Electrostatic interactions in the reconstitution of an SH2 domain from constituent peptide fragments

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Abstract

Fragment complementation has been used to delineate the essential recognition elements for stable folding in Src homology 2 (SH2) domains by using NMR spectroscopy, alanine scanning, and surface plasmon resonance. The unfolded 9-kD and 5-kD peptide fragments formed by limited proteolytic digestion of the N-terminal SH2 domain from the p85 α subunit of phosphatidylinositol 3'-kinase fold into an active native-like structure on interaction with one another. The corresponding 5-kD fragment of the homologous Src protein, however, was not capable of structurally complementing the p85 9-kD fragment, indicating that fragment complementation among these SH2 domains is sensitive to the sequence differences between the Src and p85 domains. Partial complementation and folding activity could be recovered with hybrid sequences of these SH2 domains. Complete alanine scanning of the 5-kD p85 fragment was used to identify the sequence recognition elements required for complex formation. The alanine substitutions in the p85 5-kD fragment that abolished binding affinity with the cognate 9-kD fragment correlate well with highly conserved residues among SH2 domains that are either integrally involved in core packing or found at the interface between fragments. Surprisingly, however, mutation of a nonconserved surface-exposed aspartic acid to alanine was found to have a significant effect on complementation. A single additional mutation of arginine to aspartic acid allowed for recovery of native structure and increased the thermal stability of the designed Src-p85 chimera by 18°C. This modification appears to relieve an unfavorable surface electrostatic interaction, demonstrating the importance of surface charge interactions in protein stability.

Keywords: Fragment complementation; SH2 domain; electrostatic interactions; protein stability

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Extensive site-directed mutagenesis studies of intact proteins have been used to reveal the fundamental interactions that stabilize protein structures. Buried hydrophobic interactions clearly play an essential role in stabilizing specific

folds (Richards 1977; Kellis et al. 1989; Sandberg and Terwilliger 1989; Bowie et al. 1990; Shortle et al. 1990; Lim and Sauer 1991; Eriksson et al. 1992; Jackson et al. 1993; Cordes et al. 1996; Dahiyat and Mayo 1997; Xu et al. 1998; Chen and Stites 2001), whereas the role of surface interactions is less clear (Hendsch and Tidor 1994, 1999; Meeker et al. 1996; Suckow et al. 1996; Schwehm et al. 1998; Sindelar et al. 1998; Cordes and Sauer 1999; Xiao and Honig 1999; Spector et al. 2000; Strop and Mayo 2000; Perl and Schmid 2001). Fragment complementation is another useful and sensitive phenomenon that can be used to analyze the interactions that stabilize protein structures. Certain single domain proteins retain the ability to form a folded non-covalent complex when cleaved into one or more fragments (for review, see de Prat-Gay 1996). These complexes

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Abbreviations: DTT, dithiothreitol; GST, glutathione S-transferase; HSQC, heteronuclear single quantum correlation; IPTG, isopropyl β -D-thiogalactoside; K_D , dissociation constant; MALDI TOF, matrix assisted laser desorption ionization time of flight; p85, p85 α N subunit of phosphatidylinositol 3'-kinase; SH2, Src homology II; SPR, surface plasmon resonance; Src, human Src SH2 domain.

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frequently exhibit both native-like structure and function; however, the constituent peptide fragments alone typically do not. Fragment complementation offers the opportunity to scrutinize the sequence elements necessary for productive protein folding and reveal the essential interactions necessary to stabilize the intact protein. We combine high-resolution structural data with a screen for binding activity between fragments to investigate protein stability. Fragment complementation, as a bimolecular reaction, has the advantage of providing greater sensitivity in folding studies because the complex is intrinsically less stable than the full-length protein, owing to the entropic penalty resulting from the cleavage of the polypeptide bond. In addition, it has the benefit of monitoring folding from conditions that do not require chaotropic disruption or high temperature (Berggård et al. 2001). Thus, fragment complementation is a valuable tool for the study of the specific-sequence elements that govern protein stability.

The N-terminal Src homology 2 (SH2) domain from the p85 α subunit of human phosphatidylinositol 3'-kinase (p85) can be specifically cleaved with trypsin into 5-kD and 9-kD fragments (Williams and Shoelson 1993). Reconstitution of peptide fragments results in a non-covalent complex that is essentially identical in structure to the native domain (Ojennus et al. 2001). This complex is active, albeit with reduced affinity for target peptide relative to the native protein (Williams and Shoelson 1993). The individual fragments are disordered in solution, indicating that recognition and folding of the non-covalent complex occurs through a folding-on-binding event (Ojennus et al. 2001). Because native structure is fully recovered in the complex, the interactions that stabilize the non-covalent complex also stabilize the full-length protein. Nearly 200 SH2 domains have been identified based on extensive sequence homology, allowing for identification of conserved sequence elements (see Electronic Supplementary Material). The interface between complementing fragments of the p85 SH2 domain is large ($2.36 \times 10^3 \text{ \AA}^2$ of buried surface area) and contains many highly conserved hydrophobic residues (Fig. 1).

