Dorothee Kern, Advisor

In Partial Fulfillment
of the Requirements for the Degree

Master of Science
in
Biochemistry

by
Sara G. Gelles-Watnick

May 2017
Acknowledgements

I would like to thank Dorothee Kern for her support of my undergraduate research and enthusiastic teaching. I also thank Chris Wilson, Yizhi Sun, and the other members of the Kern Lab. Thank you to my mom, my dad, and my sister for their support.
ABSTRACT

Progress towards mechanistic studies of Src tyrosine kinase using ligated protein constructs

A thesis presented to the Department of Biochemistry

Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Sara Gelles-Watnick

Tyrosine kinase c-Src is a ubiquitously expressed proto-oncogene implicated in many cancers. The Kern Lab hopes to use NMR to study the regulatory and catalytic mechanisms of c-Src. Because of the large size of c-Src, a partially labeled version of Full-length (FL) Src was required for NMR studies. To prepare the partially labelled protein, part of c-Src was isotopically labelled and then ligated to the remainder of the protein using the Sortase enzyme. Two variants of FL Src amenable to Sortase ligation (Back-ligated Src 1 and Back-ligated Src 2) were designed and generated by adding the Sortase ligation motifs at different locations in the sequence. By a coupled peptide-kinase activity assay, BL Src 1 and BL Src 2 were determined to have activity very similar to each other, and activity roughly similar to FL Src. Comparing the NMR spectra of BL Src 1 and BL Src 2 revealed that the BL Src 2 most closely mimics FL Src. Thus, BL Src 2 should be used in future NMR studies of the Src tyrosine kinase mechanism.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>6</td>
</tr>
<tr>
<td>Results</td>
<td>15</td>
</tr>
<tr>
<td>Discussion</td>
<td>33</td>
</tr>
<tr>
<td>Works Cited</td>
<td>34</td>
</tr>
<tr>
<td>Appendix</td>
<td>35</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Buffers used for purification of all proteins</td>
<td>6</td>
</tr>
<tr>
<td>Table 2</td>
<td>Concentration of reagents used in Sortase optimization trials</td>
<td>11</td>
</tr>
<tr>
<td>Table 3</td>
<td>Concentrations of reagents used in Src western blot</td>
<td>11</td>
</tr>
<tr>
<td>Table 4</td>
<td>Concentrations of reagents used in CSK phosphorylation optimization</td>
<td>12</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Linear representation of Src tyrosine kinase.</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Src conformations and its regulation by phosphorylation of tyrosines 527 and 416.</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Sortase ligation reaction to be used to create a partially isotopically labelled version of Src.</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Performing the Sortase ligation reaction on SH3/SH2 and SrcK fragments produce BL Src 1 and 2 constructs attained by ligating SH3/SH2 and SrcK.</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Sortase ligation optimization trials (Table 2) demonstrate the optimal ratios of reagents</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>After expression, purification, and ligation, Y527 in BL Src 1 is not fully phosphorylated.</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>The AP visualization system does not accurately detect differences in the amount of phospho-proteins.</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>ScanLater Western blot assay demonstrates that at sites Y416 and Y527, BL Src 2 and FL Src remain unphosphorylated after expression and purification.</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>After ATP and CSK incubation, Back-ligated Src 2 and Full-length Src are phosphorylated at site Y527, but not significantly at Y416.</td>
<td>22</td>
</tr>
<tr>
<td>10</td>
<td>Using HPLC quantification, Full-length Src and Back-ligated Src 1 phosphorylated Src optimal peptide substrate (SOPS) at similar rates compared to Src kinase 1.</td>
<td>23</td>
</tr>
<tr>
<td>11</td>
<td>Using an NADH-ATP coupled assay, Back-ligated Src 1 and Back-ligated Src 2 had similar rates, but they differed from that of Full-length Src.</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>By NADH-ATP coupled assay, Full-length Src and Substituted Src 2 have similar kinase rates, while Back-ligated Src 2 has a 3-fold lower rate.</td>
<td>26</td>
</tr>
<tr>
<td>13</td>
<td>$^{15}$N-$^1$H TROESY-HSQC NMR analysis reveals few differences between BL Src 1 with $^{15}$N-labeled SH3/SH2 domain and $^{15}$N-SH3/SH2 1 fragment.</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>$^{15}$N-$^1$H TROESY-HSQC NMR analysis reveals significant differences between BL Src 2 with $^{15}$N-labeled SH3/SH2 domain and $^{15}$N-SH3/SH2 2 fragment.</td>
<td>30</td>
</tr>
</tbody>
</table>
INTRODUCTION

Ubiquitously expressed non-receptor c-Src tyrosine kinase is involved in the regulation of the cell cycle, and therefore mutation of this proto-oncogene is the cause of certain cancers\(^1,^2\). Src plays a role in signaling cascades by interacting with various growth factor receptors, receptor protein tyrosine kinases, cytokine receptors, ligand-gated channels, gap junctions, and g-protein coupled receptors\(^3\). The mutation of Src is an important cause of breast cancer and prostate cancer\(^3,^4\). Although Src mutation plays an important role in malignant transformation, the regulatory mechanism of Src is currently unknown. This has made finding Src inhibitors for use in cancer treatment difficult. C-Src is currently not well inhibited by drugs known to inhibit Abl and other members of the Src family of tyrosine kinases (for example, Gleevec)\(^5\).

![Figure 1: Linear representation of Src tyrosine kinase.](image)

Figure 1: Linear representation of Src tyrosine kinase. Representation of the domains of c-Src tyrosine kinase. Phosphorylation sites are marked in red. Numbering from chicken Src. Adapted from Boggon 2004, Rowoski 2005.

Src is composed of a myristoyl tail, a unique SH4 domain, an SH3 domain, an SH2 domain, a flexible linker, and a kinase domain (SrcK) (Figure 1)\(^6\). Src exists in an open, active conformation and a closed, inactive conformation (Figures 2A and 2B). Crystal structures of the closed state and the open state have been determined\(^7,^8\). In the closed conformation, the SH3 domain interacts with the linker and the N-lobe of the kinase domain (Figure 2A). Also when closed, the SH2 domain interacts with the portion
of the C-terminal tail (post-SrcK) that includes the Y527 residue (all numbering will be from that of chicken Src). The catalytic site (Figure 2A) is blocked by the regulatory

**Figure 2: Src conformations and its regulation by phosphorylation of tyrosines 527 and 416.** (A) Surface representation of the open conformation of c-Src (Cowan-Jacob 2005). Adapted from Goodsell 2015. (B) Surface representation of the closed conformation of Src (Xu 1997). Adapted from Goodsell 2015. (C) Phosphorylation states of src tyrosine kinase, showing the conformations (when known) of the states.
domains (SH3 and SH2). In the open conformation, the regulatory domains do not interact with the kinase domain, and the catalytic site is unobstructed (Figure 2B).

