The handle http://hdl.handle.net/1887/20326 holds various files of this Leiden University dissertation.

Author: Somireddy Venkata, Bharat Kumar Reddy
Title: Dynamics of protein-protein interactions studied by paramagnetic NMR spectroscopy
Date: 2012-12-20
Chapter 4

The interaction of focal adhesion kinase and Src: Expression of FAK kinase domain with SH3-SH2 binding sites in insect cells and characterization of its interaction with Src homology domains
Chapter 4

Abstract

Focal adhesion kinase (FAK) is an important tyrosine kinase involved in the focal adhesions and also in signal transductions. FAK binds to the Src tyrosine kinase, thereby inducing downstream signaling events. Here the cloning, expression and purification of the focal adhesion kinase domain with its SH3 and SH2 binding sites (32k) is described. Two constructs were made differing in the positioning of the His-tag, either C-terminal or N-terminal. After purification, the activity of the kinase domain was determined by western blot using anti pTyr 397 antibodies. The interaction between the Src and 32k was compared with that of Src domains and FAK derived peptides. Chemical shift of Src domains to the 32k at its binding motif, because the binding maps of SH3 for 32k and peptide are similar. Unlike the SH3 domain, the SH2 domain did not show any binding to the 32k. These finding suggests that the pTyr motif is not accessible for binding in 32k. With the peptide containing the phosphoTyr motif binding is very clear. These findings lead to a separate model stating that SH3 binding may precede the SH2-FAK interaction and act as the initiator of the FAK-Src interaction.
Introduction

In signal transduction the complex of Src and focal adhesion kinase (FAK) plays vital roles. FAK is essential in cell migration, regulation of the actin cytoskeleton, mechanosensation, migration, proliferation, cell-cell junctions, and regulation of microtubules.\textsuperscript{177} Src is also important in cell adhesions, by regulating the actin cytoskeleton\textsuperscript{111,178}. Both of these proteins are classified as non-receptor tyrosine kinases, which transfer phosphate groups from ATP to tyrosine residues. These proteins are modular, because they have multiple domains connected by linkers, like beads on a string. Autophosphorylation of Y397 activates FAK. The cause of FAK autophosphorylation is uncertain, however, it is believed that growth factors or integrins, when binding to the receptors of the extracellular matrix, induce the catalytic activity of FAK. After autophosphorylation FAK recruits Src and other signalling proteins\textsuperscript{179}. Substrates such as paxillin\textsuperscript{180} and p130Cas\textsuperscript{111} are phosphorylated by the activated FAK/Src complex and this ultimately leads to the re-organization of the actin cytoskeleton.

Biochemical studies have revealed that overexpression of FAK leads to the development of several invasive tumours\textsuperscript{181}. Structural information about the Src:FAK complex is required for a thorough understanding of how these important proteins carry out their function.

**Figure 4.1.** Schematic representation of FAK. The linker between the FERM domain and the kinase domain carries the polyproline region for SH3 binding and Y397 for SH2 binding. Figure adopted from\textsuperscript{179}. 
FAK is about 125 kDa in size and it contains a N-terminal “band 4.1, ezrin, radixin, moesin” (FERM) domain followed by a tyrosine kinase domain and a C-terminal Focal Adhesion Targeting (FAT) domain (Fig. 4.1). The FAT and FERM domains play roles in the recognition of other proteins, hence they are known as recognition domains. The kinase domain is the catalytic domain, which contains the ATP binding site. The region between the kinase domain and the FERM domain is called the linker region. This region has a stretch of amino acid residues that forms the binding motifs of Src domains SH2 and SH3. Residue Y397 in the linker region is believed to be responsible for binding of the Src SH2 domain. Phosphorylation of Y397 increases the affinity for binding the SH2 domain dramatically. The polyproline “PxxP” sequence in the linker region represents the motif for Src SH3 binding.

The kinase domain has a typical bilobal architecture, which is conserved among many protein kinase domains. The smaller N-terminal lobe contains a five-stranded antiparallel β sheet and a single α helix. The larger C-terminal lobe is mostly α helical and contains the activation loop involved in polypeptide substrate binding. Nucleotide ligands bind in-between the lobes (Fig. 4.2A).