In this study, the recognition elements required for the association and folding of a fragment reconstituted SH2 domain are elucidated by using a complete alanine scan to systematically delineate the important recognition elements for p85 complementation and protein folding. The information obtained in the complementation assay is shown to be applicable to understanding stability of the full-length protein.

Results

Swapping fragments between SH2 domains

Like p85, the homologous human Src SH2 domain (Src) forms a non-covalent complex when cleaved at the same BC loop as the p85 non-covalent complex (Fig. 1; Williams and Shoelson 1993). The p85 and Src SH2 domains share a sig-

Trypsin Cleavage Site

Figure 1. Backbone ribbon structure of the N-terminal p85 α SH2 domain (Protein Data Bank 2PNB). Limited tryptic digestion cleaves the domain at an exposed lysine (K52) within the flexible BC loop between the β B and β C strands of the domain. The resulting 5-kD and 9-kD fragments are colored blue and gray, respectively. The backbone of amino acids strictly conserved between p85 and Src are colored yellow (5 kD) and red (9 kD); closely conserved amino acids are displayed in green (5 kD) and orange (9 kD). Hydrophobic residues (L17, W22, Y23, L36, F44, L45, V46, Y57, L59, L61, and V90) at the interface of the 5-kD and 9-kD fragments are displayed.

nificant degree of sequence similarity (29% identity over the full protein, 42% identity in the 5-kD fragment; Fig. 2). The Src 5-kD fragment was tested for complementation activity to determine if the elements needed to form a stable non-covalent complex with the 9-kD fragment from p85 were also conserved (Pardon et al. 1995; Masaki et al. 2000; Mizuguchi et al. 2000; Louis et al. 2001). The N-terminal 5-kD fragment of the Src SH2 domain and the C-terminal 9-kD p85 SH2 domain fragment do not form a folded non-covalent complex, even when complementation was initiated from either chemically or thermally denatured states. A Src-p85 chimeric fusion protein comprised of the 5-kD fragment of Src and the 9-kD fragment of p85 did not express at any temperature or cell density tested, in contrast with the wild-type p85 and Src proteins, which express well under all conditions. This indicates that the Src-p85 chimera protein is incapable of folding and is rapidly proteolyzed within the cell on expression, indicating that the missing peptide bond in the non-covalent complex was not the source of destabilization.

Design and characterization of modified complexes and proteins

Because the Src and p85 SH2 domain sequences (Fig. 2), and structures are quite similar (backbone root mean square

Figure 2. Sequence alignment of the p85 and Src SH2 domain sequences and the engineered Srcm5, Srcm10, and Srcm11 constructs. Dashes represent amino acids that are not altered. The two domains have been aligned by the method proposed by Eck et al. (1993), and conserved elements of sequence are boxed. Secondary structural elements as defined by the crystal and solution structures (Booker et al. 1992; Waksman et al. 1992, 1993; Hensmann et al. 1994; Xu et al. 1995) are shown above and below the sequences, respectively. The lysine at which trypsin digestion produces 5-kD and 9-kD fragments marked with an asterisk. Color scheme representing conservation is described in Figure 1.

deviation of 1.9 Å), we investigated whether modest changes to the Src 5-kD sequence would induce folding with the p85 9-kD peptide. Initially, five sites in the Src 5-kD fragment sequence that appeared to be important for stabilization of the core were altered to the corresponding p85 sequence (I143L, F150W, S158V, E177D, and S178A; numbering scheme of Src; Fig. 2). The resulting peptide, Srcm5, has 55% sequence identity to the p85 5-kD peptide sequence (Fig. 2) and 75% conservation of residues at the complex interface. These changes were not sufficient to recover a folded complex by NMR. Therefore, an additional five changes (L161K, L163R, and P168A and deletions of E166 and N167) were made in the Srcm5 peptide to facilitate folding. The resulting Srcm10 peptide has 63% overall sequence identity and 81% identity over the 16 residues at the interface to the p85 5-kD sequence (Fig. 2). The ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) spectrum of a mixture of Srcm10 peptide with ^{15}N -labeled 9-kD p85 protein showed no evidence of folded complex formation. In contrast, the Srcm10-p85 chimeric protein could be expressed, albeit mostly as insoluble protein present in inclusion bodies. However, a small amount of soluble protein (<10% total protein production) could be obtained by expression at 25°C. This protein is apparently folded, as many of the unique chemical shifts observed for resonances in the native p85 ^1H - ^{15}N spectrum are recovered in the Srcm10-p85 chimera (Fig. 3), indicating that the protein likely adopts an SH2-like domain fold. Broad resonances in the center of the spectrum indicate the presence of a small

amount of unfolded protein in the sample. The stability of the protein was determined by reversible temperature denaturation monitored by one-dimensional proton NMR, following well-dispersed aliphatic and aromatic resonances. The melting temperature measured for the chimeric protein ($T_m = 32^\circ\text{C}$) is reduced by 25°C relative to native p85 ($T_m = 57^\circ\text{C}$; Williams and Shoelson 1993), revealing that the Srcm10-p85 chimera is significantly destabilized relative to native protein.