There are two tyrosine residues in the kinase domain of Src, Y416 and Y527, that when phosphorylated are thought to determine the conformation (open or closed) in which Src will exist. Phosphorylation of Y527 promotes the closed form of c-Src, while the phosphorylation of Y416 promotes the open form of c-Src (Figures 2A and 2B). The phosphorylation of tyrosine 527 is performed by CSK, another Src family tyrosine kinase, while tyrosine 416 undergoes autophosphorylation. This means there are 4 phosphorylation states of Src: Y416Y527, pY416Y527, Y416pY527, and pY416pY527 (Figure 2C). In vivo studies have demonstrated that only Y416 or Y527 is

**Figure 3: Sortase ligation reaction to be used to create a partially isotopically labelled version of Src.** (A) Application of the Sortase back ligation of src tyrosine kinase to produce a partially $^{15}$N-labelled protein for NMR. (B) General mechanism of the Sortase ligation reaction. R and R' represent the remainder of the protein or peptide. X represents any amino acid.
phosphorylated at a given time\textsuperscript{6}. However, the double phosphorylated state (pY416pY527) is possible to attain in vitro\textsuperscript{11}.

The Kern lab plans to study how the relative populations of open and closed conformations of Src change with the phosphorylation state, in order to establish the regulatory mechanism of Src. To accomplish this goal, solution nuclear magnetic resonance (NMR) was used to solve the structure of Full-Length (FL) Src. Because of the large size of FL Src (it is approximately 55 kDa), peaks of the resulting $^{15}$N-$^1$H spectra of FL Src were broad and overlapped, making assignment impossible. In order to simplify the NMR spectrum while maintaining the integrity of the Src protein, we chose to express Src in two separates pieces (with one of the pieces $^{15}$N-labelled), ligate the pieces together, and then perform NMR on the partially-labelled version (Figure 3A).

To perform the ligation, the Kern group elected to use Sortase A, an endogenous \textit{Staphylococcus aureus} enzyme that reversibly ligates peptides to the cell wall\textsuperscript{12}. The Sortase A enzyme recognizes an N-LPXTG-C motif (where X is any amino acid) on Protein Y and cleaves the peptide bonds between threonine and glycine (Figure 3B). The glycine is released and an acyl-enzyme intermediate is formed, with the cysteine of Sortase A now attached to the threonine of Protein Y\textsuperscript{13}. Then, the first amino group from the N-terminus of the poly-glycine repeat on Protein Z attacks the $C_{\alpha}$ of the threonine, and cleaves the acyl-enzyme intermediate into the Sortase A apoenzyme and the Protein Y-LPXT-GGGGG-Protein Z chimera\textsuperscript{13}.
For my thesis research, I produced two Sortase back-ligated (BL Src) versions of FL Src and determined their phosphorylation states. I then performed catalytic activity assays and NMR on FL Src and two BL Src constructs to determine whether these constructs behave similarly to FL Src.
MATERIALS AND METHODS

Table 1: Buffers used for purification of all proteins

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>pH 8</th>
<th>pH 8</th>
<th>pH 8</th>
<th>pH 8</th>
<th>pH 8</th>
<th>7.5</th>
<th>6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt; Affinity Binding Buffer</td>
<td>50 mM TRIS</td>
<td>50 mM TRIS</td>
<td>20 mM TRIS</td>
<td>20 mM TRIS</td>
<td>50 mM TRIS</td>
<td>50 mM TRIS</td>
<td></td>
</tr>
<tr>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt; Affinity Elution Buffer</td>
<td>500 mM NaCl</td>
<td>500 mM NaCl</td>
<td>5% glycerol</td>
<td>1 M NaCl</td>
<td>500 mM NaCl</td>
<td>500 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>Ion exchange Binding Buffer</td>
<td>5% glycerol</td>
<td>5% glycerol</td>
<td>5% glycerol</td>
<td>5% glycerol</td>
<td>2 mM TCEP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange Elution Buffer</td>
<td>1 mM DTT</td>
<td>1 mM DTT</td>
<td>20 mM Imidazole</td>
<td>20 mM Imidazole</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP Affinity Binding Buffer</td>
<td>5 mM βMe</td>
<td>5 mM βMe</td>
<td>1 mM βMe</td>
<td>1 mM βMe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP Affinity Elution Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size Exclusion Elution Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMR Size Exclusion Elution Buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Expression and Purification of FL Src

This protocol is adapted from Seeliger 2005<sup>1</sup>. Expression vectors encoding FL Src with MBP and 6X-His affinity tags (KAN resistance) and YOPH (STREP resistance) were co-transformed into the BL21 (DE3) cell line (Appendices 1 and 2). These cells were cultured in 2 L Terrific Broth at 37°C. When an OD<sub>600</sub> of 1.2 was reached, the temperature was decreased to 20°C and the cells were induced at 0.3 mM IPTG for approximately 16 hours. The cell lysate was passed over a Ni<sup>2+</sup> affinity column and subsequently a Maltose Binding Protein (MBP) affinity column (Table 1). A Tobacco etch virus (Tev) protease cleavage reaction was performed at room temperature for 14-20 hours while dialyzing against Ni<sup>2+</sup> affinity binding buffer. A second Ni<sup>2+</sup> affinity FPLC
was performed, and the protein was subsequently purified using a size-exclusion column. Between each FPLC column, UV absorbance chromatograms and SDS-Page gels were performed to determine which fractions contained the protein.

*Expression and Purification of SrcK*

Expression vectors encoding SrcK with MBP and 6X-His affinity tags (AMP resistance) and YOPH (STREP resistance) were co-transformed into an Artic Express (DE3) cell line (Appendices 5, 9, and 2). These cells were cultured in 4 L Terrific Broth at 37°C. When an OD\(_{600}\) of 1.2 was reached, the temperature was decreased to 20°C and the cells were induced at 0.3 mM IPTG for approximately 16 hours. The cell lysate was passed over a Ni\(^{2+}\) binding affinity column and subsequently an anion exchange column (Table 1). A Tev protease cleavage reaction was performed at room temperature for 14-20 hours while dialyzing against Ni\(^{2+}\) binding buffer. A second Ni\(^{2+}\) affinity FPLC was performed, and the protein was subsequently purified using a size-exclusion column. Between each FPLC column, UV absorbance chromatograms and SDS-Page gels were performed to determine which fractions contained the protein.

*Expression and Purification of SH3/SH2*

An expression vector encoding SH3/SH2 with a 6X-His affinity tag (AMP resistance) was transformed into an Artic Express (DE3) cell line (Appendices 4 and 8). These cells were cultured in 4 L Terrific Broth at 37°C. When an OD\(_{600}\) of 1.2 was reached, the temperature was decreased to 20°C and the cells were induced at 0.3 mM IPTG for approximately 16 hours. The cell lysate was passed over a Ni\(^{2+}\) affinity column
and subsequently a size-exclusion column (Table 1). Between each FPLC column, UV absorbance chromatograms and SDS-Page gels were performed to determine which fractions contained the protein.

*Expression and Purification of $^{15}$N-labelled SH3/SH2*

Purification of $^{15}$N-labelled SH3/SH2 was performed similarly to SH3/SH2 with the following modification. Cells were cultured in M9 minimal media containing $^{15}$NH$_4$Cl. Cultures were shifted to 20$^\circ$C and induced at 0.1 mM IPTG when an OD$_{600}$ of 0.7 was reached. In addition, NMR size-exclusion elution buffer was used for the size-exclusion purification (Table 1).

