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Molecular Recognition Properties of FN3 Monobodies that Bind the Src SH3 Domain

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Summary

We have constructed a phage-displayed library based on the human fibronectin tenth type III domain (FN3) scaffold by randomizing residues in its FG and BC loops. Screening against the SH3 domain of human c-Src yielded six different clones. Five of these contained proline-rich sequences in their FG loop that resembled class I (i.e., +xxPxxP) peptide ligands for the Src SH3 domain. The sixth clone lacked the proline-rich sequence and showed particularly high binding specificity to the Src SH3 domain among various SH3 domains tested. Competitive binding, loop replacement, and NMR perturbation experiments were conducted to analyze the recognition properties of selected binders. The strongest binder was able to pull down full-length c-Src from murine fibroblast cell extracts, further demonstrating the potential of this scaffold for use as an antibody mimetic.

Introduction

In the post-genomic era, truncated antibodies such as Fab (fragment antigen binding) and scFv (single-chain fragment variable) antibody fragments, which have been produced by display technologies, have been gaining increased use as tools for studying protein function. Despite their utility, they are frequently hard to work with due to instability and low yields in bacterial expression systems. In addition, the antibody fragments contain disulfide bonds and generally do not fold properly when expressed in the reducing environment of the cytoplasm. Therefore, other proteins have been explored as alternative scaffolds for use as antibody mimetics. These proteins offer a stable framework and solvent-exposed residues responsible for ligand recognition, which can be randomized to confer novel binding activities to the parent molecule. Such scaffolds include human cytotoxic T-lymphocyte associated protein-4 (CTLA-4) [1], Z domain of *Staphylococcal* protein A [2], lipocalins [3], green fluorescent protein [4] (see Note Added in Proof), and the tenth type III domain of human fibronectin (FN3) [5].

Of these, FN3 is a monomeric, 94 residue domain, with an immunoglobulin-like fold formed by seven antiparallel β strands (Figure 1). The strands (A, B, C, D, E, F, and G) are connected by three loops on each side of the protein (i.e., loops BC, DE, and FG on one side and loops AB, CD, and EF on the opposite side). All of the loops, except the EF loop, have been shown to tolerate insertions of four glycine residues with little destabilization [6], demonstrating that five of the loops can be used as sites for grafting and/or randomization of residues. FN3 is an excellent candidate as an antibody mimetic because of its small size, high solubility, thermal stability [7], and lack of disulfide bonds, which permits intracellular expression [8]. The first FN3 library randomized five residues in each of the BC and FG loops and shortened the FG loop by three residues to give the protein a more planar binding surface [5]. Selection experiments using this M13 bacteriophage-displayed library yielded relatively modest affinity binders to ubiquitin ($IC_{50} = 5\ \mu M$), although these also crossreacted with dextran. The potential of this scaffold was later explored in a yeast two-hybrid system, in which seven residues in the FG or the AB loop were randomized and clones were selected for their ability to bind the estrogen receptor α ligand binding domain [8]. Interestingly, the screen yielded conformation-specific binders for the agonist- or antagonist-bound forms of the receptor. Finally, an mRNA display library of greater than 10^{12} individual clones was created by randomizing a total of 21 residues in the BC, DE, and FG loops [9]. A large number of high-affinity binders for TNF-α with K_d values between 1–24 nM were obtained from this library, further indicating the potential of this scaffold as an antibody mimetic. Subsequent affinity maturation experiments yielded binders with dissociation constants as low as 20 pM, showing that nanomolar binding constants are not an affinity ceiling for this scaffold [9].

To explore the potential of this scaffold further, we constructed a phage-displayed FN3 library of 2×10^9 individual clones, where the residues in the BC and FG loops were diversified. Screening the library for binders to the SH3 domain of the proto-oncogene c-Src, a non-receptor tyrosine kinase that plays a critical role in eukaryotic signal transduction, yielded six different FN3 (hereafter referred to as "monobody," as coined by Koide et al. [5]) clones [10]. Herein we report the analysis of these monobodies. Our work extends the utility of this domain as a suitable scaffold for antibody mimetics.

Results

Isolation of Monobodies that Bind to the Src SH3 Domain

We constructed an M13 phage library displaying FN3 in which five residues in both the BC loop and the FG loop were randomized. The library consisted of 2×10^9

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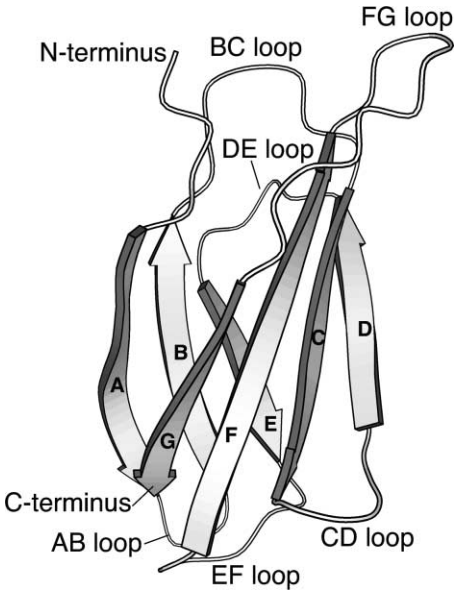

Figure 1. Schematic Drawing of FN3

The seven β sheets that comprise the domain and the connecting loops are labeled.

individual clones and displayed the FN3 variants in a monovalent fashion. The library was screened for binders to the SH3 domain of the human c-Src protein (Src SH3), which was biotinylated in vivo and immobilized on streptavidin-coated magnetic beads. After three rounds of affinity selection, 192 clones were tested for binding to the Src SH3 domain: 22 were positive and DNA sequencing revealed six unique sequences (Table 1). The 1C9 clone was recovered 15 times, the 1F11 clone occurred three times, and the other four clones were "singletons." While the sequences of the BC loops varied considerably between the clones, five of the six monobodies contained proline-rich sequences in their randomized FG loop regions. Clone 1E3 differed from the others by an FG loop sequence that was not proline-rich and contained a pair of cysteine residues. Four clones (1C9, 1F11, 1F10, and 2G10) contained two nucleotide deletions in the monobody framework, preceding the FG loop, and one nucleotide deletion in the randomized region of the FG loop, which led to the restoration of the reading frame and alteration of one or two framework residues.

