Chapter 6

The interaction of Src SH2 with the focal adhesion kinase catalytic domain studied by NMR
Abstract

The interaction of the Src SH2 domain with the catalytic domain of FAK, including the Y397 SH2 domain binding site, has been studied using NMR spectroscopy. Analysis of the chemical shift perturbations indicate that the Y397 site is not available for SH2 domain binding. The observed effects are spread over a relatively large area of the SH2 domain and the chemical shift perturbations are small, which is typical for dynamic complexes governed mainly by electrostatic interactions. Competitive binding experiments suggest that although the regular high-affinity SH2 domain binding site in FAK is not involved in the interaction, the binding affinity is still surprisingly high.
The interaction of Src SH2 with the focal adhesion kinase catalytic domain

Introduction

The interaction of FAK and Src plays a crucial role in a number of signalling pathways, regulating processes such as cell proliferation, survival and migration. The involvement of FAK and Src in many human diseases makes them important drug targets and has made Src and FAK the subject of many structural biology studies. For Src, crystal structures of isolated domains as well as of the full-length protein have been determined, elucidating the mechanism of Src activation [229-233]. Crystal structures also exist of the isolated catalytic domain, FERM domain and FAT domain of FAK [234-237]. More recently, structures of a fragment of FAK comprising both the FERM and kinase domains have been published, suggesting a mechanism of FAK autoinhibition reminiscent of the one for Src [238]. In addition to the crystal structures, NMR structures of the Src SH2 and SH3 domains exist, e.g. [39;239;240], as well as NMR structures of the FAT domain of FAK [241;242]. Examples of FAK and Src structures in the RCSB protein data bank can be found in Table 1.

To date, no NMR studies involving the catalytic domain of FAK have been reported. In spite of the large body of structural biology research on FAK and Src, in vitro studies of the FAK-Src interaction are limited to the study of peptides derived from FAK binding to the SH3 and SH2 domains of Src [139], chapters 2-4 this thesis. In these studies the SH2 and SH3 domains of Src bind to the peptide motifs present in the linker between the FERM and catalytic domains of FAK.

In the regulation of Src and FAK activity intramolecular contacts between domains keep the proteins in an inactive state. In order to investigate whether any intermolecular contacts exist between Src and FAK outside the peptide binding motifs we have studied the interaction of the Src SH2 domain with the catalytic domain of FAK including the part of the FERM domain – kinase linker that contains the Y397 SH2 binding site (Fig. 6.1). Using NMR spectroscopy we find
that although the Y397 site seems unavailable for SH2 binding in the present FAK
construct, the SH2 domain still binds the kinase domain, in a way typically seen for
dynamic complexes governed mainly by electrostatics.

