Src homology domain-mediated protein interactions

Lindfors, H.E.

Chapter 4

A dynamic intermediate state in peptide-binding to the combined Src SH3 and SH2 domains

Abstract

The interaction of peptides derived from FAK with the SH32 domain of Src has been studied using NMR and ITC. The SH3- and SH2-binding sites in FAK are separated by more amino acid residues than what is required to simply span the distance between the peptide-binding faces on the SH3 and SH2 domains. Here, the length of the linker separating the SH3- and SH2-binding sites in FAK peptides has been reduced and the effect on the interaction with the SH32 domains has been investigated. Peptides in which the distance between the SH2- and SH3-binding sites is not sufficient for simultaneous SH2 and SH3 domain-binding were expected to almost exclusively bind to the SH2 domain, due to the high-affinity nature of SH2 domain-mediated interactions compared to SH3-peptide interactions. Contrary to expectations, a significant fraction of the peptide was found to bind to the SH3 domain at low peptide-to-protein ratios. In order to explain these observations we propose a model for the peptide-SH32 interaction in which long-range electrostatic interactions between charges in the peptide and the SH2 domain help recruit the peptide into a dynamic encounter complex, from which the peptide can either bind the SH3 or SH2 domain, thereby increasing the affinity for the SH3 domain compared to peptide interactions involving the isolated domain.

This chapter will be published as part of:

Lindfors et al (2010). A dynamic intermediate state in peptide-binding to the combined Src SH3 and SH2 domains. *Manuscript in preparation.*

Introduction

A general feature of most signalling proteins is their modular architecture, meaning that they are constructed from several individually folded domains. The domains can either have catalytic functions or be interaction domains, involved in binding to other proteins, lipids or nucleic acids. Recognition of short peptide sequences by protein interaction domains plays an important role in the regulation of cellular behaviour by facilitating the assembly of the signalling protein complexes and larger protein networks involved [15;187]. The protein interaction domains do not only function to recruit the catalytic domain to its appropriate substrates in the cell, but are often also involved in the regulation of protein activity via domain rearrangements [188].

The Src family kinases (SFKs) are prototypical modular signalling proteins. These non-receptor protein tyrosine kinases are involved in the regulation of a number of cellular processes, including cell survival, proliferation, differentiation and motility [189]. SFKs share a common domain structure with an N-terminal myristoylation site, followed by a region that is unique for each family member, a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain, a tyrosine kinase domain and a C-terminal region containing a negative-regulatory tyrosine residue (Y529 in Src, using mouse Src numbering) [190]. The SH3 and SH2 domains are protein-interaction domains found in a large number of signalling proteins [191]. SH3 domains, first discovered in 1988 [192], bind to proline-rich sequences that adopt a left-handed helical conformation [15], whereas SH2 domains recognize phosphorylated tyrosine residues. Since the original discovery in 1986 [193], SH2 domains have been identified in over 100 different protein sequences in humans [155]. The SH3 and SH2 domains of SFKs are involved in the regulation of protein activity via intramolecular contacts. Phosphorylation of Y527 in the C-terminal region of the kinase leads to association of this region with the SH2 domain. Together with the interaction of the SH3 domain with residues in the linker

connecting the SH2 domain with the catalytic domain, this intramolecular binding promotes a conformation by which kinase activity is repressed [194-197]. The SH2 and SH3 domains therefore play a direct role in regulating kinase activity, and competition for SH3 or SH2 domain binding by external ligands leads to kinase activation [198;199]. Fragments encompassing the SH3-SH2 domains (SH32) from several SFKs have been characterized biochemically, structurally and computationally [139;144;200-206], because of the importance of these domains for the functioning of SFKs.

Focal adhesion kinase (FAK) can activate Src and Fyn via interactions with their SH2 and SH3 domains [139;207;208]. The linker region between the FERM and catalytic domains of FAK contains a tyrosine residue, Y397, which becomes phosphorylated in connection with FAK activation [209]. N-terminal to the tyrosine, the linker contains a proline-rich region of the sequence RALPSIPKL. These residues contain the SH3 domain binding motif PxxP [210], whereas the phosphotyrosine region with sequence pYAEI is close to the consensus sequence pYEEI for SH2 domain binding [211]. Peptides derived from FAK spanning both the proline-rich and phosphotyrosine sites have been shown to bind to SH32 fragments of Src and Fyn in a bidentate manner with equilibrium dissociation constants of 20-30 nM, which is a higher affinity than those seen for peptides containing an SH3 or SH2 binding site alone [139;212]. It has been shown that the interaction between the FAK peptide and the Fyn SH32 domains is restricted to the canonical SH3 and SH2 binding sites and that the interaction does not affect the dynamic independence of the two domains [213].