Specificity and Affinity of the Monobodies

Binding affinity and specificity of the six different clones were determined using two types of experiments. As can be seen by phage ELISA (Figure 2A), binding was specific to the Src SH3 domain, as the phage clones bound neither streptavidin nor an unrelated biotinylated protein (APC1336, an open reading frame of unknown function from *Bacillus subtilis*), which carried the same vector-encoded flanking peptide sequences as the Src SH3 domain. All of the clones, except for 1E3, appeared to have similar relative binding strengths to the Src SH3 domain. To examine whether the clones retained their binding properties outside of the phage context, we then tested these clones as N-terminal fusion proteins to a highly active variant of the *E. coli* alkaline phosphatase (AP) protein [11]. Because AP requires an oxidizing environment for the correct formation of disulfide bonds required for its stability and activity, the fusion constructs were expressed from a plasmid that includes a periplasmic localizing signal peptide sequence. As shown in an enzyme-linked binding assay (ELBA), the six clones were confirmed to bind specifically to the Src SH3 domain, but with somewhat differing binding strengths (Figure 2B). Thus, both the phage ELISA and ELBA experiments showed that the binding of the monobodies selected from the library to their target is highly specific.

To determine the binding affinity of the monobodies, the dissociation constants of the strongest and weakest binding proteins were measured by isothermal titration calorimetry (ITC). Gene fragments encoding 1F11 and 1C9 were cloned into an expression vector and purified to homogeneity. The purified proteins were dialyzed thoroughly against PBS buffer and titrated into the sample cell of an isothermal calorimeter containing the Src SH3 domain also in PBS buffer. A representative ITC experiment between Src SH3 and 1F11 is shown in Figure 3. The dissociation constants were determined to be 0.25 ($\pm$0.02) μM and 1.3 ($\pm$0.1) μM for 1F11 and 1C9 binding the Src SH3 domain, respectively. The number of binding sites (N) for 1F11 and 1C9 on Src SH3 was calculated to be approximately 1.0, as expected ($N = 0.977 \pm 0.009$ for 1F11; $N = 1.035 \pm 0.021$ for 1C9). No quantitative differences were observed in comparing the binding of the two monobodies to either biotinylated or nonbiotinylated forms of the Src SH3 domain (data not shown).

To determine the binding specificity of the monobodies, 1F11 and 1E3 were examined for their binding to

Table 1. Analysis of the Src SH3 Domain Binders

Clone	BC Loop	FG Loop	Consensus (RxxPxxP)	Frequency	Kd (μM)
1C9	DAP**SGPLD**YYR	A-Y**SLSPP**—SKP	**LSPPSKP**	15	1.3
1F11	DAP**GISQG**YYR	A-Y**SRPLP**—SKP	**RPLPSKP**	3	0.25
1F10	DAP**MALTA**YYR	A-Y**QRALP**—SKP	**RALPSKP**	1	ND
2G10	DAP**RTPKH**YYR	-LY**QLPLP**—SKP	**LPLPSKP**	1	ND
2B2	DAP**CQHNL**YYR	AVT**KRPLP**—SKP	**RPLPSKP**	1	ND
1E3	DAP**FQTIQ**YYR	AVT**LCARC**—SKP	NA	1	ND
Wild-type	DAP**AVTVR**YYR	AVT**GRGDSPAS**SKP	NA	–	ND

Amino acid sequences in the predicted BC and FG loops of the FN3 monobodies are in bold, sequences surrounding the loops are underlined, and dashes indicate deletions. Consensus amino acids in bold and underlined are essential and preferred, respectively, compared to the optimal class I peptide ligand (RPLPPLP) of the SrcSH3 domain [14]. NA, not applicable; ND, not determined.

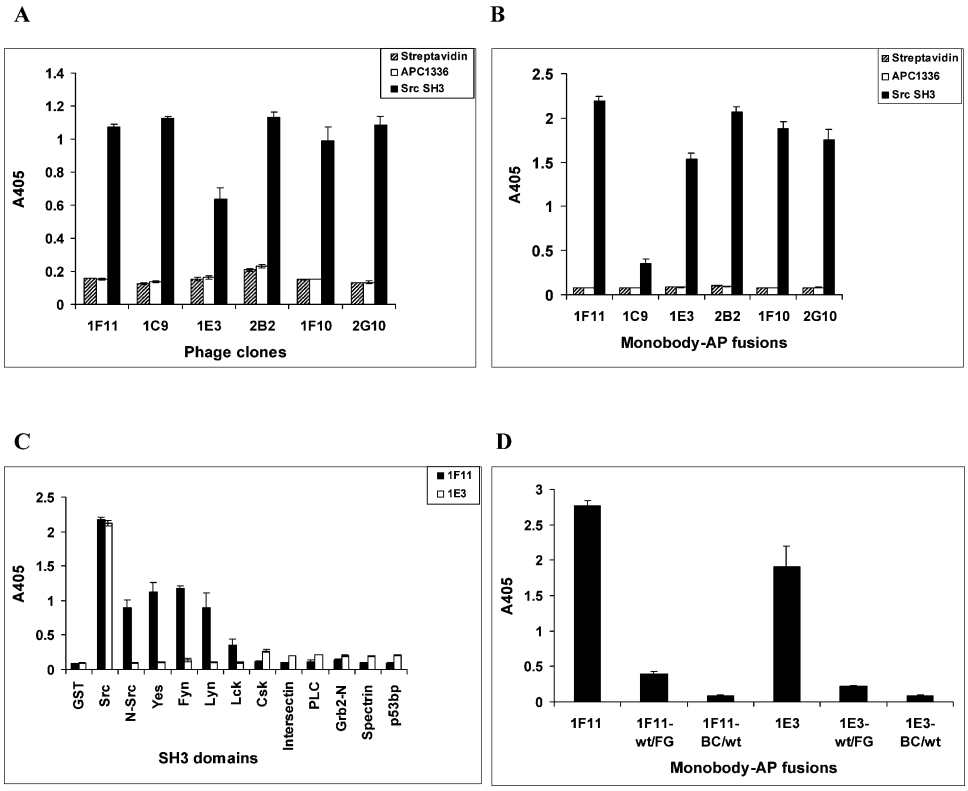

Figure 2. Analysis of the Anti-Src SH3 Monobodies

(A and B) (A), Phage ELISA; (B), ELBA. Assays were performed in duplicate for phage ELISA and triplicate for ELBA. Wells of microtiter plates were coated with 400 ng (for ELISA) or 200 ng (for ELBA) of streptavidin. Two hundred nanograms (for ELISA) or 100 ng (for ELBA) of biotinylated Src SH3 or an unrelated biotinylated protein expressed from the same plasmid as Src SH3 was immobilized in the wells. After blocking with 1% BSA, clonal phage supernatants or monobody-AP fusions were added to the wells for ELISA and ELBA, respectively. Binding was colorimetrically detected by incubation with anti-M13 antibody-HRP conjugate, followed by addition of the HRP substrate, 2′,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid, or by addition of the AP substrate, para-nitrophenyl phosphate. Streptavidin only (striped bars), control protein (white bars), Src SH3 (black bars).