Table 1. Examples of published structures of Src and FAK.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Exp. method</th>
<th>PDB</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Src kinase domain</td>
<td>X-ray diffraction</td>
<td>1YOJ, 1YOL, 1YOM</td>
<td>[243]</td>
</tr>
<tr>
<td>Src SH3, SH2 and kinase domains</td>
<td>X-ray diffraction</td>
<td>1Y57</td>
<td>[244]</td>
</tr>
<tr>
<td>Src SH2 domain</td>
<td>X-ray diffraction</td>
<td>1SPR, 1SPS</td>
<td>[245]</td>
</tr>
<tr>
<td>Src SH2, SH3, kinase domains and c-terminal tail</td>
<td>X-ray diffraction</td>
<td>1FMK</td>
<td>[246]</td>
</tr>
<tr>
<td>Src SH2, SH3, kinase domains and c-terminal tail</td>
<td>X-ray diffraction</td>
<td>2SRC</td>
<td>[247]</td>
</tr>
<tr>
<td>FAK FAT domain</td>
<td>X-ray diffraction</td>
<td>1K04, 1K05</td>
<td>[248]</td>
</tr>
<tr>
<td>FAK FAT domain</td>
<td>X-ray diffraction</td>
<td>1K40</td>
<td>[249]</td>
</tr>
<tr>
<td>FAK FERM domain</td>
<td>X-ray diffraction</td>
<td>2AEH, 2AL6</td>
<td>[250]</td>
</tr>
<tr>
<td>FAK kinase domain</td>
<td>X-ray diffraction</td>
<td>1MP8</td>
<td>[251]</td>
</tr>
<tr>
<td>FAK FERM and kinase domains</td>
<td>X-ray diffraction</td>
<td>2J0J, 2J0L</td>
<td>[252]</td>
</tr>
<tr>
<td>Src SH3 domain</td>
<td>NMR</td>
<td>1SRL</td>
<td>[253]</td>
</tr>
<tr>
<td>Src SH3 domain</td>
<td>NMR</td>
<td>1RLQ, 1PRM</td>
<td>[39]</td>
</tr>
<tr>
<td>Src SH2 domain</td>
<td>NMR</td>
<td>1HCS</td>
<td>[254]</td>
</tr>
<tr>
<td>FAK FAT domain</td>
<td>NMR</td>
<td>1KTM</td>
<td>[255]</td>
</tr>
<tr>
<td>FAK FAT domain</td>
<td>NMR</td>
<td>1PV3</td>
<td>[256]</td>
</tr>
</tbody>
</table>
The interaction of Src SH2 with the focal adhesion kinase catalytic domain

Figure 6.1. FAK domain organization. The underlined region corresponds to the fragment of FAK studied in this chapter (the catalytic domain and the linker region containing the SH2-binding site).

Experimental procedures

Protein expression and purification

FAK kinase domain was produced and purified as described in chapter 6, and non-deuterated SH2 domain was produced as described in chapter 3. For production of deuterated SH2 *Escherichia coli* BL21 cells were transformed with SH2-pET28 and incubated overnight in LB medium supplemented with 50 mg/L kanamycin at 37°C while shaking at 250 rpm. The preculture was diluted 1:100 into D\textsubscript{2}O-M9 minimal medium with 50 mg/L kanamycin (M9 salts prepared in 100% D\textsubscript{2}O, trace elements and other supplements prepared in H\textsubscript{2}O, non-deuterated carbon source) using \textsuperscript{15}NH\textsubscript{4}Cl as the sole nitrogen source. Cultures were incubated at 37°C and 250 rpm until an OD\textsubscript{600} of 0.6, the temperature was reduced to 25 °C and protein expression was induced with 0.5 mM IPTG. After 13 h cells were harvested via centrifugation. The SH2 domain was purified as described in chapter 3.

NMR sample preparation and experiments

NMR experiments were recorded at 293 K on a Bruker DMX600 spectrometer equipped with a TCI-Z-GRAD cryoprobe (Bruker, Karlsruhe, Germany). The data were processed with Azara (http://www.bio.cam.ac.uk/azara/) and analyzed using Ansig For Windows [142].
To assess the degree of deuteration of the SH2 domain a 1D NMR spectrum was recorded, from this it was estimated that 70% of the protein was deuterated (Fig. 6.2). NMR samples contained 40-100 µM $^2$H, $^{15}$N-SH2 in 20 mM HEPES pH 7, 150 mM NaCl, 4 mM MgCl$_2$, 1 mM TCEP (with 0.1 mM Na$_3$VO$_4$ for experiments with phosphorylated kinase domain). To the SH2 domain samples phosphorylated or unphosphorylated FAK kinase domain was added and 2D [$^{15}$N, $^1$H] TROSY spectra were recorded.