The SH3- and SH2- domain binding motifs in the FAK fragment are separated by a ‘spacer’ consisting of 22 aminoacids, which is approximately 10 residues more than what is necessary to span the distance between the peptide-binding faces on the SH3 and SH2 domains [214]. This allows for simultaneous association of the SH3- and SH2 domains with their respective binding sites in the peptide. If the

length of the spacer separating the two binding sites in the peptide is reduced, the peptide-protein interaction would be expected to remain unaffected as long as the peptide can span the distance between the two domains. Upon further reduction of the spacer, the peptide would be expected to predominantly bind to the SH2 domain, due to the much higher affinity of the SH2 domain for the phosphotyrosine sequence compared to the SH3 domain binding to the proline-rich sequence (chapters 2 and 3). Only after saturation of the SH2 domain binding site, would binding to the SH3 domain be expected to occur. To test this hypothesis, we have performed NMR and ITC experiments on the Src SH32 domains interacting with peptides derived from FAK, in which the spacer has been gradually reduced in length. In contrast to the hypothesis, even for short spacers simultaneous binding of both domains is observed, indicative of a SH3-peptide interaction that must have significantly higher affinity than what is observed for the SH3 domain with proline-rich peptides. We propose a model for the interaction in which electrostatic interactions of the peptide with the SH2 domain increase the effective affinity of the peptide for the SH3 domain, via the formation of an intermediate dynamic state.

Experimental procedures

Cloning, protein expression and purification

A DNA fragment encoding the mouse Src SH3 and SH2 domains (SH32), residues 85-250, was amplified by PCR from the full-length Src plasmid pUSE Src wt (kindly provided by Prof. B. van de Water). The PCR product was inserted into the expression vector pET28a using the NcoI and XhoI restriction sites and the resulting construct was verified by DNA sequencing. For production of His-tagged SH32 *Escherichia coli* BL21 (DE3) cells, transformed with SH32-pET28, were incubated overnight at 37°C and 250 rpm in LB medium supplemented with 50

mg/L kanamycin. The preculture was diluted at a volume ratio of 1:100 into fresh LB medium with 50 mg/L kanamycin for production of unlabelled protein or into M9 minimal medium with 50mg/L kanamycin using $^{15}NH_4Cl$ as the only source of nitrogen for production of ^{15}N-labelled protein. Cultures were incubated at 37°C and 250 rpm, and protein production was induced with 0.5 mM IPTG when an OD_{600} of 0.6 was reached. Cells were harvested via centrifugation after 3-5 h, resuspended in 20 mM Tris-HCl pH 8, 0.5 M NaCl and 10 mM imidazole and stored at -80°C until protein purification. Following the addition of 1 mM PMSF and 50 µg/mL DNAse, the thawed cells were lysed using a French pressure cell and the lysate was cleared by centrifugation at 40000 rpm for 30 minutes. The supernatant was loaded onto an immobilized metal affinity column (HisTrap HP, GE Healthcare) equilibrated with 20 mM Tris-HCl pH 8, 0.5 M NaCl and 10 mM imidazole. The column was washed with the same buffer containing 60 mM imidazole before elution with the same buffer containing 300 mM imidazole. The eluted protein was diluted at a volume ratio of 1:5 with 20 mM Tris-HCl pH 7.6, in order to lower the NaCl concentration to 0.1 M. Protein was loaded onto an ion-exchange column (HiTrap Q, GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.6, 0.1 M NaCl, and eluted using a gradient of 0.1 – 0.8 M NaCl. Fractions were checked by SDS-PAGE, and pure fractions were pooled and concentrated. The purity of the protein was estimated to be above 95%. The buffer was exchanged to NMR buffer for the ^{15}N-labelled protein (20 mM KPi pH 6.5, 100 mM NaCl and 1 mM DTT) and to ITC buffer for the unlabelled protein (20 mM HEPES pH 6.8, 100 mM NaCl and 1 mM TCEP). The protein concentration was determined by the absorbance at 280 nm, using a theoretical extinction coefficient of 31400 M^{-1} cm^{-1} [140].