(C) Affinity of the anti-Src SH3 monobodies 1F11 and 1E3 for other SH3 domains. ELBA assays were performed using GST fusions of SH3 domains of either Src-family or unrelated proteins as antigen. Percent amino acid identities with the Src SH3 domain are as follows: Yes, 77%; Fyn, 76%; Lyn, 53%; Lck, 49%; Csk, 38%; Intersectin SH3-A, 30%; PLCγ, 1 40%; Grb2-N, 34%; Spectrin α, 1 31%; and p53BP2, 33%. Assays were performed in triplicate using 100 ng of purified 1F11-AP or 1E3-AP per well.

(D) Contribution of BC and FG loops to target binding. ELBA with chimeric monobodies was performed as described for (B) using cell extracts, in triplicate. Replacement of the BC loops with the wild-type FN3 sequences is denoted as "wt/FG," and replacement of the FG loops with the wild-type FN3 sequences is denoted as "BC/wt."

an array of SH3 domains. ELBA experiments were performed with SH3 domains of five other Src-family proteins using purified AP-fusions of 1F11 (1F11-AP) and 1E3 (1E3-AP). 1F11-AP recognized glutathione-S-transferase (GST) fusions of SH3 domains of Yes, Fyn, Lyn, Lck, and n-Src, a form of Src that contains six additional amino acids due to alternative splicing in neuronal tissues [12], although the binding was significantly weaker in comparison to Src SH3 (Figure 2C). 1E3-AP bound only to the Src SH3 domain (Figure 2C). Both monobodies failed to bind to SH3 domains of six other proteins that do not belong to this family (i.e., Csk, Intersectin, PLCγ1, Grb2, Spectrin, and p53BP2), demonstrating that their binding is specific to the SH3 domains of Src and/or its family members (Figure 2C).

Analysis of the Binding Site of the Monobodies on the Src SH3 Domain

SH3 domains are known to bind proline-rich sequences [13], and thus it is not surprising that five of the six selected monobodies carried proline-rich sequences in their FG loops. The FG loop sequences of 1F11, 2B2, 1F10, 1C9, and 2G10 resemble the RPLPPLP consensus identified from screening combinatorial peptide libraries [14], with the R residue replaced by an L in 1C9 and 2G10. Furthermore, clones 1F11, 2B2, and 2G10 also contained the preferred residues P and L in the second and third position of the phage-display-derived consensus.

The similarity of the FG loop sequences with the Src SH3 domain peptide ligands suggested that five of the monobodies bind the Src SH3 domain at the same place. To validate this, we examined whether soluble forms of the 1F11 monobody could inhibit binding of an enzyme-linked peptide ligand (i.e., RPLPPLP) to the Src SH3 domain. Figure 4A demonstrates that the 1F11 monobody was able to block the binding of the peptide ligand to the Src SH3 domain, whereas the wild-type protein did not, indicating that 1F11 binds at or near the same region as the peptide ligand. Based on the high similarity

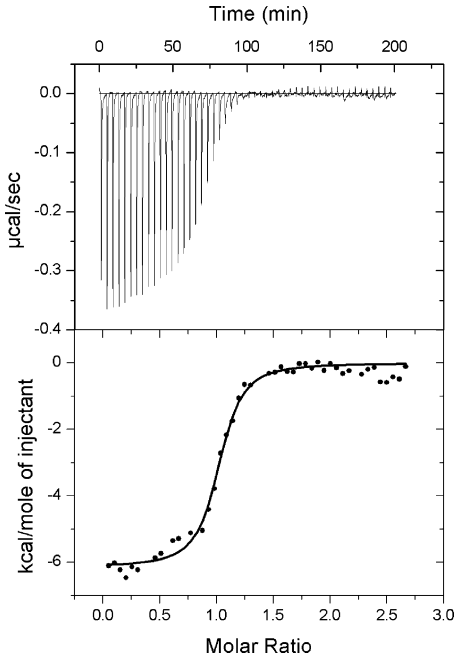

Figure 3. Measurement of the K_d between Src SH3 and the High-Affinity Monobody by ITC

Representative ITC experiment performed using Src SH3 and the monobody 1F11. The top panel shows the raw data: the heat flow generated as a result of injections of a solution of 1F11 in PBS into a solution of Src SH3 also in PBS in the sample cell. Each peak represents a single injection. The bottom panel shows the binding isotherm created by plotting the integrated heats against the molar ratio between the two proteins.

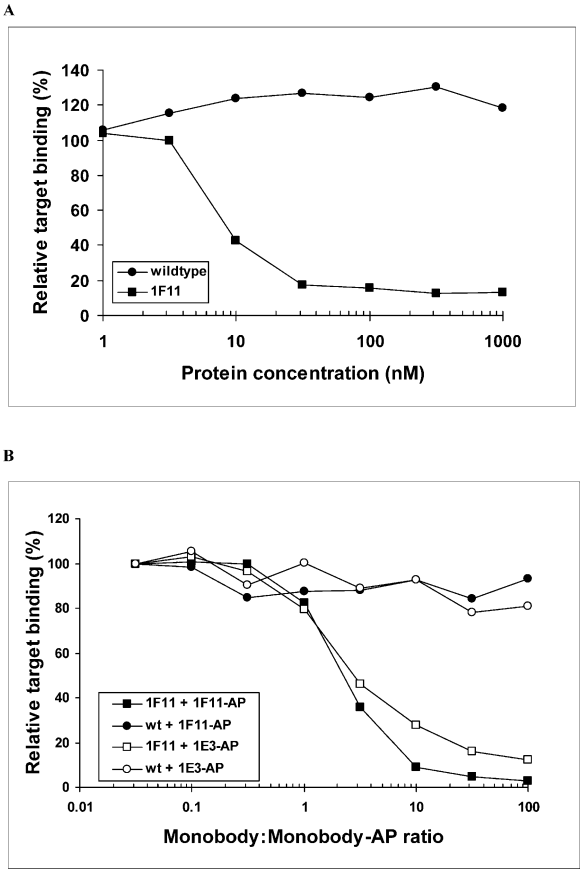

Figure 4. Competitive Binding Assays

(A) Competition between Src SH3 peptide ligand and 1F11 for Src SH3 binding. Purified Src SH3 peptide ligand-AP fusion was used to detect immobilized Src SH3 in an ELBA as described in Experimental Procedures. For competition, increasing amounts of purified 1F11 (squares) or wild-type FN3 (circles) were combined with peptide ligand-AP fusion during the binding step.