A crystal structure of a large FAK fragment showed that the binding of the N-terminal FERM domain to the kinase domain blocks access to the catalytic cleft and sequesters Y397, preventing autophosphorylation and Src recruitment. This led to the proposition of a model for the activation of FAK in which proteins like β-integrins or growth factor receptors EGFR, PDGFR, c-Met, or EphA2 bind to the FERM domain, displacing it from the kinase domain and thereby activating it. The activated kinase domain and linker region then become available for binding of Src SH2 and SH3 domains, leading to phosphorylation of the residues Tyr 576 and Tyr 577 of FAK by the Src kinase (Fig. 4.2B).
Figure 4.2. A) Crystal structure of the FAK kinase domain representing the N and C terminal lobes with the bound nucleotide ligand (PDB entry 1MP8). B) Schematic representation of FAK activation model and its interaction with Src. In an intradomain interaction of FAK the FERM domain binds to the kinase domain, sequestering the PxxP and Y397 regions and inactivating the protein. When FAK partners bind to the FERM domain, it releases the kinase domain leading to autophosphorylation of Y397 in cis or trans. Src binds to FAK with the SH2 domain to the phosphorylated Y397 and the SH3 domain to the proline-rich region. Phosphorylation of tyrosines Y576 and Y577 in the FAK activation loop by Src leads to full activation of FAK and prevents inhibition by the FERM domain. Taken from ref. Copyright (2007), copied with permission from Elsevier.
Structures of individual domains of both FAK and Src have been successfully determined by X-ray crystallography and NMR spectroscopy. The structure of the FAK/Src protein complex has not been reported. The application of NMR spectroscopy can be suitable to study the structure and dynamics of this complex.

![Figure 4.3](image)

**Figure 4.3.** Schematic representation of two constructs made in our group. The kinase domain with the short linker region containing the SH2 binding site shown in A), the extension of the construct with SH3 binding site is shown in B).

Earlier studies by our research group comparing SH2 domain binding to a FAK peptide comprising the SH2 binding motif and to the FAK kinase domain plus SH2 binding site (2k, Fig. 4.3A), showed interesting results. The studies suggested that the presence of the kinase domain prevents the specific interaction between the pY397 and the SH2 domain. Apparently, the SH2 binds the kinase domain in a non-specific fashion. The complex of these proteins seems dynamic and electrostatic interactions could play a significant role in complex formation. The residues involved in the binding of phosphorylated 2k protein are spread out over the Src SH2 surface. The chemical shift perturbations for these residues are small and similar to those for binding the unphosphorylated FAK kinase domain. In contrast, the interaction between FAK derived peptides and the Src SH2 domain showed that the peptide binds very tightly to the protein. The chemical shift perturbations of the residues affected upon binding of the
peptide containing the pY are large and reasonably localised on the Src SH2 domain. In combination, these results suggest that pY397 is not available for Src SH2 binding in the 2k construct, perhaps because it is folded back into kinase domain.

It was also clear from the earlier studies by our research group\textsuperscript{185} that a peptide containing the binding motifs of both the SH3 and SH2 sites of FAK binds to the SH3 and SH2 domains of Src, respectively, as well as to the double domain Src SH32. From these studies, it was concluded that if the peptide contained both motifs, its binding affinity for SH3-SH2 domains was only marginally greater than that for the SH2 domain alone, suggesting some anti-cooperativity of the binding. When the length of the peptide between the SH3 and SH2 binding motifs in the peptide was decreased, it was observed that the peptide binds to two SH32 domains, thus acting as dimerization linker even when the number of amino acid residues between the SH3 and SH2 binding motifs was as small as three amino acids. These findings raise the question why the SH3 binding site is present in FAK.

In order to address this question we have cloned and expressed the FAK kinase domain with the SH3 and SH2 binding motifs (32k, Fig 4.3B). The stability of this 32k construct unfortunately did not allow for extensive NMR analysis. However, biochemical and structural studies of 32k construct with the Src SH3 and SH2 domains still gave several significant insights into the interactions between these proteins.