Peptide synthesis

Peptides were synthesized as described in chapter 2 and were kindly provided by Dr. Jan Wouter Drijfhout. Peptide stock solutions were prepared by weighing out

peptide and dissolving in NMR or ITC buffer and adjusting the pH by the addition of small aliquots of NaOH or HCl.

Nuclear magnetic resonance spectroscopy

All NMR experiments were performed at 303 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].

Backbone amide resonance assignments were performed analogous to what has been described for the Src SH3 domain (chapter 2). Peptide titrations were performed through the addition of microliter aliquots of peptide stock solution with concentration ranging from 1 to 5 mM. Two-dimensional [^{15}N,^{1}H]-HSQC spectra were recorded at the start of the titration and at every step. Chemical shift perturbations were analyzed as has been described in chapter 3.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out at 303 K on a Microcal (Northampton, MA) VP-ITC microcalorimeter. Experiments were performed and data were analyzed as described in chapter 3.

Molecular graphics were generated using PyMol [150].

Results and discussion

NMR titrations were performed with peptides derived from the SH3- and SH2-domain binding sites in FAK, with gradually shorter spacers between the binding sites (Table 1). Previous ITC and NMR binding experiments have shown that a peptide derived from the SH2 binding site in FAK, p2, binds to the SH2 domain

with an equilibrium dissociation constant (K_d) of 73 nM, and that the interaction is in the slow exchange regime on the NMR time scale (chapter 3). In contrast to this high-affinity interaction, a peptide derived from the SH3 binding site in FAK, p3, was found to bind to the SH3 domain with a K_d of 56 μM in the fast-to-intermediate exchange regime (chapter 2).

Table 1. Peptides derived from FAK (SH3- and SH2-binding sequences underlined) with the length of the spacer between the SH3 binding site (RALPSIP) and the SH2 binding site (pYAEI) decreasing. The peptides containing either the SH3 domain or SH2 domain binding site are also shown. pY denotes a phosphotyrosine residue. All peptides were acetylated and amidated on the N-, and C-termini, respectively.

Peptide	Sequence	Number of residues removed compared to FAK sequence
p32	RALPSIPKLANSEKQGMRTHAVSVSETDDpYAEIIDEED	0
p-3	RALPSIPKLANSEKQGMAVSVSETDDpYAEIIDEED	3
p-7	RALPSIPKLANSEKQSVSETDDpYAEIIDEED	7
p-11	RALPSIPKLANSESETDDpYAEIIDEED	11
p-15	RALPSIPKLANTDDpYAEIIDEED	15
p-16	RALPSIPKLANDDpYAEIIDEED	16
p-17	RALPSIPKLADDpYAEIIDEED	17
p-19	RALPSIPKDDpYAEIIDEED	19
p2	ETDDpYAEIIDEED	-
p3	RALPSIPKL	-

Titrations of Src SH32 with peptide p32, which contains both the SH3- and SH2-binding motifs separated by the full-length sequence as it is present in FAK, leads to chemical shift perturbations in the SH3 and SH2 domains that are similar to those caused by peptides p3 and p2 (Fig. 4.1), indicating that the peptide-protein

interaction is confined to the SH3 and SH2 peptide binding faces on the protein. The chemical exchange behaviour for SH3 amides in the titration with p32 differs from that observed in the titration with p3. When p32 binds to Src SH32 both the resonances in the SH2 and SH3 domain show slow-exchange behaviour, whereas for p3 fast-to-intermediate chemical exchange is seen for all SH3 resonances affected by binding. This observation is illustrated in Fig. 4.2 for residues T17. Residue G155 is part of the SH2 binding site.

Peptides containing the SH2-binding region in FAK bind too tightly to the Src SH2 domain for the binding affinity to be determined with NMR (see chapter 3) and, thus, ITC was used instead. Due to solubility problems for the longest peptides p32 and p-3, no high-quality ITC data could be obtained for these peptides. For peptide p-7, which is still long enough to span the distance between the SH3 domain and SH2 domain peptide-binding faces, ITC data were fitted to a one-site binding model, yielding a K_d of 28 nM (Fig. 4.3). This agrees well with values determined with surface plasmon resonance and ITC by Thomas et al. [139] and Arold et al. [215], for the binding of the Src and Fyn SH32 domains to a FAK peptide containing both the SH3 and SH2 binding sites. Comparison with results from ITC experiments involving binding of p3 or p2 to SH32 shows that the free energy change upon binding the peptide containing both an SH3- and SH2-binding site is less than the sum of the free energy changes upon binding p2 and p3 (Table 2), indicating that the binding is anti-cooperative. This is in agreement with what has been shown for the SH32 domains of Fyn, for which it was proposed that restriction of the conformational freedom of the FAK peptide, together with possible binding-induced partial structure in the peptide, causes a small entropic penalty [216]. In the absence of favourable contributions from additional contacts outside the SH3- and SH2-binding sites, this leads to anti-cooperative binding.