(B) Competition between 1F11 and 1E3 for Src SH3 binding. Binding of 1F11-AP fusion to immobilized Src SH3 was determined in the presence of increasing amounts of purified 1F11 (closed squares) or wild-type FN3 (closed circles). Binding of 1E3-AP fusion was determined in the presence of increasing amounts of purified 1F11 (open squares) or wild-type FN3 (open circles).

of the FG loop sequences of monobodies 2B2, 1F10, 1C9, 2G10, and 1F11, we believe that they bind the Src SH3 domain in a comparable manner.

Since the 1E3 monobody lacks a proline-rich FG loop, it was possible that this monobody bound at a unique site on the Src SH3 domain other than the peptide binding groove. To determine whether 1E3 and 1F11 bind at the same or different sites, we performed competition experiments: 1F11-AP or 1E3-AP were mixed with increasing amounts of soluble wild-type protein or 1F11 monobody and incubated with Src SH3 domain immobilized in a microtiter plate well. As expected, 1F11-AP binding was inhibited by addition of soluble 1F11, whereas addition of the wild-type monobody had no effect on the binding of either 1F11-AP or 1E3-AP (Figure 4B). To our surprise, 1E3-AP binding was also inhibited effectively with the addition of soluble 1F11 (Figure 4B), demonstrating that binding of 1F11 and 1E3 monobodies to the Src SH3 domain are mutually exclusive.

We further investigated the binding site of the 1E3 and 1F11 monobodies using heteronuclear NMR spectroscopy. We prepared ^{15}N-labeled Src SH3 domain and completed sequence-specific assignments of backbone ^{1}H and ^{15}N resonances. Perturbation of amide ^{1}H and ^{15}N resonances of the SH3 domain was monitored by comparing the ^{1}H, ^{15}N-HSQC spectrum of the ^{15}N-labeled SH3 domain in the presence and absence of a binding partner. (Note that this experiment selectively measures signals from a ^{15}N-labeled component in an NMR sample, and thus signals from unlabeled monobodies and peptide are eliminated [15, 16]). In addition to the two monobodies, we also used a high-affinity peptide for Src SH3, VSLARRPLPPLP, as a control [17].

The profile of chemical shift perturbation (Figure 5) caused by the binding of 1F11 monobody was similar to that of the high-affinity peptide, confirming that 1F11 binds to the proline-rich peptide binding site. The affected residues are mainly localized near the peptide binding interface of the SH3 domain (Figure 5). We could not detect any signs of binding of the 1E3 monobody when we initially performed NMR measurements in the absence of a reducing reagent (data not shown); however, the SH3 spectrum changed when we repeated the measurements in the presence of dithiothreitol. As the 1E3 monobody contains two cysteine residues in the FG loop, this last observation suggests that these cysteines are in the reduced form in the active state of this mono-

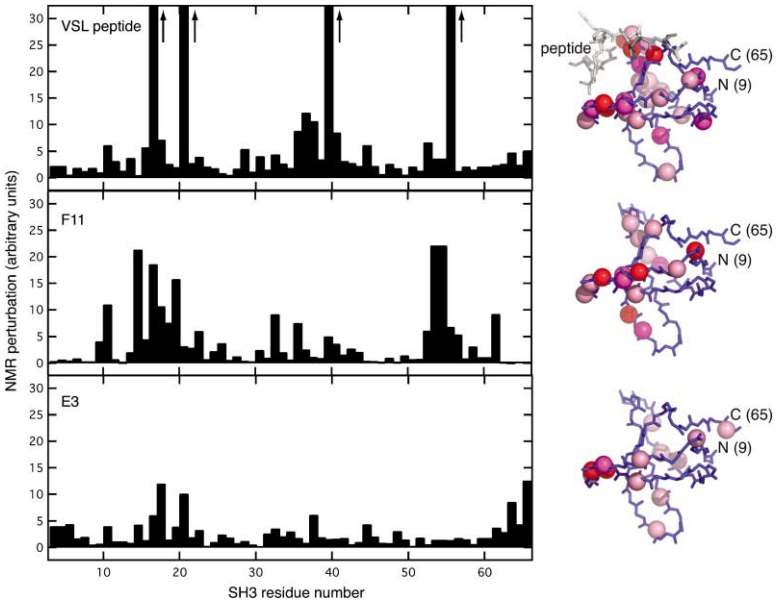

Figure 5. Analysis of the Binding Sites on Src SH3

Perturbation of NMR peak positions of the Src SH3 domain by binding of a high-affinity peptide (a), the 1F11 (b), and 1E3 (c) monobodies. The perturbation values (see Experimental Procedures) are plotted as a function of SH3 residue number. The residue numbering is according to Protein Data Bank entry 1QWF [17]. The four residues designated with an arrow in (a) had values greater than 35. SH3 residues affected by binding are mapped graphically on the right using PyMol software (www.delanoscientific.com). The amide nitrogen positions of residues exhibiting perturbation values greater than 2.5 (light pink), 5 (pink), and 10 (red) are shown as spheres in the structure of Src SH3 domain (blue) in complex with a high-affinity peptide (white), VSLARRPLPPLP (Protein Data Bank ID code 1QWF) [17]. Residues that showed significant perturbation but have solvent-accessible surface areas less than 10 Å^2 have been excluded, because perturbation of such residues is likely due to indirect effects. Note that residues 5–7 and 67–72 (residue numbering according to 1QWF) are all affected by 1E3 binding, but are not included in the figure because these residues are missing in the structure. The structure corresponds to residues 9–65. The N and C termini of the SH3 domain are marked in the figure.

body. The perturbation profile of 1E3 was distinctly different from those of the peptide and 1F11 (Figure 5). In particular, residues between 50 and 60 were not affected by 1E3 binding, while residues close to the N and C termini were more affected by 1E3 binding than by the others tested here. The distribution of SH3 residues affected by 1E3 binding (Figure 5) is clearly different from those affected by the peptide or by 1F11. (Note that the atomic coordinates used for Figure 5 do not contain residues near the termini that were affected by 1E3 binding, and thus these residues are not included.) Thus, the NMR data strongly indicate that 1E3 binds to a site that is somewhat different from the peptide binding cleft. The results also suggest a partial overlap of the binding epitopes for 1F11 and 1E3, which can explain the mutually exclusive binding of these two monobodies to Src SH3.