Analysis of complementation by surface plasmon resonance

Material limitations prevented the screening of a large number of peptides with NMR; thus, we investigated alternate techniques for assaying complementation. Macromolecular binding is readily observed with surface plasmon resonance (SPR) spectroscopy, and has been shown previously to be a valuable strategy for examining the interactions between complementing domain fragments (Kobayashi et al. 1999; Berggård et al. 2001). We set out to determine if SPR measurements of binding correlated well with the NMR data obtained on the chimeras studied thus far. Complementation between the 5-kD and 9-kD unfolded fragments of the SH2 domain from p85 was investigated by analysis of the binding of either free 5-kD fragment or the 5-kD fragment fused to glutathione S-transferase (GST) to a chip containing immobilized 9-kD fragment. Because the presence of a GST tag on the 5-kD fragment does not significantly affect the

Figure 3. ^1H - ^{15}N HSQC spectrum of the Srcm10-p85 chimera compared with wild-type p85 at 25°C. Superposition of spectra for a 50 μM sample of p85 (red) and for a 100 μM sample of Srcm10-p85 spectra (black). Peak dispersion indicates the Srcm10-p85 protein is folded.

SPR response (data not shown), all subsequent data were collected as GST-fusions of the 5-kD fragment to enhance the solubility of the peptide. The dissociation constant (K_D) for the binding of p85 9-kD fragment with the cognate GST-fusion 5-kD p85 peptide is $2 \pm 1 \mu\text{M}$, as determined by Scatchard analysis of the equilibrium SPR response as a function of analyte concentration. This apparent K_D for complementation in the low micromolar range is consistent with earlier estimates based on NMR data (Ojennus et al. 2001). As expected from the earlier NMR analysis, no binding is observed between the Src 5-kD fragment and the p85 9-kD fragment by SPR, whereas significantly reduced binding relative to wild type is observed with the p85 9-kD fragment and Srcm10 (Fig. 4). The K_D for the interaction between Srcm10 and p85 9-kD fragment is $70 \pm 5 \mu\text{M}$, which correlates well with the reduced stability of the Srcm10 chimera relative to wild-type protein. Based on these results, the SPR response at a long fixed time after injection is used to obtain relative apparent binding affinities in the alanine mutants.

Similar to the results obtained on complementation studies of calbindin D_{9k} (Berggård et al. 2001), as well as in several other systems (Corr et al. 1994; Fisher et al. 1994; O'Shannessy 1994; O'Shannessy and Winzor 1996; Sayers et al. 1998; De Crescenzo et al. 2000), the association data

could not be fit using a 1:1 binding model, precluding quantitative treatment of the association data. Effects on association and folding kinetics owing to proline isomerization have been observed in the fragment complementation systems of chymotrypsin inhibitor II (de Prat Gay et al. 1994) and thioredoxin (Chaffotte et al. 1997). Thus, the association kinetics may reflect *cis/trans* isomerization of the three proline residues present in the 9-kD fragment. In contrast, the dissociation phase is well fit by a single exponential, consistent with the dissociation arising from a single state, which has been previously characterized by NMR (Ojennus et al. 2001). The trends observed in the dissociation rates correlate very well with the equilibrium response.

Effect of alanine mutations on fragment complementation

Complete alanine scanning of the 5-kD p85 peptide was used to systematically delineate the important recognition elements for p85 complementation and protein folding. By replacing each amino acid of the p85 5-kD fragment individually with alanine and by screening for complementation activity, we were able to determine the contribution of each site to overall stability. The 5-kD fragment of the p85 non-covalent complex contains 37 residues that can be probed

Figure 4. SPR sensorgrams for binding of 20 μM samples of Src (dark blue), Srcm10 (green), and Srcm11 (red) peptides to immobilized p85 9k-His compared with wild type (light blue) 5-kD peptide. Injections were 60 μL , with flow rates of 30 $\mu\text{L}/\text{min}$ on a chip with an $R_{\text{max}} = 475$. The inset depicts the association phase for wild-type 5-kD and Srcm11 GST-fusions during a 1200-sec injection at 5 $\mu\text{L}/\text{min}$.

by alanine scanning mutagenesis. This set of single alanine mutants of the 5-kD fragment of p85 was screened for binding/complementation affinities to the p85 9-kD fragment by using SPR as a high-throughput assay. Sensorgrams for binding of each of these alanine mutants to immobilized 9k-His were acquired by using fixed injection times. The apparent binding affinity changes for single-point mutations span the full range from complete loss of binding affinity to a slight gain in activity.

The SPR data clearly reveal which residues in the p85 5-kD sequence radically affect complex stability (Fig. 5A) and are mapped onto the backbone structure in Figure 5B. Binding is completely abolished by mutation at positions W22, Y23, L36, F44, V46, R47, and D48 to alanine, whereas significant reductions (>50%) in binding activity are observed at L17, W24, S28, D41, G42, and L45. Somewhat increased apparent binding affinity is observed at sites R29 and T39.