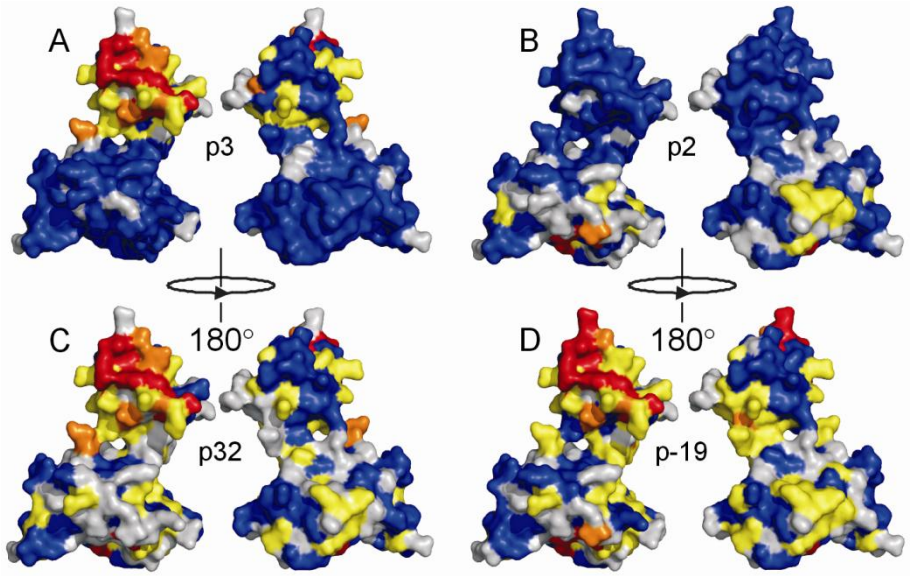

Figure 4.1. Average chemical shift perturbations ($\Delta\delta_{avg}$, Eq. 1, chapter 1) in Src SH32 caused by peptide titrations. **A)** Shifts caused by peptide p3 at a peptide-to-protein molar ratio of 10:1 **B)** shifts caused by peptide p2 at a ratio of 5:1 **C)** Shifts caused by peptide p32, ratio 1.5:1 **D)** Shifts caused by peptide p-19 at a peptide-to-protein ratio of 10:1. Chemical shift perturbations are coloured according to size and mapped onto the surface of SH32 taken from a structure of nearly full-length Src in the inactive state, PDB entry 1FMK [217]. Red: $\Delta\delta_{avg}\geq 0.3$ ppm; orange: $0.3>\Delta\delta_{avg}\geq 0.1$ ppm; yellow: $0.1>\Delta\delta_{avg}\geq 0.04$ ppm; blue: $\Delta\delta_{avg}<0.04$ ppm. Non-assigned residues are shown in grey.

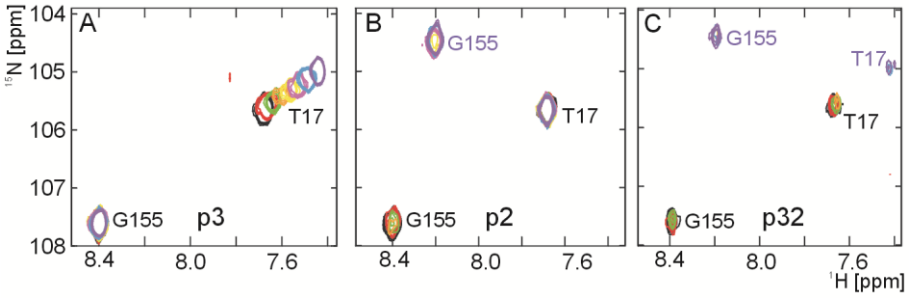

Figure 4.2. Detail from HSQC spectra of Src SH32 in titrations with peptides **A)** p3, **B)** p2 and **C)** p32. Spectra from a few titration points are shown overlaid, with starting points (free protein) in black and titration end points shown in purple.