*Expression and Purification of CSK*

Expression vectors encoding CSK with MBP and 6X-His affinity tags (KAN resistance) and YOPH (STREP resistance) were co-transformed into BL21 (DE3) One Shot cells (Appendices 3 and 2). These cells were cultured in Terrific Broth at 37$^\circ$C. When an OD$_{600}$ of 1.2 was reached, the temperature was decreased to 20$^\circ$C and the cells were induced at 0.3 mM IPTG for approximately 16 hours. The cell lysate was passed over a Ni$^{2+}$ affinity column and subsequently an MBP affinity column (Table 1). A Tev cleavage reaction was performed at room temperature for 14-20 hours while dialyzing against Ni$^{2+}$ affinity binding buffer. A second Ni$^{2+}$ affinity FPLC was performed, and the protein was subsequently purified using a size-exclusion column. Between each FPLC column, UV absorbance chromatograms and SDS-Page gels were performed to determine which fractions contained the protein.
Expression and Purification of Sortase A

An expression vector encoding Sortase A with a 6X-His affinity tag (AMP resistance) was transformed into a DH5α cell line. These cells were cultured in 1 L TB-Express Overnight Induction (Novagen) at 30°C. When an OD\text{600} of 1.2 was reached, the temperature was decreased to 20°C and the cells were induced at 0.3 mM IPTG for approximately 16 hours. The cell lysate was passed over a Ni\text{2+} affinity column and subsequently a size-exclusion column (Table 1). Between each FPLC column, UV absorbance chromatograms and SDS-Page gels were performed to determine which fractions contained the protein.

Sortase Ligation Reaction and Purification

The Sortase ligation was performed in Sortase Ligation Buffer, which is composed of 20 mM TRIS-HCl at pH 8, 150 mM NaCl, 10 mM CaCl₂, and 4 mM β-mercaptoethanol (βMe). SrcK, SH3/SH2, and Sortase were combined with a small amount of buffer in a volume of 5 mL or less and added to a dialysis cartridge. Sortase A enzyme and SH3/SH2 domains were added in excess of SrcK. Because the Sortase ligation reaction is not very favorable, a dialysis system was used to drive the reaction forward. To prevent the back reaction, the glycine-6X-His tag cleaved off during the first step of the Sortase ligation reaction was allowed to pass through a semi-permeable membrane (10 kDa pore size) while the protein reagents remained sequestered in the dialysis cartridge. The dialysis cartridge was placed into approximately 1 L of buffer. The reaction was allowed to proceed at 4°C for 3.5 days.
**Purification of BL Src and $^{15}$N-labelled BL Src**

After the Sortase ligation reaction, the reaction mixture was passed over a Ni$^{2+}$ affinity column (Table 1). The remaining reagents were collected from the Ni$^{2+}$ affinity column for use in a subsequent Sortase ligation reaction. Then, the fractions containing product are passed over a size-exclusion column.

For $^{15}$N-labelled BL Src, the same *Sortase Ligation Reaction* was performed, but using $^{15}$N-labeled SH3/SH2. During purification, the NMR size-exclusion elution buffer was used (Table 1).

**Sortase Ligation Reaction Optimization**

A procedure similar to that described in *Sortase Ligation Reaction* was used for optimization except the reactions were run at room temperature (approximately 23°C). In Trials 1 and 2, SrcK 1, SH3/SH2 1, and Sortase were combined in various concentrations with Sortase Reaction Buffer to a final volume of 200 μL (Table 2). 20 μL samples were removed and added to 30 μL of SDS-Page loading dye at 1, 2, 4, 6, 8, and 24 hours. In Trials 3-6, SrcK 1, SH3/SH2 1, and Sortase were combined in various concentrations with the Sortase Reaction Buffer to yield a final volume of 100 μL. 15 μL samples were removed and added to 5 μL of SDS-Page loading dye at 0 and 2 hours. In Trials 7-11, SrcK 1, SH3/SH2 1, and Sortase were combined in various concentrations with Sortase Reaction Buffer to a final volume of 100 μL. 15 μL samples were removed and added to 5 μL of SDS-Page loading dye at time points 0 hr and 2 hr.
Concentrations for Reactions 1-11 are shown in Table 2 below. SDS-Page gels were performed on samples for each group of trials (1-2, 3-6, and 7-11).

Table 2: Concentration of reagents used in Sortase optimization trials

<table>
<thead>
<tr>
<th>Trial</th>
<th>[SrcK 1] (μM)</th>
<th>[SH3/SH2 1] (μM)</th>
<th>[Sortase] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>150</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>150</td>
<td>17.5</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>150</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>150</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>112.5</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>75</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

Western Blot to establish phosphorylation state of BL Src: Colorimetric AP Visualization

Reactions 1-3 contained BL Src 1, MBP-CSK, and ATP in various concentrations. Below, Table 3 shows the concentrations of reagents used in each reaction. 15 μL samples were added to 5 μL loading dye at time points 5 min and 2 hours. An SDS-Page gel was performed, and then the protein was transferred to nitrocellulose membranes. The primary antibody used was Phospho-Src Family (Tyr527) Rabbit Ab (Cell Signaling Technology). The secondary antibody used was Anti-Rabbit IgG, AP conjugate (Promega). Colorimetric AP detection (BCIP/NBT) was used to visualize the Western Blot.

Table 3: Concentrations of reagents used in Src western blot

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>[BL Src 1] (μM)</th>
<th>[MBP-CSK] (mg/mL)</th>
<th>[ATP] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>0.1</td>
<td>100</td>
</tr>
</tbody>
</table>
Optimization of CSK phosphorylation of Src kinase: Colorimetric AP Visualization

BL Src 1, MgCl2, ATP, and CSK were combined in various concentrations for each reaction (Table 4). 30 μL samples were added to 10 μL of loading dye, with timepoints 30 min and 1.5 hours. The primary antibody used was Phospho-Src Family (Tyr527) Rabbit Ab. The secondary antibody used was Anti-Rabbit IgG, AP conjugate. Colorimetric AP detection (BCIP/NBT) was used to visualize the Western Blot.

**Table 4: Concentrations of reagents used in CSK phosphorylation optimization**

<table>
<thead>
<tr>
<th>Reaction #</th>
<th>[BL Src 1] (μM)</th>
<th>[MgCl2] (mM)</th>
<th>[ATP] (mM)</th>
<th>[CSK] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>20</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>20</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>20</td>
<td>5</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>20</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>20</td>
<td>5</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Western Blot to establish phosphorylation state of BL Src: ScanLater Visualization

The ScanLater method used time-resolved fluorescence to visualize western blots. The online protocol was followed (Molecular Devices). The primary antibodies used were Phospho-Src Family (Tyr416) Rabbit Ab and Phospho-Src Family (Tyr527) Rabbit Ab. The secondary anti-rabbit antibody was labelled with Europium. To visualize the secondary antibody, the membrane was scanned using the SpectraMax i3 (Molecular Devices), setting the excitation wavelength to 340/80 nm excitation.
wavelength and the emission wavelength to 616/10 nm. A “yellow hot” filter was added to visualize the bands.

*Use of HPLC to quantify of substrate phosphorylation by Src*

Src optimal peptide substrate (SOPS, Anaspec) phosphorylation by the Src constructs was used to quantify their catalytic activity. 5 mM ATP, 20 mM MgCl2, and 0.01 μM Src were added to a buffer composed of 5 mM HEPES, 500 mM NaCl, and 1 mM TCEP and adjusted to pH 8. The reaction was allowed to equilibrate for 5 minutes at room temperature prior to the addition of 2 mM SOPS. Measurements were taken at 1 minute intervals for 11 minutes, as follows. 5 μL samples were removed and combined with 10 μL 6% TCA to stop the reaction. HPLC was used to quantify the reaction progress, by the amount of phosphorylated and unphosphorylated SOPS.