For peptide titrations, a stock solution of 5 mM peptide (for synthesis see chapter 3) was prepared by dissolving peptide in 20 mM HEPES pH 7, 150 mM NaCl, 4 mM MgCl$_2$ and 1 mM TCEP, and adjusting the pH to 7 with small aliquots of 0.1–0.5 M solutions of NaOH. Titrations were performed by the addition of microliter aliquots of peptide to samples containing either 50 µM $^2$H, $^{15}$N SH2 alone or 50 µM $^2$H, $^{15}$N SH2 together with 50 µM unlabelled FAK kinase domain in 20 mM HEPES pH 7, 150 mM NaCl, 4 mM MgCl$_2$, 1 mM TCEP. [$^{15}$N, $^1$H] TROSY spectra were recorded at the start of the titration and after each addition of peptide.

![Figure 6.2. $^1$H NMR spectrum of SH2 domain purified from E.coli cultured in deuterated minimal medium (see Exp. Procedures).](image)

**Chemical shift perturbation analysis**

The average chemical shift perturbations, $\Delta\delta_{\text{avg}}$, were calculated according to Eq. 2:
The interaction of Src SH2 with the focal adhesion kinase catalytic domain

\[ \Delta \delta_{\text{avg}} = \sqrt{\left( \frac{(\Delta \delta_{\text{binding}}^N)^2}{5} + (\Delta \delta_{\text{binding}}^H)^2 \right) / 2} \]  

(1)

where \( \Delta \delta_{\text{binding}}^N \) and \( \Delta \delta_{\text{binding}}^H \) are the chemical shift perturbations of the amide nitrogen and amide proton, respectively.

For competitive binding experiments peptides were titrated into samples containing either only SH2 domain or SH2 domain and kinase domain at a 1:1 molar ratio. Chemical shift perturbations of amide resonances were plotted against the molar ratio of peptide-to-protein. A non-linear least-squares global fit to a single-site binding model [145] (Eq. 2) was performed in Origin (OriginLab corporation, Northampton, MA).

\[ \Delta \delta_{\text{binding}} = \frac{1}{2} \delta_x \left( A - \sqrt{A^2 - 4R} \right) \]  

(2)

\[ A = 1 + R + \frac{LR + U}{LUK_a} \]

In Eq. 2, \( R \) is the molar ratio of peptide-to-protein, \( \Delta \delta_{\text{binding}} \) is the chemical shift perturbation at a given ratio, \( \Delta \delta_x \) is the chemical shift perturbation at 100% bound protein, \( L \) is the initial concentration of \(^{15}\text{N}\)-labelled protein, \( U \) is the concentration of the peptide stock solution and \( K_a \) is the association constant of the complex. The error in \( K_a \) was estimated by determining the range of \( K_a \) values that produced acceptable fits given the uncertainty in \( \Delta \delta_{\text{binding}} \). For the peptide titration in the presence of the kinase domain, \( \Delta \delta_x \) values were fixed to the corresponding values obtained from the titration in the absence of the kinase domain.
Competitive binding model and chemical shift perturbation simulations

In order to estimate the dissociation constant of the kinase-SH2 domain complex, chemical shift perturbation simulations were performed and compared to data from the competitive binding experiments. Assuming a model in which the SH2 domain either binds to the peptide or to the kinase domain (Fig. 6.3) the equilibrium association constants can be written:

\[ K_1 = \frac{[SH2^*P]}{[SH2][P]} \quad (3a), \quad K_2 = \frac{[SH2^*K]}{[SH2][K]} \quad (3b) \]

where \( K_1 \) is the equilibrium association constant for the SH2-peptide complex, \( SH2^*P \) denotes SH2 domain bound to peptide, \( SH2 \) is free SH2 domain, \( P \) is free peptide, \( K_2 \) is the equilibrium association constant for the SH2-kinase complex, \( SH2^*K \) denotes SH2 domain bound to the kinase and \( K \) is free kinase domain.