Analysis of Srcm11-p85

The systematic screen of the p85 5-kD fragment revealed only one residue (D41) not incorporated into Srcm10 that had destabilizing effects of >50% of native binding when mutated to alanine, suggesting that this single amino acid is the only missing essential recognition element in folding of the Srcm10-p85 hybrid complex. The R41D mutation was therefore incorporated into the Srcm10 5-kD peptide to create the Srcm11 peptide. SPR analysis of complementation between the Srcm11 5-kD fragment and immobilized p85 9k-His shows complete recovery in complementation capability (Fig. 4). Scatchard analysis of the SPR data obtained at several analyte concentrations gives a K_D of $1 \pm 0.5 \mu\text{M}$

for the Srcm11 complementation, which is similar to the K_D of 2 μM measured on the cognate p85 complex and significantly tighter than the 70 μM K_D obtained for complementation with Srcm10.

To test if the complementation result extends to the full-length protein, the R41D mutation was introduced into the Srcm10-p85 chimera. In contrast to the other chimeric proteins cloned, the Srcm11-p85 chimera is well behaved, is completely soluble, and expresses in high yield. The ^1H - ^{15}N HSQC spectrum of the Srcm11-p85 chimera exhibits good peak dispersion and uniform line widths, characteristic of a stably folded protein (Fig. 6A). Many resonance assignments can be readily made by visual inspection and comparison to the native p85 spectrum. Reversible temperature denaturation monitored by one-dimensional ^1H spectra shows that the native-like structure melts out at 50°C (Fig. 6B). This is in dramatic contrast to Srcm10-p85, with a melting temperature of 32°C, and similar to the native p85 SH2 domain T_m of 57°C. Thus, the stability of the Srcm11-p85 chimera is approaching that of the native protein, indicating that the single additional change identified by alanine scanning indeed conferred dramatic enhancement in protein stability. A standard van't Hoff analysis of the reversible melting data for the Srcm10-p85 and Srcm11-p85 proteins gives a $\Delta\Delta G^\circ$ of -1.3 kcal/mole at 25°C for the charge-stabilizing mutation.

Discussion

Fragment complementation of homologous SH2 domains and designed peptides

Human Src and p85 are 29% identical in sequence, and exhibit 42% identity in the 5-kD segments. The human Src

Figure 5. (A) Effect of alanine substitutions on SPR response. The response after 112 sec into the injection of two runs for each mutant was averaged and normalized by the wild-type (w.t.) response. All data were collected with 20 μ M GST-5k at flow rates of 30 μ L/min. Native alanine residues are given wild-type responses and shown with open bars. (B) Mapping of the effects of alanine scanning on fragment complementation onto the p85 structure (Booker et al. 1992). The magnitude of the effects on relative wild-type affinity is indicated by color: green indicates >100% affinity; gray, >90% affinity; yellow, 50% to 90% affinity; orange, 50% to 5% affinity; and red, <5% affinity.

SH2 domain has also been shown to form a non-covalent complex when cleaved at the same BC loop as the p85 non-covalent complex (Williams and Shoelson 1993). However, this degree of similarity is insufficient to obtain a stable non-covalent complex between mixed homologs. The lack of complex formation is not because the protein is folding from fragments, as a Src-p85 chimeric protein formed by connecting the two fragments also does not fold. The inability of the Src and p85 fragments to complement is in contrast to the sequence tolerance observed in the fragment complementation of thioredoxin, which forms a stable chimeric protein between human and *Escherichia coli* homologs (Tasayco et al. 2000; Louis et al. 2001) that also have high structural similarity (backbone root mean square deviation of 1.6 Å) and similar (25%) sequence identity.

Native-like structure was observed in the thioredoxin chimera, although the stability of the resulting chimeric protein was reduced by 3.3 to 4.0 kcal/mole from the native sequences. Swapping of more highly homologous sequences is well tolerated in α -lactalbumin, in which swapping a 34-amino-acid region with 62% identity between homologous human and bovine α -lactalbumins or a 16-amino-acid region with 30% identity of bovine α -lactalbumin into human lysozyme is tolerated (Pardon et al. 1995; Masaki et al. 2000; Mizuguchi et al. 2000). The elements required for formation of a stable fold were conserved between homologs in these cases, whereas in the SH2 domains studied here, evolutionary divergence has resulted in the loss of complementarity between homologous proteins.

The SH2 domain sequence and structure alignments were used to design two derivatives of the Src 5-kD sequence potentially capable of forming a stable complex with the 9-kD p85 fragment (Fig. 2). Although the modifications improved the stability of the complex and chimeric protein somewhat, the changes were not sufficient to recover a fully stable protein. The Srcm10-p85 covalent protein chimera is clearly folded by NMR (Fig. 3); however, it is much less stable than the native protein ($T_m = 32^\circ\text{C}$ compared with 57°C for wild-type p85). Either an essential molecular recognition element needed for formation of a stable native-like non-covalent complex was lacking or a negative element had been added. The only residues that are not identical between the Srcm10 and p85 sequences are found in highly variable solvent exposed positions (Fig. 2). Consequently, a systematic approach was used to identify the missing sequence elements needed for formation of a stable chimeric protein.