*Quantification of phosphorylation of substrate by Src using NADH-ATP Coupled Assay*

In a 96 well half-area plate (Fisher Science), 4 mM PEP, 0.45 mM NADH, 2.27 μM BSA, and 2.5 μL premade PK/LDH, and 2.5 mM ATP were combined into Kinase Reaction Buffer (10X) (Omnia). Serial dilutions were used to make stock concentrations of Src constructs. Various concentrations of Src constructs were added to the wells. The reagents and Src were allowed to equilibrate in the plate reader until there was an unchanging baseline measurement. To begin the reaction, a multichannel pipette was used to add 2 mM SOPS to the wells. Alternatively, SOPS was part of the reaction mixture, and ATP was added to begin the reaction. The mixture was pipetted up and down 5 times, avoiding bubbles, and then the plate was loaded into the plate reader.
Trial 1 was performed at 25°C in a Tecan plate reader and Trial 2 was performed at 30°C in a SpectraMax i3 (Molecular Devices). The plate was shaken for 10 sec prior to reading. The absorbance wavelength was set to 340 for NADH.

**Nuclear Magnetic Resonance Spectroscopy**

200-500 µM Src construct, 10% D₂O, and 4% sodium azide, were combined in a buffer composed of 100 mM ADA and 2 mM TCEP, adjusted to pH 6.5. Measurements were collected on an Avance 800 MHz (Bruker) at 25°C and subsequently processed with NMRPipe². The ¹⁵N-¹H-TROSY-HSQC spectra were assigned and analyzed using CcpNmr Analysis version 2.4.2³. SH3/SH2 1 was assigned based on similarity to an assignment of chicken pp60 c-Src⁴ (Y. Sun, unpublished results). BL Src 1, SH3/SH2 2, and BL Src 2 peaks were assigned based on similarity to the spectra of SH3/SH2 1.
RESULTS

Designing BL Src Constructs

Figure 4: Performing the Sortase ligation reaction on SH3/SH2 and SrcK fragments produce BL Src 1 and 2 constructs attained by ligating SH3/SH2 and SrcK.
(A) Sortase Ligation using SH3/SH2 (magenta) and kinase domains (blue) (Same as in Figure 1). Sortase A enzyme is represented as a green rectangle. 6X-His tags are represented by the purple circles. The product formed is BL Src. The slashes represent the locations where the peptide bond is cleaved. The starred peptide diffuses out of the dialysis chamber, driving the reaction forward. (B) Representation of c-Src showing where the insertion (BL Src 1) or mutation (BL Src 2) of the LPETG recognition site is placed and therefore where the sequence is split for partial isotopic-labeling (red line). The arrows indicate the location in the sequence of BL Src where there is a change from FL Src. The amino acid differences between the sequences are red.
BL Src constructs were prepared by using Sortase A to ligate two protein fragments together, one containing the SH3, SH2, and SH4 domains, and the other containing the kinase domain (Figures 1 and 4A). Two Sortase-ligated constructs were designed, BL Src 1 which was composed of Sortase-ligated SH3/SH2 1 and SrcK 1 fragments; and BL Src 2 which was composed of Sortase-ligated SH3/SH2 2 and SrcK 2 fragments (Figure 4B). BL Src 1 was designed so that a Sortase recognition motif (LPETGG) was linked to a 6X-His affinity tag at the C-terminus of the SH3/SH2 1 fragment, which will result in the final BL Src 1 having a 6 amino acid insertion at position 178 (by FL Src numbering) (Figure 4B). BL Src 2 was designed so that a Sortase recognition motif (LPETGG) was linked to a 6X-His affinity tag at the C-terminus of the SH3/SH2 2 fragment, which will result in the final BL Src 2 having a 6 amino acid substitution at position 161 (by FL Src numbering). Substituted Src (Sub Src) constructs were designed to have the sequence of the back-ligated constructs after the ligation reaction. That is, sub Src 1 has the same sequence as BL Src 1, but it was expressed as a full-length protein rather than having been passed through the Sortase reaction (same for Sub Src 2 and BL Src 2).

For Sortase reactions endogenous to *Staphylococcal aureus*, the poly-G repeat is composed of 5 glycine residues, however, if the site is exposed and flexible, fewer glycine residues are necessary\textsuperscript{13,14}. For both fragments, there is a 6X-His affinity tag attached to the glycine of the recognition sequence. In the SrcK fragments, there were single glycine residues already in the sequences, so another was inserted adjacent in both SrcK 1 and SrcK 2. Because the repeat is located in the flexible linker for both SrcK 1 and SrcK 2, the Sortase reaction should still be effective. To drive the reaction
forward, we planned to dialyze the reaction against its reaction buffer so that the "G-6X-His affinity tag" fragment that is cleaved off in the first step of the Sortase reaction and subsequently diffuses out of the dialysis cartridge (Figure 4B). With this in mind, I set out to produce the BL constructs, determine their activity, and attain NMR spectra.

Optimization of Sortase Ligation Reaction

**Figure 5:** Sortase ligation optimization trials (Table 2) demonstrate the optimal ratios of reagents. (A) Coomassie blue stained 10-12% TRIS SDS-Page gel for Trial 1 (concentrations of SrcK 1, SH3/SH2 1, and Sortase: 10 μM, 100 μM, 50 μM) and Trial 2 (10 μM, 100 μM, 20 μM). (B) Coomassie blue stained 10-12% TRIS SDS-Page gel for Trials 3-6. Concentrations of SrcK 1, SH3/SH2 1, and Sortase 1 were as follows: 15 μM, 150 μM, 30 μM for Trial 3; 15 μM, 150 μM, 15 μM for Trial 4; 15 μM, 150 μM, 17.5 μM for Trial 5; and 15 μM, 150 μM, 3 μM for Trial 6. The black box covers a lane that was misloaded and is not discussed. (C) Coomassie blue stained 10-12% TRIS SDS-Page gel for Trials 7-11. Concentrations of SrcK 1, SH3/SH2 1, and Sortase were as follows: 15 μM, 150 μM, 30 μM for Trial 7; 15 μM, 112.5 μM, 30 μM for Trial 8; 15 μM, 75 μM, 30 μM for Trial 9; 15 μM, 30 μM, 30 μM for Trial 10; and 15 μM, 15 μM, 30 μM for Trial 11. All reactions were run at room temperature. All time points are written in parentheses.
To efficiently produce BL Src, it was necessary to determine the ratio of SrcK to SH3/SH2 to Sortase that would produce the most BL Src. Because the optimization required several trials, I decided to run the reactions at room temperature so it would not take as much time. Therefore, it was necessary to determine the time required for the reaction to reach completion. For Trials 1 and 2, we varied the Sortase concentration and the time (Figure 5A). Based on the intensity of the bands in the Coomassie stained gel, the most product was formed in Trial 2 at 2 and 4 hours. I concluded that the reaction reached completion after approximately 2 hours at room temperature, and proceeded to use this as the reaction duration for the following trials.