![Figure 6.3. Peptide and kinase domain competing for binding to the SH2 domain. SH2 = SH2 domain, P = peptide and K = kinase.](image)

If, at a given point in the titration, we let \( x \) be the concentration of SH2 domain bound to peptide, \( y \) the concentration of SH2 domain bound to kinase, \( A \), \( B \) and \( C \) be the total concentration of SH2 domain, peptide and kinase domain, respectively, Eqs. 3a and b can be re-written:

\[ K_1 = \frac{x}{(A-x-y)(B-x)} \quad (4a), \quad K_2 = \frac{y}{(A-x-y)(C-y)} \quad (4b) \]
Dividing Eq. 4a by Eq. 4b and solving for $x$ yields:

$$x = \frac{K_1By}{CK_2 + y(K_1 - K_2)} \quad (5)$$

In Eq. 5, $B$ and $C$ are known from the experimental parameters, and $K_1$ can be fixed to the value determined from the titration in the absence of the kinase domain. Using the value of $x$ from Eq. 5 as input in Eq. 4a, $y$ was varied until the value of $K_1$ calculated from Eq. 4a matched the fixed value used in Eq. 5. This way $x$, the concentration of SH2 domain bound to peptide, could be determined at each titration point for a given value of $K_2$, the equilibrium association constant of the SH2 domain-kinase domain complex. The chemical shift perturbation, $\Delta\delta_x$, for an SH2 domain residue at the determined value of $x$ can be predicted using Eq. 6:

$$\Delta\delta_x = \frac{x}{A} \Delta\delta_{\infty} \quad (6)$$

where $x$ is the concentration of SH2 domain bound to peptide determined using Eqs. 4 and 5, $A$ is the total concentration of SH2 domain in the sample and $\Delta\delta_{\infty}$ is the chemical shift perturbation for the residue at 100% bound protein, obtained from fitting the data from the titration in the absence of the kinase domain to the model described by Eq. 2. By varying $K_2$ the predicted chemical shift perturbations for a range of kinase-SH2 domain binding affinities could be compared to the experimental data.

**Results**

The addition of kinase domain to a sample of $^{1}H,^{15}N$ SH2 domain lead to the majority of the resonances in the HSQC spectrum being broadened beyond detection (Fig. 6.4), indicating that the proteins associate but that the protein
complex is too large to be observed using this method (approximately 48 kDa). To reduce the linewidth of the amide protons deuterated SH2 was produced and TROSY experiments were performed.

**Figure 6.4.** Overlaid HSQC spectra of free $^1$H,$^{15}$N SH2 domain (red) and $^1$H,$^{15}$N SH2 after addition of kinase domain at a ratio of 2:1 kinase to SH2 (blue). The majority of the SH2 peaks disappear upon addition of kinase, indicating binding.

Comparison of the spectrum of free SH2 domain with the spectrum of a sample containing SH2 domain and phosphorylated FAK kinase domain at a molar ratio of 1:1 reveals small chemical shift perturbations for a large number of backbone amide resonances (Fig. 6.5). Although reminiscent of a calibration problem, the chemical shift perturbations were found to be highly reproducible. The shift changes caused by the kinase domain differ from those typically caused both by nonphosphorylated (Fig. 6.6B) and phosphorylated (Fig. 6.6C) peptides binding to the SH2 domain. Chemical shift perturbations caused by the kinase domain are smaller and affect a larger fraction of the SH2 resonances. Mapping the chemical shift perturbations caused by the kinase domain onto the surface of the SH2 domain shows that the general pattern of shifts does not match that seen with peptide binding and that some SH2 domain residues that are strongly affected by peptide binding are not affected by the interaction with the kinase domain (Fig. 6.7). NMR spectra of the SH2 domain together with unphosphorylated kinase domain show a chemical shift perturbation pattern very similar to that caused by
The interaction of Src SH2 with the focal adhesion kinase catalytic domain

Figure 6.5. Overlaid TROSY spectra of $^2$H$^{15}$NSrc SH2 in the absence of kinase domain (blue) and in the presence of phosphorylated FAK kinase domain at a molar ratio of 1:1 (red).