Determination of essential sequence elements in the fragment complementation of p85

Alanine scanning of the p85 5-kD fragment and subsequent analysis of binding capability to the cognate 9-kD p85 fragment revealed the contributions to stability imparted by each residue. The effects of replacement of a side-chain with alanine on binding affinity span a wide dynamic range, varying from complete loss of binding to slightly enhanced binding affinity relative to wild type (Fig. 5A,B). Overall, the sites that are critical for stability are consistent with our understanding of protein structure. Although the 5-kD fragment is unfolded and contains no identifiable residual structure (Ojennus et al. 2001), we can not rule out the possibility of contributions from the unfolded state that are transient and can not be detected by NMR. Contributions to folding from the unfolded state have been proposed to exist in fragments of thioredoxin based on a calorimetric analysis (Georgescu et al. 2001). In addition, it has been shown that long-range electrostatic interactions can occur in the unfolded state, and could also contribute to the effects ob-

Figure 6. (A) ¹H-¹⁵N HSQC spectrum at 25°C of a 150-μM sample of Srcm11-p85 chimera (red) superimposed with the spectrum for a 50 μM sample of native p85, illustrating the similarity between spectra. Recovered cross-peaks for G42, G64, and G65 are indicated. (B) Temperature denaturation of Srcm11-p85 chimera. One-dimensional proton spectra taken from 25°C to 50°C on a 150-μM sample of Srcm11-p85. Arrows indicate well-resolved native peaks.

served here (Kuhlman et al. 1999; Pace et al. 2000; Whitten and Garcia-Moreno 2000; Wong et al. 2000).

The overall pattern of tolerance to substitution observed here correlates well with thorough scanning mutagenesis studies from a number of other proteins, including Arc repressor (Milla et al. 1994; Sauer et al. 1996), lac repressor (Suckow et al. 1996), BPTI (Yu et al. 1995), and T4 lysozyme (Rennell et al. 1991; Matthews 1996). As anticipated, the critical residues for complementation are the highly conserved hydrophobic residues packed in the core (strands βA and βB) or are buried at the interface between fragments in strand βB (Fig. 5B). For example, W22 is a strictly conserved residue whose side-chain packs in the core and makes extensive contacts with both 5-kD and 9-kD fragment residues. Y23, L36, F44, and V46 are also highly conserved and buried in the structure at the interface in the non-covalent p85 complex. R47 and D48, the only charged residues with removal that abolishes binding activity, are also located in the βB strand at the complex interface. The remaining sites in the βA and βB strands, W24 and L45, both exhibit severe loss of binding on substitution. The αA helix is more tolerant to substitution, although alanine scanning identified two sites, S28 and L36, that are important for stability. This helix does not interact significantly with residues from the 9-kD region, indicating that substitutions would be tolerated as long as the overall α-helical propensity is maintained. Alanine scanning of α helices in T4 lysozyme and λ repressor reveals a similar pattern. In these systems, sequence conservation is required for 20% to 30% of residues within an amphipathic helix (Blaber et al. 1995; Gregoret and Sauer 1998). The only site in the N-terminal tail that was found to be important for complex stability is L17. Even though it occurs before the canonical “first” resi-

due of the SH2 domain, L17 appears to stabilize the fold through close association with the surface exposed V90 and V91. Arginine 29 is an interesting case. It is strictly conserved across SH2 domains for function and is essential for phosphopeptide binding activity, as the R29 side-chain makes a cation-π interaction with the tyrosine ring of the bound peptide. Enhanced complementation was observed when R29 was replaced with alanine, implying that this residue is conserved for activity, not stability. The loops are generally tolerant to substitution, with the exception of a conserved glycine residue (G42) that occupies a restricted area of φ/ψ space (φ = 157°, ψ = 67°) and the critical D41, located in the loop between αA and βB.

Discovery of a stabilizing surface electrostatic interaction

The Src-p85 hybrid sequence design was reexamined in the context of the alanine scanning data for missing sequence elements required to stabilize the non-covalent complex. Most of the residues selected for mutation in Srcm10 were shown by alanine scanning to be important for complex formation (Fig. 2). The only mutation lacking a clear rationale for a large effect on binding is D41A. This residue is located in the solvent exposed loop between αA and βB and is an arginine in the Src SH2 domain (Fig. 7). Based on initial examination of the SH2 domain structure, it is not readily apparent why D41 is required at this position, other than it has a preference for being located in turns owing to its small hydrophilic nature. However, the additional change of an arginine to aspartate (R169D) in the Srcm11 sequence led to recovery of binding affinity of the 5-kD fragments to the immobilized 9k-His compared with the Src and Srcm10