Table 2. Thermodynamic parameters for peptides binding to Src SH32. Uncertainties in K, ΔH and ΔG are the standard deviations of three experiments, the uncertainty in TΔS was calculated using standard error propagation.

Peptide	**K_a (M^{-1})**	**ΔH (kcal*mol^{-1})**	**TΔS (kcal*mol^{-1})**	**ΔG (kcal*mol^{-1})**	**Fitting model**
p3	10.0±0.3 x 10^4	-14.0 ±0.7	-8.5±0.7	-5.6±0.1	One-site
p2	6.5±1.6 x 10^6	-9.4±0.3	0.1±0.4	-9.4±0.1	One-site
p-7	3.6±0.4 x 10^7	-20.5±0.3	-10.1±0.3	-10.5±0.1	One-site
p-19	2.4±0.1 x 10^6	-12.1±0.1	-3.2±0.1	-8.8±0.1	One-site

ITC data for peptide p-19 interacting with SH32 was fitted to a one-site binding model, yielding a K_a of 2.4 ± 0.1 x 10^6 M^{-1} (Table 2 and Fig. 4.3D). The determined equilibrium association constant is comparable to the affinity of the SH32 domains for peptide p2, containing only the SH2-binding site. In addition to the one-site binding model a two-site binding model was tried, but this did not improve the fit.