After establishing the proper reaction duration, I wanted to determine the optimal Sortase A enzyme concentration. Therefore, for Trials 3-6, I used 150 μM SH3/SH2 1 and I varied the concentration of the Sortase (Figure 5B). Based on the intensity of the bands in the Coomassie stained gel, the most BL Src 1 was formed for Trial 3, which indicated that the optimal Sortase concentration was 30 μM. Once the optimal Sortase concentration had been ascertained, it was necessary to determine the optimal concentration of SH3/SH2 1 fragment. For Trials 7-11, I used 30 μM Sortase and varied the concentration of SH3/SH2 1 (Figure 5C). The most intense product band was formed in Trial 7 (150 μM SH3/SH2 1), closely followed by the product band in Trial 10 (30 μM SH3/SH2 1). All of the BL Src 1 bands are very similar in intensity, although there are very different SH3/SH2 1 concentrations. This suggests that the Sortase concentration is a more important factor in the efficiency of the reaction than SH3/SH2 1 concentration. This experiment allowed for identification of the optimal ratio of reagents that should be used to perform the Sortase ligation reaction: 15 μM SrcK 1, 150 μM
SH3/SH2 1, 30 μM Sortase (or a ratio of 1:10:3). The BL Src used for research in the remainder of my thesis was produced using optimal reagent concentrations, was run for 3.5 days at 4°C to prevent protein denaturation.

**CSK Phosphorylated State of BL Src**

![Figure 6: After expression, purification, and ligation, Y527 in BL Src 1 is not fully phosphorylated.](image)

We hypothesized that after expression and purification of BL Src 1, the protein would be unphosphorylated. To test this, an anti-phospho-Y527 primary antibody western blot visualized with AP was performed on BL Src 1 (Figure 6). I decided to compare untreated BL Src 1 alone and BL Src 1 treated with ATP to BL Src 1 treated
with ATP and CSK. When BL Src 1 is treated with ATP and CSK, it will be fully phosphorylated at the Y527 position. Comparing BL Src 1 alone to pY527 BL Src 1 will allow us to determine whether or not BL Src 1 is phosphorylated. Comparing BL Src 1 with ATP to pY527 BL Src 1 will allow us to confirm that CSK is necessary to phosphorylate Y527 (i.e., autophosphorylation does not occur). The product bands representing pY527 BL Src 1 were all approximately the same intensity after 5 minutes (Figure 6A). However, after 2 hours, the band for Reaction 3 (2 hours) was more intense while the other two bands remained at approximately the same intensity (Figure 6B). I concluded that Reaction 1 and Reaction 2 (both after 2 hours) do not contain fully phosphorylated Y527 BL Src 1. In this experiment, a negative control was not performed. Therefore, we can conclude that the purified BL Src 1 is less phosphorylated.

**Figure 7:** The AP visualization system does not accurately detect differences in the amount of phospho-proteins. (A) Coomassie blue stained 10-12% TRIS SDS-Page gel of reactions 1-7. Reaction 1 was composed of 2 μM BL Src 1 and 20 mM MgCl₂. Reaction 2 was composed of 2 μM BL Src 1, 5 mM ATP, and 20 mM MgCl₂. Reaction 3 was composed of 2 μM BL Src 1, 500 μM ATP, and 20 mM MgCl₂. Reaction 4 was composed of 2 μM BL Src 1, 5 mM ATP, 2 μM CSK, and 20 mM MgCl₂. Reaction 5 was composed of 2 μM BL Src 1, 5 mM ATP, 0.2 μM CSK, and 20 mM MgCl₂. Reaction 6 was composed of 2 μM BL Src 1, 15 mM ATP, 0.02 μM CSK, and 20 mM MgCl₂. Reaction 7 was composed of 2 μM BL Src 1, 5 mM ATP, 0.004 μM CSK, and 20 mM MgCl₂. 30 μL samples were added to 10 μL of loading dye, with timepoints 30 min and 1.5 hours. (B) Western blot using an anti-phospho-Y527 rabbit primary antibody and AP visualization, Reactions 1-7, timepoints at 30 min and 1.5 hr.
than the fully-phosphorylated BL Src 1 at tyrosine 527, however we cannot be certain that BL Src 1 is completely unphosphorylated. We can also conclude that BL Src 1 does not undergo significant autophosphorylation. Additionally, the experiment demonstrates that the CSK phosphorylation reaction is mostly completed after 2 hours. This experiment demonstrated that after expression, purification, and ligation, the Y527 site on BL Src 1 is not fully phosphorylated.

After assessing the phosphorylation state of BL Src 1, I attempted to optimize the CSK phosphorylation of tyrosine 527, by varying concentrations of CSK and ATP incubated with BL Src 1. As in Figure 6, a western blot was performed using anti-phospho-Y527 primary antibody and AP visualization; BL Src 1 alone and BL Src 1 plus ATP were included as negative controls (Figure 7). However, the result shown in Figure 2 was not reproducible. The former was a control to account for any background signal due to unspecific binding of antibody. Regardless of the reaction composition, the BL

![Figure 8: ScanLater Western blot assay demonstrates that at sites Y416 and Y527, BL Src 2 and FL Src remain unphosphorylated after expression and purification.](image)
Src 1 bands were all approximately the same intensity as the negative controls. One possible explanation is that the AP visualization system was not able to accurately detect the differences in the amounts of phosphoproteins.

To attempt to solve this problem, I performed additional western blots using a different visualization method. The blots were similar to those in Figure 7, however the visualization technique used was time-resolved phosphorescence (ScanLater) and an anti-phospho-Y416 Ab was used in addition to the anti-phospho-Y572 Ab used earlier (Figures 8B and 8C). FL Src, which is not phosphorylated after expression and purification, was used as a negative control. For both of the western blots shown in

![Figure 9: After ATP and CSK incubation, Back-ligated Src 2 and Full-length Src are phosphorylated at site Y527, but not significantly at Y416. (A) Coomassie blue stained 10-12% TRIS SDS-Page gel of pBL Src 2 and pFL Src. (B) Western blot of pBL Src 2 and pFL Src using an anti-phospho-Y416 rabbit primary antibody and an anti-rabbit ScanLater secondary antibody. (C) Western blot of pBL Src 2 and pFL Src using an anti-phospho-Y527 primary rabbit antibody and an anti-rabbit ScanLater secondary antibody.](image-url)
Figure 4, there were either no or very light bands corresponding to BL Src 2 and FL Src. The western blots were repeated for BL Src 2 and FL Src after incubation with CSK and ATP (Figures 9B and 9C). In the pY527 western, FL Src and BL Src 2 product bands were of similar dark intensity. In the pY416 western, faint bands were visible for both FL Src and BL Src 2, as opposed the lack of corresponding bands when no CSK and ATP were present (Figure 8B). It is possible that a tiny fraction of FL Src and BL Src 2 have both tyrosine residues phosphorylated. Based on the similarity in intensity, it seems that there is the same fraction of pY416 for both BL Src 2 and FL Src, so the constructs are still comparable.

The Coomassie blue stained gels of the BL Src 2 preparation show that there is no detectable contamination by unligated SH3/SH2 or SrcK 2 fragments (Figure 8A). Interestingly, after CSK and ATP incubation, SrcK 2 bands appear in the BL Src 2 lanes on both pY416 and pY527 westerns (Figure 9B and 9C). This indicates that unligated

![Graph A](image1)

**Figure 10:** Using HPLC quantification, Full-length Src and Back-ligated Src 1 phosphorylated Src optimal peptide substrate (SOPS) at similar rates compared to Src kinase 1. (A) Assays measuring the phosphorylation of SOPS by SrcK 1 (blue diamonds), BL Src 1 (orange squares), and FL Src (grey triangle) at room temperature, quantified by HPLC. (B) Calculated rates and standard deviations (N=3) of SOPS phosphorylation by SrcK 1, BL Src1, and FL Src.
SrcK 2 is present in amounts too small to see in the Coomassie blue stain, but is detectable in western blots when it is phosphorylated. Alternatively, this band could reflect a reversal of the ligation or proteolysis that occurring during CSK+ATP treatment.