\textbf{Materials and methods}

\textit{Cloning of 32k in the baculovirus expression system}

The portion of the mouse FAK gene encoding the kinase domain with its SH3 and SH2 (32k) binding sites (GenBank accession number: M95408.1) was amplified by PCR with the forward and reverse primers A and B respectively (Table 1). After one cycle of denaturation of the template (10 min at 94°C), 30 cycles were performed comprising of the following steps, denaturation at 94°C for 1 min, annealing at 58°C for 2 min,
elongation at 72°C for 2 min. Finally, one cycle of elongation at 72°C for 10 min was applied. The amplified 32k gene fragment was cloned into vector pET28a (Novagen) using EcoRI and XhoI restriction enzymes. A construct containing the 32k together with a C-terminal 6 histidine tag from the pET28a vector (32k-H6) was amplified by PCR using the primer A and C as forward and reverse primers, respectively (Table 1). The amplified product was then cloned into the pFastBacDual transfer vector (pFBD, (Invitrogen)) using the restriction enzymes EcoRI and SpeI.

**Table 4.1. Primers used for cloning 32k constructs.**

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequence</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><code>5’CTATGCAAGAATTCTATGCAGAAAGAAGGTGAACGG3’</code></td>
<td>EcoRI</td>
</tr>
<tr>
<td>B</td>
<td><code>5’GATGCAACTCGAGCTCCAGGATTGTGCTGAGCTGAGC3’</code></td>
<td>XhoI</td>
</tr>
<tr>
<td>C</td>
<td><code>5’CGGCTGCAGACTAGTGCCGGATTCATCTAGTGTTGG3’</code></td>
<td>SpeI</td>
</tr>
<tr>
<td>D</td>
<td><code>5’CTATGCAACATATGCAAGAAGGTGAACGG3’</code></td>
<td>NdeI</td>
</tr>
<tr>
<td>E</td>
<td><code>5’GATGCACTCGAGCTCCAGGATTGTGCTGAGCTGAGC3’</code></td>
<td>XhoI</td>
</tr>
</tbody>
</table>

The 32k construct was also cloned from the pET28a derivative into vector pFBD (Invitrogen) containing an N-terminal deca histidine tag and a factor Xa protease cleavage site (H10-32k). The forward primer D containing an NdeI restriction site, and the reverse primer E containing an XhoI restriction site, were used for PCR amplification according to the same protocol as described above.

Both 32k fragments (of about 1.1-kb) in pFBD were transferred to the AcNPV genome. This was achieved by transformation of the 32k gene into *E. coli* strain DH10EMBacY. The presence of a fluorescent marker in the virus DNA in DH10EMBacY cells is advantageous compared to normal DH10Bac cells (see below). DH10EMBacY 32k
transformed cells were plated on LB agar medium containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. Recombinant bacmids were identified by blue/white colony screening, because the transposition of the construct results in the disruption of the \( lacZ \) gene on the bacmid. The recombinant bacmid was isolated by using the solutions I, II and III of the QIAprep spin mini prep kit (QIAGEN), but the DNA was not purified using the provided columns. Instead, the DNA was precipitated using isopropanol, washed with 70% ethanol, dried, and dissolved in sterile water. The sequences of the 32k constructs were confirmed by sequencing of PCR products generated with a gene specific forward and M13 reverse primer (Fig. 4.4). The purity of Bacmid-32k was determined by UV spectroscopy, giving a 260 nm/280 nm of 1.8.

**Figure 4.4.** PCR amplification of the 32k construct in the recombinant bacmid. On the left, an agarose gel with in lane 1 the marker and in lane 2 the amplification product using a gene specific forward and M13 reverse primer. The expected size 1603 bp (1000(32k)+458+145). On the right, schematic representation of the transposition region.