Figure 6.6. A) Detail of TROSY spectrum of free SH2 domain (blue) overlaid with spectrum of a 1:1 mixture of SH2 and FAK kinase domain (red). Spectra recorded at T=293 K and pH=7.0. B) Detail of overlaid HSQC spectra from titration of SH2 domain with peptide $\text{ETDDYAEIIDEED}$, T=303K and pH=6.5. C) Detail of overlaid HSQC spectra from titration of SH2 domain with peptide $\text{ETDDpYAEIIDEED}$, T=303K and pH=6.5.
the phosphorylated kinase domain, but with generally larger shift changes (Figs. 6.8 and 6.9).

A competitive binding experiment was performed where the unphosphorylated peptide ETDDYAEIIIDEED (chapter 3) was titrated into either a 1:1 mixture of SH2 and phosphorylated kinase or into SH2 alone. Titration of peptide into SH2 domain causes significant chemical shift perturbations of some SH2 resonances (Fig. 6.10A), while addition of equal amounts of peptide to the SH2–kinase mixture produced much smaller chemical shift changes (Fig. 6.10B). Some peaks that are shifted in the SH2:kinase complex relative to free SH2 are not affected by peptide binding to SH2 only. When peptide is added to the SH2:kinase complex these resonances start to shift towards the corresponding position in free or peptide bound SH2 (Fig. 6.10C). Both these observations indicate that the peptide is competing with the kinase domain for binding to the SH2 domain. Fitting of the chemical shift perturbations for the titration of SH2 domain with peptide to a 1:1 binding model yields a dissociation constant of $0.17 \pm 0.03$ mM (Fig 6.11A). This value agrees within the error margins with the previously reported value (chapter 3), despite differences in buffer, pH and temperature. For the titration of SH2 domain with the same peptide in the presence of kinase domain it was assumed that the chemical shift perturbation for fully bound protein, $\Delta \delta_x$, for each residue would be the same as in the titration without the kinase domain. By fitting the titration data using fixed $\Delta \delta_x$ values obtained from the fit in Fig. 6.11A, an apparent $K_d$ of $2.2 \pm 0.4$ mM was determined (Fig 6.11B). Including the kinase domain at a ratio of 1:1 with the SH2 domain thus leads to an apparent affinity of the SH2 domain for the peptide that is 13-fold lower than the binding affinity without the kinase domain present.
The interaction of Src SH2 with the focal adhesion kinase catalytic domain

Figure 6.7. Chemical shift perturbations of SH2 domain residues upon addition of phosphorylated FAK kinase domain (top panel) and peptide ETDDpYAEIIDEED (bottom panel), mapped onto the surface of the SH2 domain (PDB structure 1HCS [257]). Residues are coloured according to the size of the average chemical shift perturbation, $\Delta\delta_{\text{avg}}$. Red: $\Delta\delta_{\text{avg}} \geq 0.3$ ppm; orange: $0.3 > \Delta\delta_{\text{avg}} \geq 0.1$ ppm; yellow: $0.1 > \Delta\delta_{\text{avg}} \geq 0.04$ ppm; blue: $\Delta\delta_{\text{avg}} < 0.04$ ppm. Non-assigned residues are shown in grey.

Figure 6.8. Chemical shift perturbations of SH2 domain residues upon addition of unphosphorylated FAK kinase domain at a kinase:SH2 ratio of 1:1, mapped onto the surface of the SH2 domain (PDB structure 1HCS [258]). Residues are coloured according to the size of the average chemical shift perturbation, see Fig. 6.7.
Figure 6.9. Detail of overlaid TROSY spectra of the SH2 domain, showing free SH2 (black), SH2 in complex with phosphorylated kinase (red) and unphosphorylated kinase (blue).