**Activity of BL Src Constructs**

We next wanted to check that the kinase activity of BL Src 1 mimicked that of FL Src. I first used HPLC to measure the ability of BL Src 1, FL Src, and SrcK 1 to phosphorylate Src optimal peptide substrate (SOPS). I chose to also test SrcK 1 because it lacks regulatory domains and therefore behaves as if it is the FL Src construct in a completely open confirmation. The rates of SrcK 1, BL Src 1, and FL Src, were found respectively to be $31.37 \pm 2.92 \text{ s}^{-1}$, $18.41 \pm 1.10 \text{ s}^{-1}$, and $14.58 \pm 1.77 \text{ s}^{-1}$ (Figure 10). This indicates that the BL Src 1 has activity more similar to FL Src than SrcK 1. Therefore, BL Src 1 is being regulated by the ligated SH3/SH2 domains.

Because this method of analysis (HPLC) was time consuming and somewhat unreliable, we repeated these Src phosphorylation rate of SOPS measurements using NADH-ATP coupled assays. In Trial 1 (25°C), FL Src was found to have a rate of $6 \pm 2 \text{ s}^{-1}$, and pFL Src to have rate of $0.21 \pm 0.01 \text{ s}^{-1}$, both at 2 mM SOPS (A. Temesgen and R. Agafonov, unpublished results) (See Figure 11C). In Trial 2 (30°C), FL Src was found to have activity $10.3 \pm 0.7 \text{ s}^{-1}$, and pFL Src to have rate $0.48 \pm 0.01 \text{ s}^{-1}$, both at 2 mM SOPS (C. Wilson and S. Gelles-Watnick, unpublished results). BL Src 1 to have activity of $14.7 \pm 1.6 \text{ s}^{-1}$, and pBL Src 1 to have a rate of $1.05 \pm 0.50 \text{ s}^{-1}$, both at 2 mM SOPS (C. Wilson, unpublished results). The activity of BL Src 2 was found to be $12.0 \pm 1.1 \text{ s}^{-1}$ and the activity of pBL Src 2 was found to be $1.01 \pm 0.03 \text{ s}^{-1}$, both at 2 mM SOPS.
(Figure 11B). The Trial 2 activity ratios of Y416Y527 (unphosphorylated) Src to Y416pY527 (phosphorylated) Src for FL Src, BL Src 1, and BL Src 2 were as follows: 23.6 ± 1.4, 11.8 ± 1.5, and 12.4 ± 0.8, respectively (Figure 11D). The approximately 2-
fold increase in activity between Trials 1 and 2 for both the FL Src and pY527 FL Src activities may be explained by the difference in temperatures (25°C and 30°C, respectively) at which the experiments were conducted.

The SOPS phosphorylation activities of BL Src 1, BL Src 2, and FL Src measured at 30°C were compared. For both BL Src 1 and BL Src 2, the unphosphorylated and phosphorylated versions have higher rates than the unphosphorylated and phosphorylated versions FL Src. Also, both BL Src 1 and BL Src 2 activity ratios of unphosphorylated Src to phosphorylated Src rate are smaller than for FL Src (Figure 11D). Because both forms of back-ligated Src are more active, we hypothesize that the modified linker alters the domain interactions such that there is less regulation. I propose that in the BL Src, either (a) there is a larger portion of protein in

![Figure 12: By NADH-ATP coupled assay, Full-length Src and Substituted Src 2 have similar kinase rates, while Back-ligated Src 2 has a 3-fold lower rate. (A) NADH-ATP coupled assay measuring the phosphorylation of 2 mM SOPS by 24.3 μM FL Src at 33°C. (B) NADH-ATP coupled assay measuring the phosphorylation of 2 mM SOPS by 18.3 μM BL Src 2 at 33°C. (C) NADH-ATP coupled assay measuring the phosphorylation of 2 mM SOPS by 31 μM Sub Src 2 at 33°C. (D) SOPS phosphorylation rates by FL Src, BL Src 2, and Sub Src 2, at 2 mM SOPS and 33°C.](image-url)
the open conformation or (b) the closed version of the protein is more accessible to the substrate so the reaction can proceed. Another possibility is that the Sortase reaction itself causes changes to the interactions between the regulatory and catalytic domains of BL Src that alters its kinase activity.

To check that the Sortase reaction itself did not cause the change from FL Src to BL Src 1 and BL Src 2 activity, we decided to compare the activity of FL Src, BL Src 2, and Sub Src 2 (Figure 12). These measurements were performed at 33°C. The rate of FL Src was found to be 28.5 ± 2.69 s⁻¹. The rate of BL Src 2 was found to be 9.19 ± 2.09 s⁻¹. The rate of Sub Src 2 was found to be 29.26 ± 2.11 s⁻¹.

The rates of FL Src and Sub Src 2 are the same within error. Surprisingly, the phosphorylation rate of BL Src 2 was approximately 3-fold smaller, even though it has the same amino acid sequence as Sub Src 2. The difference in activity between BL Src 2 and Sub Src 2 raises the possibility that some of the protein might be damaged or denatured during over the Sortase Ligation process. One of the important issues with BL Src is that it takes a much longer time to prepare and purify than does Sub Src 2 or FL Src (see Discussion).

**NMR on BL Src constructs**

After optimizing the Sortase ligation reaction, determining the phosphorylation state of BL Src, and demonstrating its activity, I then produced ¹⁵N-labeled SH3/SH2 1 and ¹⁵N-labeled SH3/SH2 2 fragments, then used Sortase A to ligate them to the corresponding unlabeled SrcK fragments. ¹⁵N-¹H-TROSY-HSQC spectra were taken of
these partially-labeled BL Src constructs and then assigned based on similarity to the SH3/SH2 1 assignment (Y. Sun, unpublished results).

NMR was used to determine whether the interactions between the regulatory and catalytic domains were present for the BL Src constructs. Spectra were taken of the
back ligated constructs with only the SH3/SH2 fragment $^{15}$N-labeled and corresponding $^{15}$N-labeled SH3/SH2 fragment (Figure 13A and 14A). The SH3/SH2 1 fragments alone behaves as the completely open conformation (i.e., it is all in this conformation) of the FL Src, because its regulatory domains cannot bind to anything and instead just tumble in solution. If BL Src has the same regulatory interactions as FL Src, part of the population should be closed and part should be open, which would shift some BL Src peaks away from the completely open position (like the peaks for SH3/SH2 fragment). Therefore, I expected that there would be many peak shifts between the SH3/SH2 fragment and the BL Src spectra. From an overlay of the spectra of BL Src 1 and SH3/SH2 1, it is ascertained that the peaks in the BL Src 1 and SH3/SH2 1 spectra match up very well (Figure 13). Therefore, I conclude that BL Src 1 does not have interactions between the regulatory and catalytic domains that are present in FL Src.

There are some small shifts between the spectra, which can be viewed on the structure (See Figure 13B) to be localized to a specific location on the SH3/SH2 domains. This is most likely because in the SH3/SH2 1 fragment, there is the insertion of the Sortase recognition motif (LPTETG) and in the SH3/SH2 domain of BL Src 1 there is the connection to the SrcK domain. Consistent with this interpretation, most of the shift differences are localized to the region of the Sortase recognition sequence insertion (Figures 13B and 13C). Based on these data, it appears that BL Src 1 does not accurately model the conformational equilibrium of FL Src.