Contribution of the BC and FG Loops to Target Binding

To determine to what extent each of the randomized loops contribute to binding, we swapped each of the loop sequences in the monobodies with those of the wild-type monobody and compared the relative binding strengths of the chimeric proteins to the Src SH3 domain. Replacing the FG loop with the wild-type sequence completely abolished binding of the 1F11 monobody to the Src SH3 domain (Figure 2D), as anticipated because of the high level of similarity of the FG loop sequence to the peptide ligand. Surprisingly, replacing the BC loop with the wild-type sequence also diminished binding of 1F11 to the Src SH3 domain (Figure 2D). A similar result was observed with the two chimeric constructs of 1E3. These observations suggest that both loops contribute to the interaction between the Src SH3 domain and the selected monobodies.

Utility of the Monobodies

As we wanted to clarify whether we could use the engineered monobodies as affinity reagents, the strongest binder, 1F11, was tested in Western blotting and pull-down experiments. Ten micrograms of purified GST, GST-Src SH3, GST-Yes SH3, and GST-Intersectin SH3 were resolved by SDS-PAGE, blotted onto nitrocellulose, incubated with 1F11-AP, and protein complexes were detected by chemiluminescence (Figure 6A). 1F11-AP bound to the filter-blotted Src SH3 domain with little or no binding to the other blotted proteins, despite the large amounts of protein present. Some binding to Yes SH3 was detected with increasing incubation times (data not shown). However, 1F11-AP was unable to detect full-length c-Src in extracts of A431 cells in Western blot (data not shown); presumably, its affinity was not high enough for detecting small amounts of c-Src present on the blot.

Next, we determined whether 1F11 could be used to pull down full-length c-Src from mammalian cell extracts. Soluble forms of the wild-type and 1F11 monobodies were immobilized on Ni-NTA agarose beads and incubated with cell extracts of a mouse fibroblast cell line expressing c-Src. As seen in Figure 6B, beads loaded with 1F11 recovered full-length c-Src from a cell extract, while wild-type FN3 (or beads alone) did not, demonstrating that the monobodies can be used successfully in pull-down experiments.

Discussion

From a phage-displayed library of FN3 domains, in which a total of ten amino acids were randomized on two-surface exposed loops, we successfully isolated affinity reagents for the SH3 domain of the human oncogenic protein, c-Src. Twenty-two positive clones, corre-

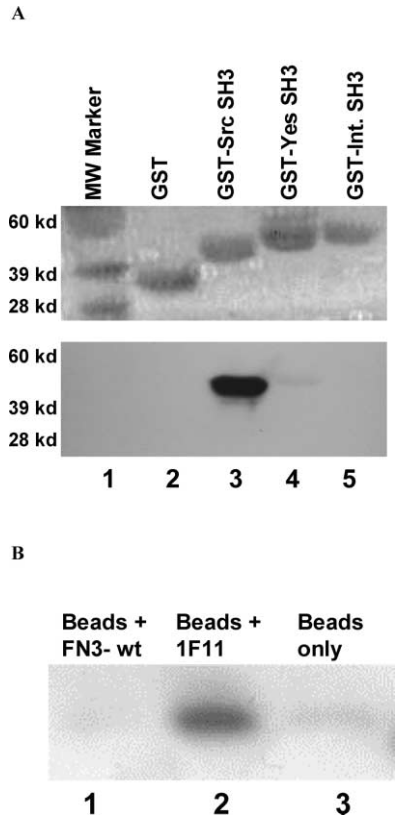

Figure 6. Utility of the Monobody in Western Blots and Pull-Down Assays

(A) Western blot. Ten micrograms of each of the proteins (GST or GST fusions to the SH3 domains of Src, Yes, or Intersectin) was separated by SDS-PAGE on two gels. One was stained with Coomassie blue (top gel); the other was used for Western blotting (bottom gel) with a 100 ng/ml solution of purified 1F11-AP protein.

(B) Pull-down. Cellular Src protein was isolated from cell extracts of mouse embryonic stem cells using 1F11. Assay was performed as described in Experimental Procedures using Ni-NTA agarose beads alone (lane 1), loaded with 1F11 (lane 2), or loaded with wild-type FN3 (lane 3). Beads were washed, boiled, and subjected to SDS-PAGE followed by Western blotting with a mouse monoclonal anti-Src antibody GD11.

sponding to six unique sequences, were isolated from a library of two billion recombinants after three rounds of affinity selection. Interestingly, five out of the six unique clones had proline-rich FG loop sequences that resembled the class I peptide ligand sequence preference (i.e., +xxPxxP) of the Src SH3 domain. It is generally agreed that proteins have a limited number of regions for interaction with other proteins. Such binding sites have been termed "hot spots" [18] in which a small number of amino acids, typically hydrophobic and disordered, contribute the bulk of the binding energy. The hot spot of most SH3 domains consists of two shallow pockets that accommodate each proline in the PxxP motif and a third pocket that seats the positively charged residue in the +xxPxxP or PxxPx+ motif of class I or class II ligands, respectively [19, 20]. Thus, the majority of the monobodies appear to "home in" on the same hot spot of the SH3 domain, as do combinatorial peptides [14, 19, 21, 22]. In the case of two of the monobodies, the positive charge of the class I motif has been replaced by a leucine, which in one case (clone 1C9) led to a reduction in binding strength as expected; however, in another case (clone 2G10) binding strength was unaltered. This is somewhat surprising, given that the positively charged residue in the peptide ligand sequence contributes significantly to binding.

Mutagenesis experiments have confirmed that in two of the clones tested (1F11 and 1E3), both of the randomized loops contributed significantly to binding. In the case of 1F11, this was unexpected. Since the entire proline-rich binding motif is contained in the FG loop, replacing the BC loop with the wild-type BC loop sequence was not expected to cause a drastic reduction in the binding affinity of this clone to Src SH3. These results indicate that there are determinants for binding the Src SH3 domain in both loops, and that the BC loop either directly takes part in the intermolecular interaction with the Src SH3 domain or stabilizes the binding indirectly through intramolecular interactions. Given that, in the three-dimensional structure of FN3, the BC and FG loops are located adjacent to each other, both of these explanations are plausible [23]. This observation also agrees with analysis of an engineered monobody that binds ubiquitin [5], in which alanine replacement of each residue in the randomized loops led to a reduction in binding to ubiquitin. Further structural analysis of monobody-SH3 complexes will reveal the exact nature of these interactions.