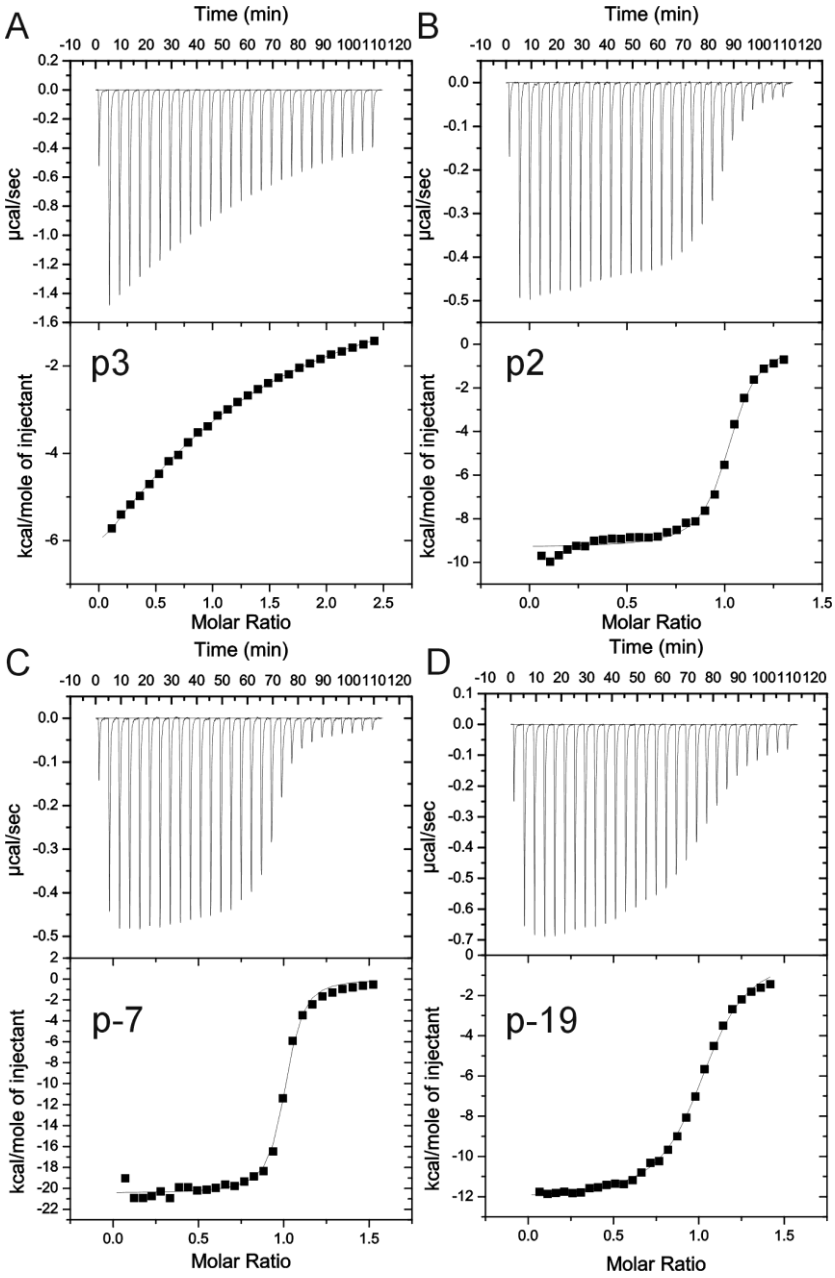

Figure 4.3. Representative isothermal titration calorimetry curves for the binding of Src SH32 to peptides A) p3, B) p2, C) p-7 and D) p-19. Top panel: raw data after baseline correction. Bottom panel: integrated data corrected for the heat of dilution of the peptide. The solid line in the bottom panel represents the best fit to a one-site binding model.

Examination of the exchange characteristics of SH2 and SH3 resonances in NMR titrations with gradually shorter peptides into an SH32 sample shows that for all peptides containing the phosphotyrosine the SH2 domain resonances remain in slow exchange. For the longer peptides the SH3 resonances also show slow exchange between free and bound forms, but for peptides with more than 15 amino acid residues removed from the linker the exchange becomes faster. For peptide p-16 slow-to-intermediate exchange was observed, for peptide p-17 the SH3 resonances are in intermediate-to-fast exchange, and for the shortest peptide, p-19, fast exchange behaviour is observed for the SH3 resonances (Fig. 4.4).

For the shorter peptides, such as p-19, the distance between the SH3- and SH2-binding sites is too small for the peptide to bind to both domains simultaneously, causing the domains to compete for peptide binding. Positioning the SH3 domain and SH2 domain binding faces close enough together for simultaneous binding would require substantial domain rearrangements, which would be reflected by chemical shift perturbations of the linker residues. The chemical shift perturbations for these residues are all small, however, indicating that the relative orientation of the domains remains unchanged. In light of the large difference in binding affinity between the two domains (about 3 orders of magnitude), it would be expected that virtually all available peptide would bind the SH2 domain until the SH2 domain is saturated, and only then would the excess peptide bind to the SH3 domain. This would yield a sigmoidal binding curve for the SH3 domain. The chemical shift perturbations for a few SH3 resonances in the titration with p-19 are plotted against the molar ratio of peptide to protein in Fig. 4.5.