*Virus production*

The recombinant bacmid was transfected into *Spodoptera frugiperda* insect cells (Sf9) using the liposome-mediated transfection reagent, fugene (Roche chemicals). Sf9 cells
were seeded (2x10^6 per mL) in serum free TMN-FH (Sigma chemicals) medium, together with 50 µg/mL penicillin and 50 µg/mL streptomycin in a T25 flask. The cells were incubated for 45 min at 27°C. The cells were transfected with 5.2 µg Bacmid-32k with liposome fugene and then incubated at 27°C for 5 h. To remove the bacmid – fugene mixture, the cells were washed gently with the above media twice. After washing, the cells were supplemented with growth media containing antibiotics and 5% (v/v) heat-inactivated fetal bovine serum (FBS). The supernatants were harvested when a 75% cytopathic effect (CPE) was observed, by monitoring Polh driven enhanced yellow fluorescent expression. The virus was harvested by centrifugation of the cells at 1200 rpm for 10 min, and supernatant was stored at 4°C for further passage (P1 stock). High-titer virus stocks were prepared and stored at 4°C and used for infection. For this purpose suspension cultures were infected with P1 stocks until a multiplicity of infection (MOI) of 5 was reached, as judged from the production of YEP.

32k production
For protein production, Sf9 cells were grown in 1.5 L erlenmeyer flasks in an incubator shaker at a speed of 90 rpm at 27°C. The cells were infected at 2x10^6 per mL measured from the cell counter (Bruker) with recombinant baculovirus at a multiplicity of infection (MOI) of 5. The cells were harvested 72 h post infection. The time of harvesting was further optimised by monitoring of enhanced yellow fluorescence expression. The cells were harvested by centrifugation at 1800 rpm at 4°C. The 500 mL cell pellets (2x10^6 cells) were resuspended in 20 mL of the lysis buffer containing 50 mM Tris-HCl pH 8, 10 mM imidazole, 500 mM NaCl, 0.1% triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, and a protease inhibitor cocktail tablet (Roche). The resuspended pellets were flash frozen using liquid nitrogen and stored at -80°C.

32k purification and phosphorylation treatments
All purification steps were undertaken at 0-4°C, either on ice or in a cold room. The resuspended cell pellets were thawed on ice, lysed by adding 0.5-2% Triton X-100 (w/v) and incubated for approximately 1 hour. The cell lysate was cleared by ultracentrifugation at 40,000 rpm for 45 min. The cleared lysate was further filtered using
The interaction of focal adhesion kinase and Src

a 0.4 µm filter. The supernatant was loaded on a pre-equilibrated Ni column. The equilibration buffer contained 50 mM Tris-HCl pH 8, 500 mM NaCl and 10 mM imidazole. The protein was eluted using a gradient from 10 mM to 400 mM imidazole. The protein eluted at 200 mM imidazole for 32k-H6 construct, whereas for H10-32k construct protein eluted at 300 mM. Pooled fractions were dialysed against a buffer containing 50 mM HEPES pH 7, 300 mM NaCl and 1 mM DTT. The dialysis was performed overnight, using a membrane with a 12 kDa cutoff. The salt concentration was brought down to 100 mM by adding 50 mM HEPES pH 7. Then protein was incubated with phosphatase (YoPH) with 1:20 molar ratio of protease:total 32k for 2-3 hours at room temperature. Imidazole and MgCl2 were added to a final concentration of 20 mM and 2 mM respectively. The phosphatase was removed using a NTA column and the protein was eluted by applying a gradient of imidazole (see above). For autophosphorylation, the protein was dialysed for 3h against phosphorylation buffer 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 4 mM MgCl2 and 1 mM DTT, and incubated overnight with 2 mM ATP and 1 mM Na3VO4 (phosphatase inhibitor). The full length and cleaved phosphorylated proteins of 32k-H6 (see results), were separated by anion exchange chromatography (HiTrap Q-column, GE Healthcare). Before applying the protein to the column, the salt concentration was brought down to 100 mM by dilution with buffer containing 20 mM Tris-HCl pH 7.6 and 1 mM Na3VO4. The full length and cleaved phosphorylated protein were separated by applying a gradient from 100 to 400 mM of NaCl. The anion exchange chromatography was also used in the case of 32k-H10 which helped to remove some of the impurities.