Figure 7. Electrostatic surface maps for p85 (A) and Src (B) generated by using GRASP (Nicholls et al. 1991). Regions of positive potential are colored blue; regions of negative potential, red. The loop between α A and β B in both SH2 domain structures is shown to illustrate molecular contacts in the structures of p85 (C) and Src (D). Key neighboring residues of D41 in the p85 structure (Booker et al. 1992) and neighboring residues of R169 in the Src structure (Xu et al. 1995) are indicated.

peptides (Fig. 4), as well as a dramatic increase in the thermal stability (T_m) of the Srcm11-p85 chimeric protein relative to the Srcm10-p85 protein (50°C versus 32°C). A van't Hoff analysis of the melting data indicate that the Srcm11-p85 chimeric protein is stabilized by -1.3 kcal/mole relative to the Srcm10-p85 protein.

The charge swapping mutation has a pronounced effect on the recovery of structure in the protein. Evidence for recovery of native structure on arginine-to-aspartic acid mutation at position 169 in Src (corresponding to position 41 in p85) is readily apparent by comparison of the ^1H - ^{15}N HSQC spectra of the Srcm10-p85 and Srcm11-p85 proteins. The spectrum for the chimeric Srcm11-p85 protein, shown in Figure 6A, displays many features that indicate that the overall structure is more like that of the native p85 structure than that of Srcm10-p85 (Fig. 3). For example, the cross-peaks of G64, G65, and G42, all of which are close in the structure to D41, are either too weak to be observed or are dramatically shifted in the Srcm10-p85 spectrum. Based on these well-dispersed cross-peaks, it seems that the presence of an arginine at this site in Srcm10 introduces changes in the local protein architecture that are destabilizing. However, these cross-peaks reappear at their native chemical shifts in the Srcm11-p85 spectrum indicating a more native-like conformation in the Srcm11-p85 chimera.

The structures of p85 and Src were reexamined in the region of position 41 to investigate the nature of the interactions rendering this site critical to complex stability. The side-chain of D41 in p85 and the corresponding R169 in Src are solvent exposed and in a loop that does not form any obvious interactions that would stabilize the non-covalent complex (Fig. 7). The presence of a much larger side-chain (van der Waals volume of R is $148 \text{ \AA}^3$ and of D is $91 \text{ \AA}^3$) might simply perturb the structure at the surface of the p85 9-kD fragment in the non-covalent complex in a way that is accommodated in Src. However, the reduced fragment

complementation activity of the D41A p85 5kD peptide argues that charge is critical because alanine is more comparable in volume to aspartate than to arginine (van der Waals volume of A is $67 \text{ \AA}^3$). The large stability conferred by the charge-swapping mutation indicates that an electrostatic interaction is important. Indeed, there are several positively charged residues (R62, K63, K68, and K108) near D41 in p85 (Fig. 7C). In particular, R62 and K63 are within $8 \text{ \AA}$ of the D41 carboxyl side-chain. The inherent flexibility of the loop and side-chains may allow D41 to interact even more closely with these residues than is apparent in the average NMR structure. A negative charge may be required in this location of p85 to balance the overall positive charge of the nearby surface area. Alternatively, the positive charge of arginine introduced with the Src 5-kD fragment in the swapping experiment may introduce an unfavorable interaction. Such an unfavorable interaction would explain the inability of Srcm10 to form a high-affinity complex and the lower thermal stability of the Srcm10 chimeric protein. In the Src structure, R169 packs closely to D190 and D192 on the surface of the protein (Fig. 7D). Interestingly, the negatively charged D190 and D192 in Src correspond in position to the positively charged K63 and R169 in p85. The arginine-to-aspartate charge swap may satisfy an electrostatic interaction that is conserved yet covaries between the Src and the p85 SH2 domains.

Several studies have addressed the contribution of surface exposed salt bridges to protein stability. Until recently, it was believed that electrostatic interactions derived from charged surface residues contributed minimally to the overall stability of protein folds, based on studies in T4 lysozyme, barnase, staphylococcal nuclease, and myoglobin (Daopin et al. 1991; Sali et al. 1991; Meeker et al. 1996; Ramos et al. 1999). The small contribution of surface electrostatic interactions was thought to be caused by the unfavorable entropic and desolvation penalties associated with salt-bridge formation (Hendsch and Tidore 1994). In

recent years, however, a growing number of surface electrostatic interactions that contribute energetically to the protein fold have been reported (Anderson et al. 1990; Marqusee and Sauer 1994; Vetriani et al. 1998; Grimsley et al. 1999; Ibarra-Molero et al. 1999; Loladze et al. 1999; Xiao and Honig 1999; Strop and Mayo 2000; Takano et al. 2000; Martin et al. 2001; Sanchez-Ruiz and Makhataadze 2001). Typically, as in the case of T4 lysozyme, proteins can be stabilized by 3 to 5 kcal/mole by the addition of a partially exposed salt bridge (Anderson et al. 1990). Indeed, proteins surfaces may be optimized for electrostatic interactions (Spasov et al. 1994). Recently, the relief of unfavorable surface charge interactions has been shown to have a significant effect on stability. Modification of a surface-exposed arginine in the peripheral subunit binding protein, located in an area of overall positive potential, increased stability of the protein even when replaced by a hydrophobic residue (Spector et al. 2000). In a related finding, the *Bacillus caldolyticus* cold-shock protein was stabilized by a glutamic acid mutation. The source of stabilization was a combination of additional hydrophobic interaction and the relief of a pair-wise Glu–Glu interaction on the surface of the protein (Perl and Schmid 2001). Finally, ubiquitin has been shown to be alternately stabilized or destabilized by judicious modification of surface charges (Loladze et al. 1999).