The same NMR analysis was performed on SH3/SH2 2 and BL Src 2. In contrast to the results for BL Src 1, there were large shifts in the majority of peaks between
SH3/SH2 2 and BL Src 2 (Figure 14A and 14B). Figures 14B and 14C demonstrate that the shifts are not localized to a specific region. Instead, they are widely distributed, including locations where in the closed conformation, there would be interactions between the regulatory domains and the catalytic domains. Thus, the spectra are

**Figure 14:** $^{15}$N-$^1$H TROESY-HSQC NMR analysis reveals significant differences between BL Src 2 with $^{15}$N-labeled SH3/SH2 domain and $^{15}$N-SH3/SH2 2 fragment. (A) NMR spectra overlay of BL Src 2 (purple peaks) and SH3/SH2 1 (green peaks) taken at 25°C. (B) Shift differences between BL Src 2 and SH3/SH2 2 for each amino acid presented as a bar graph. (C) Shift differences superimposed onto the crystal structure of Src (PDB: 2SRC). SH3 and SH2 domains are shown as ribbons; the kinase domain is shown as a surface.

- **A**
- **B**
- **C**

- 0.035-0.100 ppm chemical shift
- shifted to unknown location
- or = Missing assignment, or unclear
consistent with the hypothesis that BL Src 2 has interactions between the catalytic and regulatory domains, like those in FL Src.
DISCUSSION

Analysis of FL Src by NMR has been complicated by its large size. The Kern Lab conceived of using the Sortase ligation method to partially isotopically label Src. In this thesis, I describe the design and purification of BL Src 1 and BL Src 2, two Sortase-ligated constructs. I determined that the BL Src constructs are unphosphorylated after expression, ligation, and purification. We measured the catalytic activity of BL Src, however the measurements were not found to be reproducible. NMR spectra overlays of the BL Src constructs and the SH3/SH2 fragments demonstrated that BL Src 2 has interactions between regulatory and catalytic domains, while BL Src 1 does not. From these results, I conclude that point mutations in the flexible linker do not interfere with the interactions between the regulatory and catalytic domains, however, lengthening the linker does.

Activity assays that quantified rates of SOPS phosphorylation by BL Src produced inconsistent results. This may be caused by the instability of BL Src coupled with the length of time required for its purification. Purifying the SrcK fragment requires 4 FPLC columns, and can take 3-5 days. Purifying the SH3/SH2 fragment requires 2 columns, and takes 1-3 days. It takes 3-5 days to run the ligation reaction. Finally, purifying the back-ligated product takes 2 columns, and 1-3 days. In total, the time required is from 9-15 days or longer. Purification of different batches of Src took place over variable amounts of time, and several batches of BL Src were used in our coupled
assays. Our inconsistent results, therefore, may be the result of Src deterioration during the purification process.

These experiments demonstrate the utility of Sortase ligation in isotopically labeling parts of large proteins for analysis by NMR. However, care must be taken in placement of the Sortase recognition sequence to preserve protein activity because modification of the sequence may alter the function of the protein. The recognition sequence should be engineered into an exposed region of the protein and a region that does not play an important role in the regulation or activity under study. An example of such a region is the linker of Src. Furthermore, the protein function must be carefully assessed to determine whether its activity is preserved after modification and ligation.

This work suggests a series of future experiments. Because NMR spectra demonstrated that BL Src 2 most closely models FL Src, BL Src 2 should be used for future studies. To determine the open and closed populations of Src in each phosphorylation state, NMR must be performed on pY416pY527 BL Src 2 and pY416Y527 BL Src 2. We also plan to perform NMR for the duration of CSK and autophosphorylation reactions on BL Src 2. The Kern Lab will also use single molecule FRET to observe the opening and closing of BL Src in real time. When these experiments are completed, it is our expectation that these results will make an important contribution to understanding the regulatory mechanism of c-Src.
WORKS CITED


APPENDIX

Apexdx 1: FL Src construct (Kanamycin resistance)
MKHHHHHHPMKIEEGKLVIWINGDKYNGLAEVGKKEKDTGKVTVEHPDKEEKFPQVAATGDGPDIIIFWAHDFGGGAQSSGLAEITPDKAFQDKLYPFTWDARVYNGKLIAYPIAVEALS1YKDDNLPPPKEYAIPALDKELKAKGKSALMFNLQEPYFTPWPLAADDGGYAFKYENGKDYIKDVKVAGNAKAGLTLVDDIKNKHMNAATDYSIAEAAFNKGETAMTIMGWAWSNIDSKVNYGTVLPFTFJKQPSKFPFVGLSAGINAAASPKELAKEFLENYLLTDEGLEAVNNDGPLAGAVLKSYESELAKDRPRAATMENAQKGEIMPENIQMSAFWYAVRTAVINASGRQTVDEALKDAGTNSSSSNNNNNNNNNNNPMENSELYFGQAMAVTTFVAYDYESRTTEDLSFKKGERLQIVNNTEGDWWLWALSHLSTGQTGYIPSLOYEEAPSDESQAAEQEYFGIKITRRERELLNLNENPRGTFLVERSETTTKGAYCLSVDNDAKGLNHVHYKIRKLDGGFYITSQFNSQLQVLVAAYSHADGHRLLTTPSKPTQQLGLAKDAEWIPRESLRLEVKKLGQGCFEWMGTNGTWRVAIKTLKPGTMSPEAFLEAQAVMKKLHHEKLVQLYAVVSEEPIYVTEYMSKGLDLLFLKGETGKYRLPQLVDMAAQISGMAYVERMINYVHRDLRAAENLVGENLCKVADFLARLIEDNEYTAGGAKFPIKWTAPAALYGRFTIKSDWVSGILLTGLTOKGRVYPGVMNVENQLDQERVERGMRMPCPPECPELGHDLMCQCWKEPEERPTFEYLQAFLEYFSTEPQYQPGENL

Composed of:
- His-MBP tag
- TEV site
- Src-FL sequence
- pETM-41 vector

Apexdx 2: YOPH construct (Streptomycin resistance)
pCDFDuet-1 vector

Apexdx 3: CSK construct (kanamycin resistance)
HHHHHHHPMLVPRGSMSAIQAAWPSGTCEIAKYNFHGTAEQDLPFCGKDVTLITAVTKDPSNWYAKAKNVGREGIIPANYVQKREGVKAGTкалслслсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмсмs

35
Molecular weight: 52,365.2 Da
Extinction coefficient (280 nm): 74425

**Appendix 4: SH3/SH2 1 construct** (ampicillin resistance)
MVTTFVALYDYESRTETDLSFKGERLQIVNNTEGDWLHSLSTGQTGYIPSNYVAP
SDSIQAEWWFYGKTRRESERLLNAENPRGTFLVRESETTKGAYCLSVDNDNAKGL
NVKHYKLDSGSGFYITSRTQFNSLQLVAYYSKADGLCHRLTTVCPTSKPQTGQL
AKDALPETGGHHHHHG

Highlighted = removed during sortase ligation
Underlined = changed from Wt

Contains a His tag.