Production of Src 2H-15N SH2 and 2H-15N SH3 domains
Deuterated 15N labelled Src SH2 and SH3 domains were produced in *E. coli* BL21. Approximately 50 ng pET28 vector containing the genes coding for the Src SH2 or SH3 domain was transformed into competent cells. Pre-cultures were obtained with LB medium and then cells were serial diluted into D2O-M9 minimal medium. The M9 medium, trace elements, vitamins, antibiotics and glucose were all made in D2O. 15NH4Cl was used as the sole nitrogen source and the glucose was not deuterated. The cell cultures were incubated in 1.5 L erlenmeyer flasks in an incubator shaker at 200 rpm
and 37°C. Gene expression was induced with 0.5 mM IPTG at an OD₆₀₀ of 0.5-0.6. The cells were harvested 13 h after induction by centrifugation at 6000 rpm for 20 min. The cell pellet was suspended in buffer containing 20 mM Tris-HCl, pH 8, 500 mM NaCl and 10 mM imidazole, flash frozen using liquid N₂ and stored at -80°C until protein purification.

**Purification of Src²H⁻¹⁵N SH2 domain**

The pellets were quickly thawed and 1 mM PMSF (protease inhibitor) and 50 µg/mL DNase were added and the mixture was incubated for 30 min on ice. The cells were lysed by using a French press. The lysate was cleared by ultracentrifugation at 35,000 rpm for 45 minutes at 4°C. The supernatant was filtered over a 0.4 µM filter before loading on a NTA column. The column (HiTrap HP, GE Healthcare) was pre-equilibrated with buffer containing 20 mM Tris-HCl pH 8, 500 mM NaCl and 10 mM imidazole. The protein was eluted with a gradient of 10-500 mM imidazole. The purified protein was concentrated and diluted with 20 mM Tris-HCl, pH 7.6, to achieve a final NaCl concentration of 100 mM. The protein was loaded on an anion exchange column (HiTrap Q, GE Healthcare) pre-equilibrated with 20 mM Tris-HCl pH 7.6, 100 mM NaCl, and the protein was eluted with a gradient of NaCl from 100 mM - 1M. The purity of the protein was verified by SDS-PAGE. The percentage of deuteration was assessed by mass spectrometry and a 1D ¹H spectrum. It was found that about 80% of the protein was deuterated.

**Purification of Src²H⁻¹⁵N SH3 domain**

The purification method for the Src SH3 domain is described in chapter 2.

**Peptide synthesis**

Peptide synthesis is described in chapter 2

**Western blotting**

About 5-50 µg of protein per lane was loaded on a 15% SDS-PAGE gel, which was run at 100 V for 10 min and then at 160 V for 65 min. To blot the proteins, a sandwich of PVDF membrane and the SDS-PAGE gel between fiber pads was put at 90 V for 120
The interaction of focal adhesion kinase and Src min. The PVDF membrane was incubated with primary antibodies, being either anti-FAK pY397 (Biosource) or anti-His₄ (Qiagen), both diluted 1:1000. After incubating for an hour, the membrane was washed twice with PBS buffer with 0.05% Tween 20 for 15 min to remove the unbound antibodies. The membrane was then incubated for an hour with horseradish peroxidase-conjugated to mouse (α- His₄) and rabbit (α-pY397) as the secondary antibody and washed twice. The signal was detected with ECL (GE health care).

NMR spectroscopy
NMR samples contained the Src domains and 32k proteins in 20 mM HEPES pH 7, 150 mM NaCl, 4 mM MgCl₂, 1 mM TCEP and 6% D₂O. For the phosphorylated 32k proteins 1 mM Na₃VO₄ was also added in order to inhibit possible phosphatase activity during the titrations. The pH of the samples was checked before and after the NMR measurements. The interaction of the Src domains with 32k was studied with TROSY HSQC experiments at 293 K, on a Bruker DMX600 equipped with a TCI-Z-GRAD cryoprobe. 1024 and 90 complex points were acquired in the direct and indirect dimensions, respectively, with a interscan delay of 1 s. The data were processed using nmrPipe software¹⁵⁴ and analysed using CCPN Analysis 2.1.5.¹⁵⁵ The amide backbone assignments for the Src SH3 and Src SH2 were kindly provided by Dr Hanna Lindfors. For peptide titrations, stock solutions of 5-20 mM peptide were prepared by dissolving peptides in 20 mM KPi, pH 6.5, 0.1 M NaCl, and adjusting the pH to 6.5 with small aliquoted of 0.1-0.5 M solutions of NaOH or HCl. Titrations with unlabelled peptides were performed by addition of microliter aliquotes of peptide to sample containing 0.2 mM ¹⁵N SH3 or SH2 in 20 mM KPi pH 6.5, 0.1 M NaCl, 1 mM DTT. [¹⁵N, ¹H] HSQC spectra were recorded after addition of each aliquot of peptide. In every titration, the spectrum of free protein was also recorded.