In the present case of complementing peptides from SH2 domains, the Src-p85 complementation experiment introduced an unfavorable electrostatic interaction at the surface by introducing a positive charge from Src into a positively charged region of the p85 protein. The native aspartate residue appears to be necessary to satisfy electrostatic interactions at the surface of the protein because the change at this site covaries between the Src and p85 sequences. Knowledge of the stabilizing effect D41 has on complex formation allowed for the rescue of the designed Srcm10 peptide. The single R41D change in the Srcm10 sequence improved the binding affinity dramatically, indicating that the electrostatic contribution of this surface exposed residue is essential for stable complex formation. This dramatic stabilization extended to a chimeric Srcm11-p85 protein, in which the single mutation R41D recovered both native structure and thermal stability. This study demonstrates the ability of fragment reconstitution to probe interactions important in stabilizing protein folds and reveals an important example of the contribution of surface electrostatic interactions to protein stability.

Materials and methods

Materials

Primers for site-directed mutagenesis were purchased from Invitrogen. *E. coli* cell stocks were obtained from Novagen. All solu-

tions and media were prepared with chemicals purchased from Fisher and Sigma. The affinity- and metal-chelating columns were purchased from Pharmacia. Reversed-phase columns were obtained from Vydac. All reagents for peptide synthesis were purchased from NovaBiochem or AP Biosystems. Materials for surface plasmon resonance studies were purchased from Biacore. Centrifugation devices for concentration were obtained from Amicon. Vitamin mix for growths in minimal media was purchased from GIBCO BRL.

Generation and purification of p85 peptide fragments

His-tagged p85 protein and fragments were prepared as previously described (Ojennus et al. 2001). Full-length His-tagged protein was purified on a metal-chelating column (Pharmacia) charged with nickel. Fragments were generated by limited proteolysis with trypsin, purified by high-pressure liquid chromatography on a reversed-phase semi-preparative C18 column, and lyophilized for storage.

Synthesis of peptides

The 5-kD peptides of p85, Src, and Src mutants were synthesized, as previously described (Ojennus et al. 2001), on an ABI 433A peptide synthesizer by using standard 9-fluorenylmethoxycarbonyl chemistry for batch-wise solid-phase peptide synthesis on a 9-fluorenylmethoxycarbonyl–Lys(Boc)–Wang resin. Final deprotection of side-chains and cleavage from the resin were performed by treatment of the resin with trifluoroacetic acid. The peptides were then purified by reversed-phase high-pressure liquid chromatography on a C18 semi-preparative column. Fractions were lyophilized, and the mass of the peptides was confirmed by matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry.

Construction, expression, and purification of a Src-p85 and Srcm10-p85 chimera

Standard recombinant techniques were used to create a pET21a plasmid coding for the N-terminal 5-kD fragment of the Src SH2 domain and the C-terminal 9-kD fragment of the p85 SH2 domain. The Src insert was ligated into the plasmid containing the p85 9-kD sequence by using *Nde*I and *Nsi*I restriction enzyme sites. *E. coli* BL21(DE3) cells were transformed with the plasmid, and single colonies were grown to an O.D.₆₀₀ of 0.4, 0.7, or 1.0, at which time they were induced with 1 mM isopropyl- β -D-thiogalactoside (IPTG). Time points were collected over the course of 16 h at 16°C, 25°C, and 37°C and analyzed for induction of protein expression by SDS-PAGE.

Standard mutagenesis techniques were used to insert the N-terminal tail of seven amino acids of Src into the Src-p85 chimera and to make point mutations required in the design of Srcm5 and Srcm10. *E. coli* BL21(DE3) cells were transformed with the protein expression plasmids, and single colonies were grown to an O.D.₆₀₀ between 0.5 and 0.9, followed by induction with 1 mM IPTG and growth at 25°C. Time points during protein expression were collected at 0, 2, and 4 h and overnight. Cells grown for 4 h after induction were lysed by French press, and insoluble material was removed by centrifugation. The cell lysate was passed over a Pharmacia 5 mL Sp Sepharose cation exchange column in 20 mM potassium phosphate buffer (pH 7.2), 0.25 mM Na₂EDTA, 0.02% Na₃, and 20 mM NaCl. Protein was eluted from the column at 1

mL/min with a salt gradient up to 1 M NaCl in the same buffer. Fractions were combined, dialyzed, and concentrated into NMR buffer (see below).