Analysis of selected monobodies showed that they bound the target with modest affinity. By isothermal titration calorimetry, the dissociation constants for the weakest (1C9) and strongest (1F11) binders to the Src SH3 domain are estimated to be 1.3 and 0.25 μM, respectively. Such measurements paralleled the differences in binding of AP fusion proteins for the two binders, rather than the phage ELISA measurements, suggesting that the ELBAs are more representative of binding strength. The ability of the monobody scaffold to yield high-affinity reagents has been demonstrated by Xu and coworkers [9], who isolated binders from an mRNA display library that bound TNF-α and leptin with nanomolar dissociation constants. The complexity of our library was approximately three orders of magnitude smaller than this mRNA display library. We suspect that larger phage-displayed libraries may yield tighter affinity reagents, as there is a direct correlation between the size of the display libraries and the affinity of the binders isolated from said libraries [24]. In addition, since both loops in the monobodies contribute to binding, it will take large libraries to find their optimal sequence contribution. Nevertheless, the affinities of the selected monobodies are stronger than that of peptides (i.e., 3 μM) and are comparable to natural interacting partners. For example, the HIV Nef protein binds the Hck SH3 domain with a K_d of $\sim$250 nM [25]. Despite their modest affinity, the strongest monobody was suitable for displacing natural interacting partners of the SH3 domain in pull-down experiments. The monobodies also showed excellent specificity for their targets. Neither of the two monobodies tested bound non-Src family SH3 domains, although the 1F11 clone did bind somewhat to SH3 domains from other members of the Src family of tyrosine kinases. On

the other hand, clone 1E3 was highly specific for the Src SH3 domain. This is a level of specificity that has been difficult to achieve with combinatorial peptide ligands [26]. Therefore, these monobodies can potentially be used for selective detection, coimmunoprecipitation, or intracellular perturbation of c-Src and some of its family members.

Since their introduction by Koide and coworkers, the potential of monobodies to yield useful reagents has been explored briefly by only two previous studies. In the first, monobodies specific for agonist- or antagonist-bound forms of the estrogen receptor have been used to probe the conformational state of this protein in yeast cells by Koide and coworkers [8]. In the second, high-affinity binders have been isolated against TNF-α and leptin by Xu and coworkers [9], which were subsequently used to construct self-assembling protein arrays. We have explored the potential of this scaffold further to show that it can be used for pull-down experiments as soluble protein and for binding assays as a fusion to AP. We have also shown that monobodies can yield very specific binders for proteins with a high degree of similarity, such as the SH3 domains used in this study.

Because one of the monobodies was able to bind the full-length c-Src, it can potentially be used as an intrabody for selective intracellular manipulation of c-Src. Furthermore, AP or green fluorescent protein fusions of these monobodies can be used for colorimetric and fluorescence detection and/or localization of their respective antigens. In the future, it should be possible to select highly specific reagents for other SH3 domains as well either from this library or from other focused libraries that can be constructed with this scaffold.

Significance

The utility of the monobody, as an antibody mimetic based on the tenth type III domain of human fibronectin, was demonstrated with the isolation of two classes of monobodies that bound to the SH3 domain of the human oncogenic protein, c-Src. One class contained proline-rich sequences similar to class I peptide ligands for Src SH3 and bound at the peptide binding pocket, as shown by competitive binding and NMR perturbation experiments. The second class, which contained only one member, lacked proline-rich sequences and bound Src SH3 at a site somewhat separate from the peptide binding site. The first class of monobodies bound to the SH3 domain of other members of the Src family, and the second class was highly specific and bound only to the SH3 domain of Src. The strongest binder had a dissociation constant of 0.25 μM for Src SH3, which is comparable to the affinity of an SH3 domain with its cellular interacting partner. The utility of the best monobody was evidenced by its ability to pull down c-Src from murine fibroblast cell extracts, demonstrating that monobodies can be used in coimmunoprecipitation experiments. In the future, highly specific monobodies like the ones isolated here could be expressed inside cells to interfere with protein function and thus serve as a valuable tool to study protein function. Our work supports the use of monobodies as affinity reagents in research and diagnostics.

Experimental Procedures

Reagents

The *E. coli* strains TG1 and BL21(DE3) (Novagen, Madison, WI) were used for plasmid propagation and protein expression, respectively. All restriction endonucleases and DNA modification enzymes except for T4 DNA Ligase (Promega, Madison, WI) were from New England BioLabs (Beverly, MA). *Pfu* Turbo DNA Polymerase was from Stratagene (La Jolla, CA). The phagemid pHEN4::cAbLys3 was a gift of Dr. Serge Muyldermans (Vrje Universieit, Belgium). The plasmid containing the coding sequence for APC1336 was a gift from Dr. Frank Collart (Argonne National Laboratory, Argonne, IL). T24E mouse embryonic fibroblasts were a gift from Dr. Marsha Rosner (University of Chicago, Chicago, IL). The mouse monoclonal anti-Src antibody GD11, goat anti-mouse HRP-conjugate, and A431 cell extracts were obtained from Upstate (Lake Placid, New York). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was from Sigma (St. Louis, MO). HRP-conjugated anti-M13 antibody was from Amersham Biosciences (Piscataway, NJ). DNA sequencing and oligonucleotide synthesis were performed by MWG Biotech (High Point, NC).

FN3 Gene Construction

The human fibronectin tenth type III domain (FN3) gene was constructed essentially as described by Koide et al. [5], with several modifications. Briefly, six oligonucleotides, approximately 60 bases each, with 16 nucleotides of complementarity at their 5′ and 3′ termini were assembled into double-stranded DNA with the Klenow fragment of the *E. coli* DNA polymerase. The product was amplified by PCR using *Pfu* DNA polymerase and cloned into pBluescript SK (Stratagene). To maximize expression of the FN3 domain in *E. coli*, the coding region was designed with codons frequently used in *E. coli*. Also, a D7K mutation was incorporated to increase stability of the FN3 domain at neutral pH [27]. The assembled FN3 open reading frame was then amplified by PCR with two sets of primers, denatured and annealed to generate heteroduplexes with sticky ends, and cloned into the phagemid vector, pHEN4 [28], as a fusion to gene III of bacteriophage M13. This construct, pHEN4::FN3, was confirmed by sequencing and transformed into TG-1 cells for viral packaging with the helper virus, M13K07. Display of wild-type FN3 on the phagemid particles was confirmed by ELISA with a polyclonal anti-fibronectin antibody from Sigma (St. Louis, MO).