Chemical shift perturbation analysis
The method of determination of averaged chemical shift perturbations are described in chapter 1, Eq. 1.3.
Results

The aim of this work was to determine the interaction of Src SH3 and SH2 domains with their FAK binding sites attached to the kinase domain (32k). The Src SH3 domain recognizes the polyproline motif, whereas the Src SH2 domain requires the pY397 residue on the linker region of FAK. Dr Hanna Lindfors studied the interaction between FAK and the Src SH2 domain. She cloned the kinase domain of FAK together with the Src SH2 binding site (2k) and studied the interactions between 2k and the Src SH2 domain. NMR experiments revealed that the Src SH2 domain binds to 2k in a non-specific way, contrary to isolated peptides containing pY397 or Y397, which exhibited specific interactions. These results raise the question whether the SH2 domain alone is enough to initiate the complex formation of Src and FAK. Alternatively, the Src SH3 domain could also play an important role in the initiation of complex formation. To address this question, the SH3 binding site was added to 2k and the interaction between the Src SH3 and SH2 domains with 32k was studied. For this purpose a fragment of FAK, comprising the SH3 and SH2 binding sites and the kinase domain of the mouse protein was subcloned.

First, a construct, coding for 32k with a C-terminal hexa-histidine tag was generated, cloned into the pFBD vector and transposed into the virus bacmid in *E. coli* DH10EMBacY cells, encoding also the yellow fluorescence protein. The recombinant bacmid was transfected to the SF9 cells. Co-expression of the yellow fluorescent tag with 32k functioned as a monitor, not only for determining the progress of viral infection (Fig. 4.6), but also to determine the time to harvest the infected cells on the basis of its yellow fluorescence.
The interaction of focal adhesion kinase and Src

Figure 4.5. The 32k-H$_6$ construct. A) Schematic representation. B) Amino acid sequence. The SH3 and SH2 binding sites are indicated by the bold PISP and Y397 residues, respectively. The approximate site of cleavage is indicated.

Figure 4.6. Fluorescent microscope images of transfected insect cells expressing YFP. A) Titer 1; B) Titer 2; C) Titer 3.
The protein 32k was produced and could be purified using metal affinity chromatography. However, SDS-PAGE and Western blot results showed that the protein was susceptible to cleavage at N-terminus. On SDS-PAGE two bands were observed, both reactive toward α-His$_4$ antibodies. Although the site of cleavage has not been precisely identified, the size difference places the cleavage site between the SH3 and SH2 binding sites (Fig. 4.5). The full length protein is 37.4 kDa and the cleaved one around 34 kDa. The two forms of the protein consistently appeared during the purification and biochemical characterization.

![Figure 4.7. SDS-PAGE (15%) analysis of the fractions of a HiTrap Q column showing partial separation of the full length and cleaved 32k-H$_6$. Lane 1-11 are column fractions, from low to high salt. Lane 12, sample before loading on the column.](image)

They were partially separable using anion exchange column (Fig. 4.7). The protein was treated with the YopH phosphatase to remove the phosphate on Y397, and could be readily re-phosphorylated upon incubation with ATP, demonstrating kinase activity. Western blots for both the full length and cleaved protein were performed using α-His$_4$ and α-pY397 (antibodies raised against pY397). Surprisingly, α-pY397 antibodies gave a signal only for the cleaved protein, not for the full length protein (Fig. 4.8). The cleaved product clearly still retains pY397. We do not understand why the full length protein is not phosphorylated at Y397.
Figure 4.8. A western blot of the purified 32k-H6 stained with α-His$_4$ (A) and pY397 (B) as primary antibodies. Lane 1, protein treated with phosphatase YopH. Lane 2, marker. Lane 3, phosphatase treated protein that was subsequently incubated with ATP.