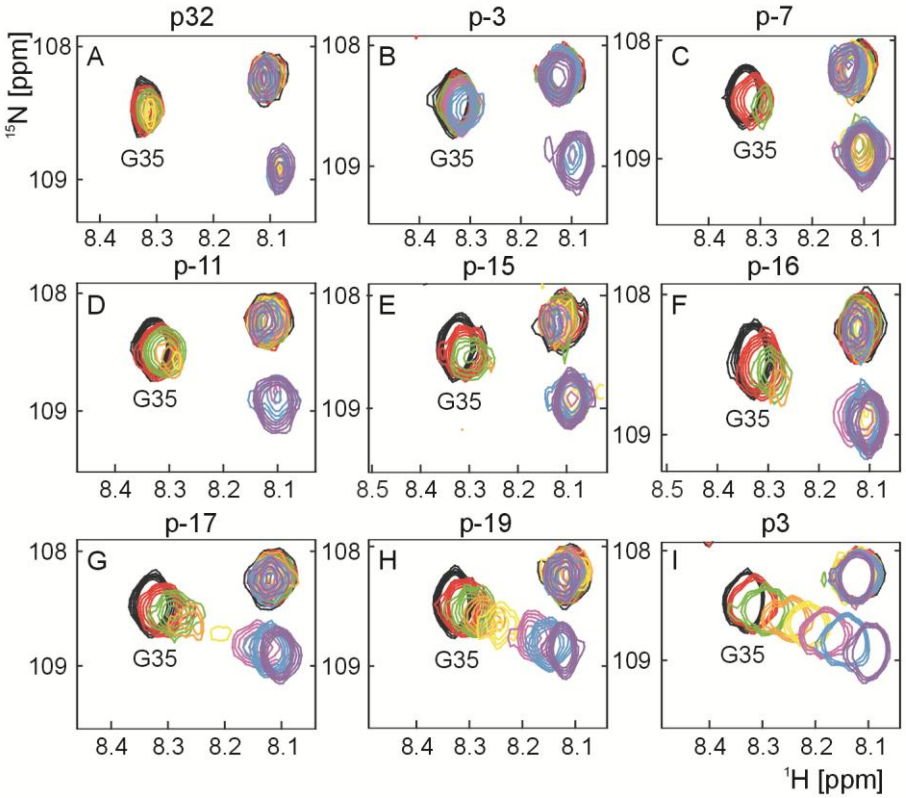

Figure 4.4. Detail from HSQC spectra of Src SH32 in titrations with peptides in which the distance separating the SH2- and SH3-binding sites has been gradually decreased from A) to H). For comparison the titration with peptide p3, containing only the SH3-binding site, is shown in I). Spectra from a few titration points are shown overlaid, with starting points (free protein) in black and titration end points shown in purple.

In contrast to what was expected, the SH3 binding curve for peptide p-19 does not display a sigmoidal binding curve, but chemical shift perturbations are observed for SH3 resonances already at low concentrations. This means that a significant fraction of the peptide binds to the SH3 domain, to an extent that would indicate a considerably larger binding affinity than what has been determined for the proline-rich sequence binding to the SH3 domain. In Fig. 4.5 a fit of the chemical shift perturbations to a 1:1 binding model is shown. It was assumed that the SH2 domain competes for the peptide with the SH3 domain, effectively decreasing the

concentration of the peptide available for binding the SH3 domain, at least at peptide-to-protein ratios smaller than 1. On the basis of this model, a correction factor for the peptide concentration was introduced as an additional parameter (for details see chapter 3). The best fit yields a correction factor of 3.6, with a binding constant of 1.0 ± 0.4 x 10^5 M^{-1} for the peptide interacting with the SH3 domain within SH32. This could suggest that about 30% of the peptide binds to the SH3 domain and 70% to the SH2 domain, which would imply a remarkably high affinity of the p-19 peptide for the SH3 domain, only about 2.5-fold less than for the SH2 domain.

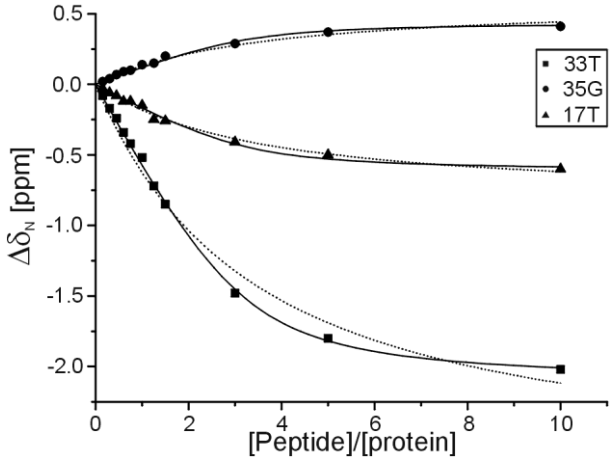

Figure 4.5. Chemical shift perturbations for a few SH3 domain amides within the SH32 tandem upon titration with peptide p-19. The solid line represents a fit to a 1:1 binding model, yielding a correction factor for the peptide concentration of 3.6, K_a = 1.0 ± 0.4 x 10^5 M^{-1}. For comparison the best fit without a correction factor is shown (dotted line), K_a = 1.3 x 10^3 M^{-1}.

The data suggest that the apparent affinity of the SH3 domain for its binding motif within the short peptide containing both an SH2- and SH3-binding site, p-19, is higher than the affinity of the SH3 domain for peptide p3, which contains only the SH3 binding site. Thus, the presence of the SH2 binding site in the peptide affects the binding of the SH3 motif to the SH3 domain within the context of the SH32 protein. The increased affinity of peptide p-19 for the SH3 domain cannot be

explained by interactions of the SH3 domain with the SH2 binding site in the peptide because the NMR experiments showed that peptide p2, containing the SH2 binding motif only, does not interact with the SH3 part of SH32. This conclusion is further supported by the fact that chemical shift perturbations in the SH3 domain caused by the interaction with peptide p-19 are very similar to those caused by binding of peptide p3, which does not contain the SH2-binding motif (Fig. 4.1).