**Appendix 5: SrcK 1 construct** (amp resistance)
MAGSHHHHHHGMASMTGGQOMGRSGDDDDKELYFOGGWEIPRESLRLERKLGQ
GCFGEVWMGTVNTGTTVAIKTLKPGTMSPEAQVMMKLHREKLVQLYAVVSEEP
PIYIVTEMSKGLDFLKGETGYLRPQLLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADVFLARLIEDNEYTARQGAKFPWIKWTAPEAALYGRFTIKSDVWSFGLLDTELETGRPVYPMVREVLQVERGYRMPCEPCELQSHDLMCQCWARKEPEERTFEYLQAFLEDYFTSTEPQYPQGEL

Highlighted = removed during sortase ligation

Contains a MBP tag and a His tag.

**Appendix 6: BL Src 1 sequence** (post-ligation)
MVTTFVALYDYESRTETDLSFKGERLQIVNNTEGDWLHSLSTGQTGYIPSNYVAP
SDSIQAEWWFYGKTRRESERLLNAENPRGTFLVRESETTKGAYCLSVDNDNAKGL
NVKHYKLDSGSGFYITSRTQFNSLQLVAYYSKADGLCHRLTTVCPTSKPQTGQL
AKDALPETGGEIPRSELRLERKLGQGCFGEVWMGTVNTGTTVAIKTLKPGTMSPE
FLQEAMKKLRHEKLVQLYAVVSEEPYIVYTEMSKGLDFLKGETGYLRPQLLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADVFLARLIEDNEYTARQGAKFPWIKWTAPEAALYGRFTIKSDVWSFGLLDTELETGRPVYPMVREVLQVERGYRMPCEPCELQSHDLMCQCWARKEPEERTFEYLQAFLEDYFTSTEPQYPQGEL

Underlined = changed from Wt

**Appendix 7: Sub Src 1 construct**
MVTTFVALYDYESRTETDLSFKGERLQIVNNTEGDWLHSLSTGQTGYIPSNYVAP
SDSIQAEWWFYGKTRRESERLLNAENPRGTFLVRESETTKGAYCLSVDNDNAKGL
NVKHYKLDSGSGFYITSRTQFNSLQLVAYYSKADGLCHRLTTVCPTSKPQTGQL
AKDALPETGGEIPRSELRLERKLGQGCFGEVWMGTVNTGTTVAIKTLKPGTMSPE
FLQEAMKKLRHEKLVQLYAVVSEEPYIVYTEMSKGLDFLKGETGYLRPQLLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADVFLARLIEDNEYTARQGAK
FPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLDQVERGYRM
PCPPECPESLHDLMCQCWRKEPEERPTFEYLQAFLEDYFTSTEPQYQPGENL

Underlined = changed from WT

Contains a his tag and a MBP tag.

**Appendix 8: SH3/SH2 2 construct** (ampicillin resistance)
MVTTFVALDYESRTETDLFSFKGERLQIVVNTEGDWWLASHSLSTGQTGYIPS
Y VAPSIDIQAEWYFGKTRRESERLLLNAENPRGTFLVRESETTKGAYCLSVSDFDNA
KG
LNVHYKIRKLDGSGFYITSRTQNSLQQLVAYYSHADGLCHRLTLPETGH

Highlighted = removed during sortase ligation
Underlined = changed from WT

Contains 6X-His tag

**Appendix 9: SrcK 2 construct** (ampicillin resistance)
GDGGDKENLYFGGKQPTQGLAKDAEIPRESLRLEVKLGQGCGFEGVWMGTWNGT
TRVAI
KTLKPGTMSPEAFLQEAQVMKLRHEKLVLQLYAVVSEPIYIVTEYMSKGLLDFLKGE
T
GKYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCVKVADFGLARLIE
DN
EYTARQGAKFPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLD
Q
VERGYRMPCPPECPESLHDLMCQCWRKEPEERPTFEYLQAFLEDYFTSTEPQYQPGENL

Highlighted = removed during sortase ligation

Contains 6X-His tag and MBP tag.

**Appendix 10: BL Src 2** (post-ligation)
MVTTFVALDYESRTETDLFSFKGERLQIVVNTEGDWWLASHSLSTGQTGYIPS
Y VAPSIDIQAEWYFGKTRRESERLLLNAENPRGTFLVRESETTKGAYCLSVSDFDNA
KG
LNVHYKIRKLDGSGFYITSRTQNSLQQLVAYYSHADGLCHRLTLPET
GGKPTQGLAKDAEIPRESLRLEVKLGQGCGFEGVWMGTWNGTTRVAI
KTLKPGTMSPEAFLQEAQVMKLRHEKLVLQLYAVVSEPIYIVTEYMSKGLLDFLKGE
T
GKYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCVKVADFGLARLIE
DN
EYTARQGAKFPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGMVNREVLD
Q

37
Appendix 11: Sub Src 2 construct
MVTTFVALYDYESRTETDL5FKKERLQIVNNTEDDWWLAHSLSTGQTGYPNSNY
VAPSDSIQEEWYFGKITRRESERLLLNAENPRGTFLVRESEETTKGAYCLSVSDFDNAG
KN
LNVKHYKIRKLDSSGFYIITSRTQFNSLQLVAYYSKHADGLCHRLELPET
GGKPQTPQGLAKDAWEIPRESLRLEVKLGQGCFGEVMGTWNGTTRVAIG
KTLKPGTMSPEAFLQEAQVMKLRHEKLVLQYAVVSEEPYIVTEYMSKGSLLDFLGKET
GKYRNLQVLVMAAQSAGMYVVERMNYVHRDLRAANILVGENLVCKVADVFLARLIE
DN
EYTARQGAKFPIKWAPEAALYGRFTIKSDVWSFGLLTELTTKGRVPYPMVNREVLD
Q
VERGRMPCPCPESLHDLMCQCWRKEPEERPTFEYLQAFLEDYFTSTEPQYQPG
ENL

Appendix 12: Purification Yield from various proteins, expressed normally or

<table>
<thead>
<tr>
<th></th>
<th>TB Media (mg/L)</th>
<th>M9 Media (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH3/SH2 1</td>
<td>20</td>
<td>8.7</td>
</tr>
<tr>
<td>SrcK 1</td>
<td>1.6</td>
<td>n/a</td>
</tr>
<tr>
<td>SH3/SH2 2</td>
<td>3.9</td>
<td>3.6</td>
</tr>
<tr>
<td>SrcK 2</td>
<td>1.3</td>
<td>n/a</td>
</tr>
</tbody>
</table>

\[^{15}\text{N}-\text{labeled}\]
Appendix 13: SrcK 2 Purification results (A) Ni$^{2+}$ affinity column chromatogram and Coomassie blue stained SDS-Page gel of collected fractions. (B) Anion exchange column chromatogram and Coomassie blue stained SDS-Page gel of collected fractions. (C) Ni$^{2+}$ affinity column chromatogram and Coomassie blue stained SDS-Page gel of collected fractions. (D) Size exclusion column chromatogram and Coomassie blue stained SDS-Page gel of collected fractions.
Appendix 14: SH3/SH2 2 Purification results (A) Ni\(^{2+}\) affinity column chromatogram and Coomassie blue stained SDS-Page gel of collected fractions. (B) Size exclusion column chromatogram and Coomassie blue stained SDS-Page gel of collected fractions.
Appendix 15: Post-ligation BL Src 2 Purification results (A) Ni²⁺ affinity column chromatogram and Coomassie blue stained SDS-PAGE gel of collected fractions. Black box obstructs irrelevant samples. (B) Size exclusion column chromatogram and Coomassie blue stained SDS-PAGE gel of collected fractions.