An alternative construct was also produced to circumvent the difficult purification of the non-cleaved product. To enable easy removal of degraded 32k, the His tag was placed on the N-terminus (H$_{10}$-32k). In this way, the full length protein was readily purified using the N-terminal His tag (Fig. 4.9). The western blot shows that residue Y397 can be dephosphorylated using the YopH phosphatase and phosphorylated by incubating the protein with ATP (Fig. 4.10). A small degree of C-terminal degradation was observed for H$_{10}$-32k.
TROSY NMR experiments were performed to study the binding of the SH3 and SH2 domains ofSrc to 32k. An overlay of the spectrum of the free $^2$H,$^{15}$N SH3 domain with that of SH3-32k 1:1 mixture is shown in Fig. 4.11. The chemical shift perturbations of the amide protons of the SH3 domain in the presence of 32k suggested that the two proteins bind. The largest chemical shift perturbations are observed for the residues 28, 30, 33, and 38. Residues 14, 18, 19, 37, 41, and 53 are broadened beyond detection.
In another experiment, reported in chapter 2, a peptide that contains the Src SH3 binding site of FAK (RALPSIPKLA, with the N and C termini acetylated and amidated, respectively) was titrated into a solution with $^{15}$N-labelled SH3. Chemical shift perturbations were monitored upon addition of the peptide. An overlay of the spectra recorded during the titration is shown in the Fig. 4.11C. The resonance positions shift with increasing peptide concentration, demonstrating binding in the fast exchange regime for most residues. The maximum chemical shift perturbation of SH3 domain is observed for the residues 16, 19, 33, and 38. The residues 37 and 52 are broadened beyond detection. The $K_d$ of the complex was determined to be $63 \pm 3$ µM (Fig. 2.5, chapter 2).

The chemical shift perturbations for the SH3-32k and SH3-peptide complex are qualitatively similar, with the peaks shifting in the same direction in the HSQC plane. The $K_d$ for the SH3-32k complex was estimated to be $200 – 250$ µM, by assuming that the average perturbations of the 100% bound SH3 domain are of similar size as those observed for the SH3-peptide complex. The average perturbations ($\Delta\delta_{avg}$) that are thus obtained are shown in Figure 4.12 and the binding maps in Figure 4.13. There is a striking similarity between the binding patterns, suggesting that the SH3 domain binds the 32k in a similar fashion as the peptide, albeit with slightly reduced affinity.
Figure 4.11. Overlaid TROSY spectra of free $^2$H-$^{15}$N Src SH3 (blue) with 1:1 Src SH3:32k domain (red). A) Full spectrum. B) Detail showing the perturbations of residues Asn137 and Ser 142. (C) The perturbations of the same residues as in B) in a titration with peptide RALPSIPKLA observed in HSQC spectra.
Figure 4.12. The average chemical shift perturbations of amide nuclei of the Src SH3 domain bound to 32k or peptide, extrapolated to 100% bound state assuming $K_d$ values of 63 and 220 µM, respectively. *residues broadened beyond detection.
Figure 4.13. Binding maps of the Src SH3 domain (PDB entry 1RLQ\(^{158}\)) based on the averaged chemical shift perturbations upon addition of 32k (top) and peptide (bottom). The residues are coloured according to the size of the average amide chemical shift perturbation. Red: \(\Delta \delta_{\text{avg}} \geq 0.15\) ppm; orange \(0.15 > \Delta \delta_{\text{avg}} \geq 0.1\); yellow \(0.1 > \Delta \delta_{\text{avg}} \geq 0.05\); blue \(\Delta \delta_{\text{avg}} < 0.05\). Magenta: Residues broadened beyond detection. Unassigned and proline residues are coloured grey.