Binding of SH32 to short peptides with bindings sites for both domains can be described by a thermodynamic cycle for the free protein and peptide, two 1:1 complexes and the 2:1 peptide-to-protein complex (Fig. 4.6A). Based on the experimental observations it can be concluded that the ratio of K_1 and K_3 is merely 2.5. The values of K_2 and K_4 remain as yet undetermined.

The data indicate a much tighter affinity for the SH3 domain for the peptide with the SH2 binding site than for p3. It is proposed that long-range electrostatic interactions between the negative charges surrounding the SH2 core binding motif and charges in the SH2 domain help recruit the peptide to the SH32. Such interactions have previously been shown to be important for SH2 domain-peptide interactions, increasing the affinity of the SH2 domain for the peptide more than 30-fold and, at the same time, resulting in a dynamic mode of interaction in spite of the high affinity (see chapter 3). Recruitment of the peptide to the SH32 domains via long-range charge-charge interactions leads to a dynamic encounter state, from which the peptide may associate with either the SH2 or the SH3 domain (Fig. 4.6B). Thus, SH3 binding profits from the formation of a relatively stable encounter complex, as has been observed for other complexes [100;218], increasing the affinity of the interaction. In this way the SH3 domain is able to compete effectively with the SH2 domain for peptide binding. The association constants for a second peptide will differ from the first one, because the positive charges on the SH2 domain will be shielded by negative charges on the peptide.

SH2 domains interact with their phosphotyrosine target sequences with a considerably higher binding affinity than SH3 domains interact with proline-rich sequences. For isolated domains the binding affinity of peptides for the SH3 domain-interaction can be around a thousand-fold lower than for the SH2 domain (see chapters 2 and 3). In light of this large difference in binding affinity one might raise the question how much the SH3 domains contributes to the interaction of Src with target proteins. The results presented here show that via a dynamic encounter state, the SH3 domain affinity can be higher in the context of a multidomain protein than what is observed for individual domains.

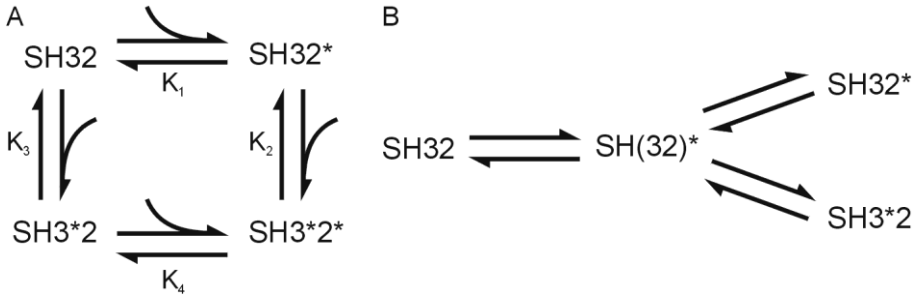

Figure 4.6. A) Model of interaction of Src SH32 with a peptide containing both SH2- and SH3-binding sites in which the distance between the sites is too large for a single peptide to bind simultaneously to both domains. In this model, a peptide first binds the SH32 tandem either via the SH3or the SH2 domain, followed by a second peptide binding the other domain. An asterisk symbolizes the domain bound to peptide, and K_1 to K_4 denote the equilibrium association constants. B) Electrostatic interactions between positively charged residues in the SH2 domain and negatively charged residues at the SH2-binding site in the peptide help recruit the peptide into an encounter complex with the SH32 domains. From the encounter complex the peptide can either bind to the SH2 or the SH3 domain, increasing K_3 compared to peptide binding to an isolated SH3 domain.

Conclusions

Including both the SH3- and SH2- binding motifs in peptides derived from FAK increases the affinity for binding to Src SH32, compared to the individual domains interacting with their respective binding sites. If the distance between the SH3- and

SH2-binding motifs is decreased to a point that the length separating them is no longer sufficient for the peptide to interact simultaneously with the SH3 and SH2 domains, the SH3 domain is still able to compete with the SH2 domain for peptide binding, in spite of the large difference in binding affinity for the isolated domains. We propose a model in which long-range electrostatic interactions between the negative residues surrounding the SH2-binding site and positive charges in the SH2 domain help recruit the peptide into a dynamic encounter complex, from which the peptide can bind either the SH3 or SH2 domain, increasing the affinity of the SH3 domain-peptide interaction. The distance between SH2- and SH3-binding motifs in Src target proteins differ, and, thus, such a dynamic intermediate may be a more general feature of SH3- and SH2 domain-mediated interactions.