Expression and purification of GST-5-kD peptide fusions

Standard cloning techniques were used to make a GST fusion of the 5-kD fragment of p85. The insert coding for the 5-kD fragment (residues 12–52 of p85) was cloned into the expression vector pGEX-2T with *Bam*HI and *Eco*RI restriction enzyme sites. BL21 *E. coli* cells were transformed with the plasmid and grown at 37°C to an O.D.₆₀₀ between 0.4 and 0.7. Protein expression was induced by addition of 1 mM IPTG, and the cells were grown for 2 to 4 h at 37°C.

GST-fusion proteins were purified from the soluble fraction of cell lysates by affinity chromatography using a 5 mL glutathione affinity column. Protein was eluted in five column volumes of 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. The GST-fusion proteins were dialyzed into 50 mM potassium phosphate (pH 7.4), 50 mM NaCl, and 2 mM dithiothreitol (DTT) and concentrated by using Centricon-10 spin concentrators. Fusion proteins were then lyophilized for storage. The samples were readied for use by resuspension in the same volume of buffer as before lyophilization with 1 mM DTT.

NMR experiments

Uniformly ¹⁵N-isotopically labeled protein was produced by expression in minimal media containing the following: 6.7 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1.5 g/L NaCl, 2 g/L glucose, 10 mL/L Basal Medium Eagle Vitamin solution, 1.62 μg/L FeCl₃, 2.86 μg/L H₃BO₃, 15 mg/L CaCl₂ · 2H₂O, 40 μg/L CoCl₂ · 6H₂O, 200 μg/L CuSO₄ · 5H₂O, 200 mg/L MgCl₂ · 6H₂O, 2 μg/L MoO₃, 200 μg/L ZnCl₂, 50 mg/L ampicillin, and 1.5 g/L (¹⁵NH₄)₂SO₄. All NMR samples were prepared in 50 mM potassium phosphate buffer (pH 5.8), 50 mM NaCl, 0.02% NaN₃, and 10% D₂O unless otherwise specified. Experiments were run on a Unity^{inova} 500-MHz spectrometer, and spectra were processed with NMRPipe software using a cosine apodization function and one round of zero-filling (Delaglio et al. 1995). Typical ¹H-¹⁵N HSQC spectra were obtained with 2048 points and 128 t₁ increments by using a gradient sensitivity-enhanced pulse sequence (Silver et al. 1984; Kay et al. 1992a,b; Farrow et al. 1994; Bendall 1995). Watergate (Piotto et al. 1992) one-dimensional proton spectra were obtained with 2048 points.

Surface plasmon resonance analysis

All SPR analysis was performed at 25°C on a BiacoreX system using Biacore CM5 research grade sensor chips. Immobilization of the His-tagged 9-kD p85 peptide was achieved by amine coupling of the fragment. Attachment of the 9-kD peptide to the chip via an engineered unique C-terminal cysteine residue yields similar data. The running buffer for immobilization was Biacore HBS-N buffer (10 mM HEPES at pH 7.4, 150 mM NaCl), and the 9-kD fragment was in 50 mM potassium phosphate buffer (pH 5.8) and 50 mM NaCl. Immobilization of between 1000 to 2000 response units was achieved by a 1-min injection of 3 μM 9-kD fragment at 5 μL/min. Running buffer for all binding experiments was 50 mM potassium phosphate (pH 7.4), 50 mM NaCl, 2 mM DTT, and 0.005% P20 surfactant. Complete regeneration of the 9-kD p85 immobilized

chip was achieved with a 30 μL injection of 8 M urea at 30 μL/min. Concentrations of GST-fusion proteins were calculated assuming complete formation of GST dimers. Alanine scanning runs were performed by using analyte injections of 60 μL at 30 μL/min. All alanine mutants were screened sequentially on the same sensor chip, and runs were fully reproducible. To monitor the integrity of the chip throughout the screen, data on the wild-type peptide were recollected after every seven mutant runs, and a total of just 3% loss in signal was observed by the end of the mutant screen. Equilibrium data were collected for several concentrations of analyte with injections of either 60 μL or 100 μL at flow rates of 5 μL/min. Data from a reference cell was collected during all injections and subtracted from SPR data. The kinetics of the association and dissociation phases were measured at several flow rates from 5 to 75 μL/min. The kinetic rates measured were not affected by flow rate, demonstrating that the system is not mass-transfer limited. The relative binding affinity was examined by comparing the responses for each mutant to that of wild type at 112 sec into a 60 μL injection run at 30 μL/min.

Electronic supplementary material

The supplementary figure depicts the sequence alignment of 58 human SH2 domains generated by ClustalW (Thompson et al. 1994). Identical residues to the human p85 sequence are shaded dark yellow; similar residues, light yellow. The p85 numbering scheme, which follows the numbering scheme for the solution structure of the bovine p85 SH2 domain (Booker et al. 1992; Ojennus et al. 2001), is above the alignment.

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