Similarly, the interactions between the Src SH2 domain and 32k were studied by TROSY NMR experiments. An overlay of the spectrum of the free \(^2\text{H},^{15}\text{N}\) SH2 domain with that of 1:1 SH2-phosphorylated mixture is shown in Fig. 4.14. Fig. 4.15 shows the same for the sample with unphosphorylated 32k. The residues of Src SH2 domain showed negligible chemical shift perturbations upon addition of either the phosphorylated or unphosphorylated 32k. This suggests that Src SH2 domain did not bind to the 32k. For comparison, a peptide derived from FAK either phosphorylated or unphosphorylated...
The interaction of focal adhesion kinase and Src

(ETDDpYAEIIDEED or ETDDYAEIIDEED with N and C termini acetylated and amidated, respectively), was titrated to the $^{15}$N- labelled SH2 domain (Fig. 4.14C and Fig. 4.15C). Unlike the 32k protein, the peptides showed binding to the Src SH2 domain. The chemical shift perturbations of the Src SH2 residues with the phosphorylated peptide exhibited a slow exchange behaviour, whereas for the unphosphorylated peptide fast exchange behaviour was observed. These NMR results suggest that the SH2 binding site of 32k may be not accessible for the binding of Src SH2.
Figure 4.14. TROSY spectrum of free $^2$H-^{15}N Src SH2 (blue) overlaid with the spectrum of the Src SH2 domain and phosphorylated 32k (1:1) (red). A) Full spectrum. B) Detail showing the perturbations of residues Ser 191 and Tyr 204. C). The perturbations of the same residues of Src SH2 domain titrated with peptide ETDDpYAEIIIDEED followed with HSQC spectra. Panel C was taken from ref.
Figure 4.15. TROSY spectrum of free $^2$H-$^{15}$N Src SH2 (blue) overlaid with the spectrum of the Src SH2 domain and unphosphorylated 32k (1:1) (red). A) Full spectrum. B) Detail showing the perturbations of residue Asp 215. C) The perturbations of the same residue of Src SH2 domain titrated with peptide ETDDYAEIIDEED followed with HSQC spectra. Panel C was taken from Dr. Lindfors$^{182}$
Conclusions

The aim of the study was to determine the interactions between the Src domains and 32k. The kinase domain with the linker region containing the SH2 and SH3 binding sites was cloned and expressed in insect cells. Two constructs were made, differing in the position of the histidine tag. Purification of the protein and biochemical characterization such as phosphorylation and dephosphorylation of Y397 were carried in this work. NMR experiments were performed to study the interaction of 32k with the SH3 and SH2 domains of Src. The results were compared with SH-domain binding to FAK-derived peptides with the SH binding motifs.

The chemical shift perturbation data show that the Src SH3 domain binds 32k in a similar fashion as the peptide, although with a slightly reduced affinity (3-4 fold). The SH2 domain, however, showed negligible binding to the phosphorylated and unphosphorylated 32k, in stark contrast with the FAK based peptides, that showed high affinity and very specific binding to the SH2 domain.

Earlier research on the 2k construct showed significant but non-specific binding to the Src SH2 domain. The chemical shift perturbation data for the SH2 domain upon binding to 2k suggests that SH2 domain is not binding in a specific region on 2k, because the shift changes are small and spread out over the entire surface, typical for a dynamic complex. It is not clear where the SH2 binds on 2k but there is no specific interaction with pY397. For some reason it appears to be abolished in 32k, perhaps due to differences in electrostatic potential between 2k and 32k. It remains an interesting question why the SH2 cannot bind the pY397 in the same fashion as it binds to peptide containing pY397. Though the protein was susceptible to cleavage western blots on the used NMR samples showed that the pY397 residue was still present (data not shown).

All NMR experiments reported here are with the H_{6}-32k. Due to the fact that the stability of the protein was poor for this construct, performing similar NMR experiments with the H_{10}-32K construct would be advisable. The presence of protease cleavage site in H_{10}-32K
construct makes it possible to remove the histidine tag if necessary. Then the question can be addressed whether binding of SH2 to pY397 in 32k is enabled by the interactions of SH3 with the SH3-binding motif. This would support a role for the SH3 domain in initiating the interaction with FAK.