Library Construction

A library was constructed that randomized five codons in both the BC (residues 26–30) and FG loops (residues 77–81) coding regions and contained a three amino acid deletion in the FG loop, similar to that previously reported [5]. NNK codons were used to randomize the ten positions, where N is an equimolar mixture of all nucleotides, and K is an equimolar mixture of G and T. This codon set encodes all of the amino acids and one stop (TAG) codon that is suppressed in TG1 cells by the *supE* gene product by the insertion of glutamine. Briefly, the FN3 coding region was amplified in two halves, using primers P1 (forward) and P2 (reverse) for the N-terminal half and P3 (forward) and P4 (reverse) for the C-terminal half of the protein. (P1 anneals to a region in the vector upstream of the cloned FN3 gene. P2 encodes a 15-base NNK stretch flanked by 25 nucleotides complementary to the FN3 gene on either side of the region encoding the BC loop. P3 is complementary to the 25 nucleotides at the 5′ end of P2. P4 is similar to P2, except that it anneals to the region encoding the FG loop.) The resulting fragments were gel purified and assembled through overlap PCR, making use of the 25 nucleotide long complementarity in their sequence. The resulting full-length, randomized gene was gel purified yielding 130 ng of DNA (2.6×10^{11} molecules), amplified a few cycles with another set primers to yield sticky ends as described elsewhere [29], purified using the Qiagen PCR product purification kit (Valencia, CA), and ligated into NcoI/NotI-digested pHEN4. This material was transformed by electroporation into *E. coli* TG1 cells. The library size was determined to be 2×10^9 individual clones, with a vector background of $\sim$0.1%. For affinity selection experiments, the library of FN3 plasmids was rescued with the helper phage M13K07, and the resulting viral parti-

cles were concentrated by precipitation with a final concentration of 6% polyethylene glycol and 0.3 M NaCl.

Target Preparation and Affinity Selection Experiments

The human c-Src SH3 domain coding region was amplified by PCR and cloned into the expression vector pMSCG16 [39] using ligation independent cloning (LIC) [30]. This vector encodes an N-terminal six-histidine tag, a substrate sequence (AviTag) for biotin ligase [31], a GSGS linker, a cleavage site for the tobacco etch virus (TEV) protease, and an LIC site. This construct was transformed into the *E. coli* strain BL21 (DE3) (Novagen) carrying the pBIRAcm plasmid (Avidity, Denver, CO). This plasmid encodes the biotin ligase required for in vivo biotinylation of the target protein. Expression of the SrcSH3 domain was induced at an optical density (600 nm wavelength) of 0.7 with 1 mM IPTG for 5 hr at 30°C; at the time of IPTG addition, the cells were also supplemented with 50 μM D-biotin (Sigma). Cells were harvested and the Src SH3 domain was purified using Ni-NTA resin (Qiagen) according to manufacturer's instructions. Biotinylation was confirmed by binding to streptavidin-coated magnetic Dynabeads M-280 (Dynal Biotech, Lake Success, NY).

Affinity selection experiments were performed using 100 nM Src SH3 on streptavidin-coated magnetic beads as follows. One milliliter of the library, containing 10^{12} phagemid particles, was precleared on 100 μl Dynabeads and then incubated with the biotinylated target on the beads at room temperature (with tumbling) for 2 hr. Beads were washed three times with 1 ml Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.05% Tween-20 (TBST). Bound phage were eluted with 500 μl of 50 mM glycine-HCl (pH 2) and neutralized with 100 μl of 1 M Tris-HCl (pH 8). Three hundred microliters was used to infect exponentially growing TG1 *E. coli* cells by incubation with gentle shaking at 37°C for 1 hr. Cells were plated on 2×YT plates supplemented with 50 μg/ml carbenocillin and 1% glucose (2×YT-AG) and incubated overnight at 30°C. The next day, colonies were scraped into 5 ml 2×YT, 200 μl ($\sim 6 \times 10^9$ cells) of which was inoculated into 10 ml 2×YT-AG liquid medium, grown for 1 hr at 30°C, and infected with $\sim 6 \times 10^9$ helper phage, M13K07. After an additional hour of incubation at 37°C, the cells were pelleted and resuspended in 10 ml 2×YT with 50 μg/ml carbenocillin and 25 μg/ml kanamycin. After overnight growth at 30°C, cells were pelleted by centrifugation, and the phage was precipitated from the supernatant in 6% polyethylene glycol-0.3 M NaCl and resuspended in 500 μl phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). One hundred microliters of this material was used for the second round of selection, as described above.

After the third round of selection, cells were plated at limiting dilution, and 192 colonies were picked for verification of binding by phage ELISA. Biotinylated Src SH3 (200 ng) was immobilized in 96 microtiter plate wells that were coated with 400 ng of streptavidin per well. The control for the initial screen consisted of streptavidin-coated plates only. The positive clones were confirmed in duplicate by a second phage ELISA, which included an unrelated biotinylated protein (i.e., APC1336) as a negative control.

Construction of Alkaline Phosphatase Fusions and Their Use in Enzyme-Linked Binding Assays

The regions encoding the six unique Src SH3 binding monobodies were amplified using *Pfu* DNA polymerase and cloned into the alkaline phosphatase (AP)-fusion vector, pEZ707 [11], by LIC. This vector allows expression and export of proteins into the periplasmic space as N-terminal fusions to AP, which carries six histidines at the C terminus for rapid purification by immobilized metal affinity chromatography (IMAC). The AP protein encoded by this construct is a variant carrying two mutations (D153G/D330N), which is approximately 16 times more active than the wild-type enzyme [11]. Because the BL21 (DE3) strain does not contain a *supE* gene, the TAG codons in 1F10, 1E3, 2G10, and 2B2 were converted to CAG, which codes for glutamine, by site-directed mutagenesis. For purification of the monobody-AP fusion proteins, cultures were grown in LB at 30°C until they reached an optical density of 0.7 and induced by 0.5 mM IPTG and grown overnight at 30°C. Six-histidine-tagged proteins were purified from whole-cell extracts, following Qiagen's instructions.

Binding assays for the monobody-AP fusions were performed with 96-well microtiter plates that had been coated with 200 ng of streptavidin and 100 ng of biotinylated Src SH3 domain. In some cases, 1 μg of glutathione-S-transferase (GST) fusions to different SH3 domains was immobilized directly in the wells. Monobody-AP fusions were added either as purified proteins at 100 ng/well or as extracts of bacterial cells lysed with Bugbuster detergent (Novagen). Cell extracts were prepared as follows. Two milliliters of cultures were grown overnight in LB, cells were recovered by centrifugation, and the pellets were resuspended in 100 μl of Bugbuster reagent with 0.1 μl of Benzonase (Novagen). After 30 min incubation at 25°C, cell debris was pelleted by centrifugation for 15 min. AP activity in the supernatant was measured and normalized. Approximately 1–3 μl of the supernatant was used per well, and bound enzyme was detected with the chromogenic substrate para-nitrophenylphosphate (Sigma). For competition experiments, either the peptide ligand-AP fusion or monobody-AP fusions were mixed with different amounts of purified soluble monobody before addition to the wells. All experiments were performed in triplicate, and averages of the data points were plotted.