Figure 6.10. Competitive binding experiments, detail of overlaid TROSY spectra of the SH2 domain. A) Titration with peptide ETDDYAEIIDEED in the absence of kinase domain. Molar ratios of peptide to SH2 domain are indicated in the figure. B) Titration with peptide ETDDYAEIIDEED, in the presence of FAK kinase domain. Molar ratios of peptide to SH2 domain are indicated in the figure. C) Black: free SH2 domain. Purple: SH2 domain in presence of peptide, molar ratio 20:1 peptide to protein. Blue: SH2 domain in the presence of kinase domain, molar ratio 1:1. Red: SH2 domain in the presence of kinase domain, molar ratio 1:1 and peptide added at a molar ratio of 20:1 peptide to protein. Resonances that are not affected by peptide binding (black and purple peaks) but that are shifted in the presence of kinase domain (blue peaks) start to shift towards the position they have in free and peptide-bound SH2 domain upon addition of a large excess of peptide (red peaks).
To estimate the affinity of the SH2 domain for the kinase domain, chemical shift perturbation simulations were performed using a competitive binding model (see Exp. Procedures for details). Chemical shift perturbations for a residue in the SH2 domain upon peptide titration in the presence of the kinase domain were simulated for a range of SH2-kinase domain binding affinities (Fig. 6.12). A comparison with the experimental data indicates that the kinase domain binds the SH2 domain with a $K_d$ close to 1 µM (RMSD = 0.03 ppm).

**Discussion**

The fact that the chemical shift perturbations seen for the interaction of kinase domain with SH2 domain differ completely from the shifts seen for SH2 binding to peptides derived from FAK, suggests that the interaction of the kinase domain with the SH2 domain is independent of the Y397 binding site. The shift changes are small and spread out over a larger portion of the SH2 domain. This is typically observed for dynamic complexes where electrostatics is the main force driving the interaction [259]. The chemical shift perturbations observed for unphosphorylated kinase domain binding to the SH2 domain show the same pattern of shifts, only slightly larger. This difference in size of the chemical shift perturbations may be due to the additional charges on the phosphate group influencing the electrostatics of the interaction. It was already demonstrated that electrostatics play an important role in SH2 domain interactions (chapter 3).
Even at a 20:1 molar ratio of peptide to SH2, the chemical shift perturbations of the SH2 amides in the kinase-SH2 mixture are significantly smaller than those seen for the sample with SH2 domain only. In other words the kinase domain is efficiently competing with peptide for binding the SH2 domain, and the SH2 domain displays a considerably higher affinity for the kinase domain than for the unphosphorylated peptide. The $K_d$ of 0.5-1 µM estimated from the chemical shift perturbation simulations indicates a surprisingly high binding affinity, which is unusual for highly dynamic electrostatic complexes. However, previous studies involving the Src SH2 domain binding to peptides have shown that the high-affinity nature of some SH2 domain interactions does not imply that the interaction is static (chapter 3).

There are several possibilities as to why the Y397 binding site is not available for SH2 domain binding. The kinase domain is capable of autophosphorylation of Y397 both in cis and trans in vitro and it is thus possible that the linker is bound to the active site of the kinase domain of the same molecule or of another kinase molecule.
Figure 6.12. $^{15}$N chemical shift perturbations of SH2 residue Y204 upon titration with peptide ETDDYAEIIDEED in the presence of the kinase domain (filled circles), together with chemical shift perturbations for the same residue in the presence of the kinase domain simulated for different dissociation constants of the kinase-SH2 domain complex. For comparison, chemical shift perturbations for Y204 upon titration with the same peptide in the absence of the kinase domain are shown as filled squares.

**Conclusions**

The Src SH2 domain is capable of binding with a high affinity to the catalytic domain of FAK, in a way that does not involve the Y397 region in FAK, and the interaction appears to be highly dynamic and mainly governed by electrostatics. The kinase domain is able to effectively compete with peptides derived from the Y397 binding site for binding to the SH2 domain. The fact that the SH2 domain interacts with the kinase domain with a high affinity independently of the Y397
binding site, suggests that there is still much to be learnt about the interaction of FAK and Src.