Cellular Expression and Purification of FN3

To generate soluble protein for competition, ITC, and NMR experiments, genes encoding the monobodies and the wild-type FN3 were cloned into pET28 (b) (Novagen), an intracellular expression vector that adds a six-histidine tag to the C terminus of the protein. For competition and ITC experiments, cells were grown in LB, and protein expression was induced, at an OD_{600} of 0.7, with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 hr at 30°C. Proteins were purified by immobilized metal affinity chromatography using Ni-NTA beads (Qiagen), according to the manufacturer's instructions. Purified proteins were analyzed for quantity, molecular weight, and purity using a BioAnalyzer 2100 (Agilent, Palo Alto, CA). Protein yields were typically 100–200 mg per liter of culture.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed at 30°C using a VP-ITC (MicroCal, Northampton, MA). F11, C9, and Src SH3 were purified as described above and dialyzed thoroughly against PBS to ensure equivalent pH and salt concentration of the samples. Proteins were filtered to remove any aggregates and degassed prior to use. Src SH3 (30 μM, 1.4 ml) was placed in the calorimetric cell, and F11 or C9 (300 μM, 0.4 ml) was placed in the titration syringe. Titration was conducted with injection of 3 μl aliquots each, at a default injection rate of 300 s intervals. The heat of association was calculated from the difference between the integrated heat of reaction and the corresponding heat of dilution. Data were analyzed using the Origin software (version 7.0), with embedded calorimetric fitting routines, to determine N (number of binding sites), ΔH (change in binding enthalpy, cal/mole), ΔS (change in binding entropy, cal/mole/deg), and K_d (dissociation constant).

NMR Spectroscopy

^{15}N-labeled Src SH3 protein was prepared by growing the expression strain in M9 minimal media supplemented with ^{15}N-ammonium chloride as the sole nitrogen source. The protein was purified as described above, and the N-terminal tag was cleaved off with TEV protease, which itself contained a six-histidine tag. The uncleaved protein, tag, and protease were removed by IMAC. The cleaved protein was concentrated and buffer exchanged into 50 mM sodium phosphate buffer (pH 6.0) containing 150 mM NaCl and 10 μM EDTA in 95% H_2O/5% D_2O using an Amicon ultrafiltration cell (Millipore, Billerica, MA). The final protein concentration was 1.2 mM. ^1H, ^{15}N-HSQC, ^{15}N-edited TOCSY, and ^{15}N-edited NOESY [32, 33] were performed on a Varian INOVA 600 spectrometer using pulse sequences in the BIOPACK supplied by Varian. The HSQC spectra for chemical shift perturbation analysis were recorded on ~0.2 mM ^{15}N-labeled Src SH3 samples that contain ~30% molar excess of a binding partner. For measurements of the Src SH3-1E3 complex, experiments were performed in the presence and absence of 5 mM dithiothreitol. NMR data were processed using the NMRPipe suite [34] and analyzed using the NMRView program [35] on a Linux

workstation. Backbone resonance assignments of Src SH3 were made using standard procedures [36, 37].

Chemical shift perturbation was defined using the "minimal movement" method [15]. We first picked HSQC cross peaks in the free and complex spectra. We identified a cross peak in the complex spectrum that is the closest to a given cross peak in the free spectrum. Our search algorithm uses a particular cross peak only once, prohibiting sharing of a cross peak in the complex spectrum for multiple cross peaks in the free spectrum. After matching cross peaks, we calculated the perturbation value, δ, according to the following equation:

$$\delta = 100\sqrt{(\Delta H/range_H)^2 + (\Delta N/range_N)^2},$$

where ΔH is the difference in the ^{1}H chemical shift between the free and complex cross peaks, ΔN is the difference in the ^{15}N chemical shift, and range$_H$ (3.591) and range$_N$ (26.26) are normalization coefficients for the ^{1}H and ^{15}N dimensions derived from the chemical shift dispersions of the free SH3 spectrum. Solvent-accessible surface areas were calculated with a probe radius of 1.4 Å using the CNS program [38].

Western Blot Using 1F11-AP Fusion

Ten micrograms of pure GST, GST-Src SH3, GST-Yes SH3, and GST-Intersectin SH3 (A) protein were resolved by SDS-PAGE in 10% Tris-glycine mini gels (Invitrogen, Carlsbad, CA) and blotted onto nitrocellulose membrane using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The blot was blocked for 1 hr with 100 ml of blocking buffer (3% non-fat dry milk in PBS) and then incubated with 1:5,000 dilution of a 0.5 mg/ml purified 1F11-AP solution in 100 ml of blocking buffer. After a 2 hr incubation, the blot was washed three times with 100 ml of blocking buffer containing 1% Tween-20 and rinsed thoroughly with deionized water. Binding of the AP fusion protein to the blot was revealed with the chemiluminescent substrate Lumi-Phos (Pierce, Rockford, IL) and imaged by X-ray film autoradiography.

Pull-Down Assay

Approximately 3×10^7 T24E cells (mouse embryonic fibroblasts) were lysed in 1.3 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF) by incubation on ice for 20 min. Cell debris was pelleted by centrifugation at 4°C for 10 min. Four hundred microliters of the supernatant was mixed with 50 μl of Ni-NTA beads (50% suspension in lysis buffer), either alone or preloaded with 20 μg of six-histidine-tagged 1F11 or wild-type FN3 protein, and tumbled overnight at 4°C. The next day, beads were washed three times with 1 ml lysis buffer, 20 μl of loading buffer was added to the beads, and the mixture was boiled for 5 min. Beads were recovered by centrifugation, the supernatants were resolved in a 10% Tris-glycine mini gel (Invitrogen), and blotted onto nitrocellulose membrane using a Trans-Blot Semi-Dry Transfer Cell. The blot was blocked for 1 hr and tumbled overnight at 4°C with a 1:500 dilution of the mouse monoclonal anti-Src antibody GD11 in blocking buffer. The next day, the blot was washed four times in TBST and incubated with a 1:10,000 dilution of goat anti-mouse HRP-conjugate at 25°C for 2 hr. The blot was washed four times in TBST, rinsed in deionized water, incubated with the chemiluminescent substrate Supersignal West Pico (Pierce) for 5 min, and exposed to X-ray film.

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Note Added in Proof

The article in reference 4 was retracted in May, 2004.